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In vitro propagation of *Eria tomentosa* (Koen.) Hook. f.: An important indigenous medicinal orchid of Bangladesh

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Abstract
Stem and leaf segments of aseptically grown seedlings of *Eria tomentosa* (Koen.) Hook. f. gave response to both PGR supplemented agar solidified and liquid MS media and PM media. Both the explants underwent direct organogenesis producing shoot primodia like structures (SPSs) on MS medium fortified with 1.5-3.0 mg/l BAP or Kn either alone or in combination with 0.5-1.0 mg/l IAA or NAA. However, liquid MS + 3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP took lesser time and was effective for induction of shoot primordial than agar solidified PM. Liquid media were found more effective in SPSs induction. It was revealed that MS medium was more effective than PM medium for SPSs induction. The shoot primordia developed from stem and leaf segments underwent elongation when cultured on both liquid and agar solidified MS elongation media supplemented with different concentrations and combinations of PGRs and the maximum rate of elongation was achieved in MS (liquid) + 2.0 mg/l BAP + 1.0 mg/l IAA. The elongated shoots were produced aerial roots in agar solidified MS media supplemented with different PGRs. Thus an efficient protocol was developed for mass scale propagation of the selected orchid species.

Keywords: *Eria tomentosa*, *in vitro* culture, medicinal orchid

1. Introduction
Orchidaceae is one of the largest families in angiosperms. Orchids make up the largest species of plants in the world and are cosmopolitan in distribution. They have been found growing in practically every corner of the earth - from the Russian tundra to the treetops of the tropical rainforests. They grow in all sorts of diverse and difficult conditions. There are over 25,000-35,000 orchid species in approximately 850 genera and another 100,000 hybrid varieties. Orchids are well known for their medicinal value as well as the economic importance and widely cultivated for ornamental purposes. It is estimated that approximately 1200 species of orchids belonging to 174 genera occur in China. More than 250 species (or over 20% of total species) in approximately 100 genera are already recorded in Chinese herbals as having medicinal usage. In Bangladesh, the Orchidaceae family represents a total of 178 species and a variety under 70 genera. Out of 178 taxa listed, 116 are epiphytic, 60 are terrestrial, *Vanilla parishii* Rchb. f. is a hemi-epiphyte whereas *Luisia viridiflora* (Bl.) Lindl. shows both epiphytic and terrestrial habits. More than 26 species are reported for ethnobotanical and therapeutical usage against 30 types of diseases in the ethnic communities [1]. Herbal medicine is practised worldwide, and not in Asia alone. In many continents, orchids have been used to nourish or to heal. *Salep* derived from terrestrial Mediterranean orchids and extracts of North American *Cypripediums* featured in European and American pharmacopoeia well into the later nineteenth century [2]. Nevertheless, using orchids as medicine is most entrenched in China, which has a well-documented and the longest history of continuous usage, as well as the longest list of orchids in medicinal use. When we study the history of ancient Alternative systems of medicine Ayurveda and Traditional Chinese Medicine (TCM) are on the forefront [3]. Asthavarga is important ingredient of various classical Ayurvedic formulations like Chavyanprasa [4]. Out of eight constituents of Ashtavarga, four have been reported to be orchids. Traditional Chinese medicine widely utilizes orchids in medicines. A few of them have been subjected to phytochemical and pharmacological studies. In India, work has been carried out on chemical analysis of some medicinally useful orchids. *Eulophia campestris*, *Orchis latifolia*, *Vanda roxburgii* are some important plants to mention [5].

Dendrobium macraei is another important orchid from Ayurvedic point of view as it is reported to be source of Jivanti [6]. *Cypripedium parviflora* is widely used as aphrodisiac and nervine tonic in Western Herbalism [2, 3]. *Vanilla planifolia* is commercially important orchid as it is source of vanillin used as a foodstuff flavoring [7]. 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 [8]. This figure undoubtedly indicates the necessity of production and improvement of orchids.

Many medicinal orchids are reported to contain alkaloids. Antimicrobial activities of some orchids have been suggested although detailed investigations are still warranted [9]. Recent works have reported isolation of anthocyanins, stilbenoids and triterpenoids from orchids. Orchinol, hircinol, cypripedin, jibantine, nidemin and loroglossin are some important phytochemicals reported from orchids.

Eria tomentosa (Koen.) Hook. f. is an epiphytic orchid, was found to occur in Chattogram, Sylhet, Rangamati (Kaptai, Bangchhari) and Bandarban (Alikadam) districts of Bangladesh. Pseudobulbs are large, ovoid, form a very stout woody rhizome. Leaves 3-4, elliptic oblong, very thick, nerveless, smooth, spongy and contracted into a very thick petiole. Inflorescence terminal, as thick as goose-quill or less, base with short coriaceous sheaths, stout, brown-villous, bracts large, oblong or lanceolate, not reflexed, coarsely veined with a broad thickened roughened central area from the base to the apiculate tip. Flowers brown tomentose, sepals coriaceous, falcate-lanceolate, mentum large incurved; petals oblanceolate. Lip oblong, strongly nerved with wide claw; midlobe clawed, subquandrately cordate. Flowering time is May. The Plant is used as aphrodisiac [1]. Pseudobulbs and leaves are washed and pasted properly, then juice is extracted and this juice is taken as aphrodisiac, chyawanprash and as linament in treating body swelling, inflammation and rheumatism and leaf paste also used in treating epilepsy and poisonous boil by Chakma. Juice is also used to treat eye disease, leucorrhoea, irregular menstruation (by Tanchangya) and diabetes by bengali people. They also mix the whole plant paste with garlic and mustard oil to treat rheumatism. The Marma people use the pseudobulb's juice to treat constipation and leaf juice to cure liver and kidney diseases. Pseudobulb and leaf are used as medicinal ingredients to treat hypertension, diabetes, heavy menstruation and leucorrhoea by Rakhain.

At present, the orchids also figure prominently in the Red Data Book prepared by International Union for Conservation of Nature (IUCN). In fact, the entire family is now included in Appendix-II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where the international trade is strictly controlled and monitored. However, mass multiplication can be achieved through micropropagation from *ex vitro* and/or *in vitro* explants which would be very useful to restoration of valuable and Red listed orchids in nature. Regeneration of shoot buds or protocorms like bodies (PLBs) and plantlet development in *in vitro* necessitate an exogenous supply of auxins and/or cytokinins for many orchid species [11]. Moreover, individual treatments of cytokinins, auxins and natural supplements have been recorded to be the most important factors to promote and improve the plant development from PLBs [12-15]. Hence, in order to obtain an efficient regeneration system with high frequency, *in vitro* seed derived protocorms with leafy shoot initials was experimented on cytokinins, natural additives and auxins either individually or in combination. The main goal of this research was to establish efficient protocol for *in vitro* rapid propagation of *Eria tomentosa* an indigenous medicinal orchid species of Bangladesh.

2. Materials and Methods

2.1 Collection and identification of plant materials

The plant (*Eria tomentosa*) was collected from Dhopachari of Chattogram district, Bangladesh. The orchid species was identified and authenticated by consulting relevant literature and critical examination of herbarium. The voucher specimen has been preserved at the Herbarium of Chittagong University (HCU). Capsule of disease free and fresh plant were used for *in vitro* culture. Collected fresh samples were cultured in aseptic condition for mass scale propagation following tissue culture method in the laboratory of Plant Tissue Culture and Biotechnology, Department of Botany, University of Chittagong.

2.2 Culture of plant material

Protocorms with leafy shoot initials raised from *in vitro* green pods seeds of the selected species cultured on Murashige and Skoog (MS) and Phytamax (PM) basal medium, were used as the explants. As the protocorm explants were obtained through *in vitro* cultures, no sterilization was required.

2.3 Effect of plant growth regulators and additives on shoot growth

Protocorms were inoculated on 1/2 MS medium supplemented with different concentration and combination of cytokinins [6-benzylaminopurine (BAP), kinetin (Kn)], auxins [Indole-3-acetic acid (IAA), Indole- 3-butyric acid (IBA), a-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram (Pic)] and of natural additives [banana pulp (BP) and coconut water (CW)] for shoot growth.

2.4 Culture condition

The basal 1/2 MS medium was fortified with 2% sucrose, 100 mg/l myo-inositol and will be solidified with 0.7% (w/v) agar. The pH was adjusted to 5.8 ±0.2 either with 0.1 N NaOH or HCl prior to autoclaving at 121 °C and 1.5 kg/cm^2 for 30 min. All cultures were aseptically maintained at 25± 2 °C under 16/8 h (light/dark) photoperiod with a light intensity of 40 µmol m^{-2} s^{-1} by white fluorescent light.

2.5 Experimental design and analysis of data

Protocorm explants were cultured in 100 ml culture bottles with five explants, each treatment was comprised of three bottles and the experiment will be repeated twice. Regular observations of cultures were made once a week. Total number of protocorms responding with healthy shoots was recorded after 5 weeks and the total number and length of shoots as well as roots was recorded after 15 weeks of culture. The percentage of the explants regenerating a shoot and the average shoot number and length was calculated by the following formulas:

$$\text{Percentage of response} = \frac{\text{Total number of explants with a shoot}}{\text{Total number of explants cultured}} \text{ X } 100$$

$$\text{Average number or length of shoot} = \frac{\text{Total number of } \frac{\text{shoots}}{\text{roots}} \text{ or sum of length of } \frac{\text{shoots}}{\text{roots}}}{\text{Total number of explants cultured}}$$

The average number, length of shoots were analysed statistically using one-way analysis of variance (ANOVA), and the mean±standard error (S.E.) of triplicates was represented and compared using Duncan's Multiple Range Test at 5% level of significance using SPSS statistic program software version 22.0.

3. Results and Discussion

Seeds of *Eria tomentosa* were cultured on MS medium without any PGRs. Cent percent germination rate was recorded. Greenish seedlings were produced. Mass scale propagation was done with the use of shoot primodia like structures (SPSs). When the masses of the *in vitro* grown tiny seedlings of these species were subcultured in both agar solidified and liquid MS & PM media supplemented with different kinds of PGRs those produced SPSs at the base. These SPSs were used for mass scale production of seedlings. In order to achieve rapid growth and regeneration of plantlets these SPSs were further grown individually on the elongation media. Eighteen different kinds of solid & liquid media with various combinations and concentrations of PGRs were used for the purpose. Of these, nine were prepared using MS basal medium and the rest nine were prepared using PM basal medium. Liquid media were more effective in SPSs induction. *Eria tomentosa* SPSs formation took place in liquid MS + 3% (w/v) sucrose and 0.5 mg/l Pic + 1.0 mg/l BAP; 0.5 mg/l Pic + 1.0 mg/l BAP; 1.0 mg/l IAA + 1.0 mg/l BAP; 1.0 mg/l Pic + 1.0 mg/l BAP; 0.5 mg/l Pic + 1.0 mg/l BAP; 0.5 mg/l Pic + 1.0 mg/l BAP; 0.5 mg/l NAA + 1.0 mg/l BAP; 0.5 mg/l NAA + 1.0 mg/l BAP; 0.5 mg/l Pic + 1.0 mg/l BAP; 0.5 mg/l Pic + 1.0 mg/l BAP. The maximum number of SPSs produce on MS+0.5 mg/l Pic + 1.0 mg/l BAP medium. Within 60d the SPSs turned into plantlets on the same medium. Comparison of the efficiency of media combination in terms of SPSs induction revealed that liquid media were more effective than agar solidified media. It was further revealed that MS medium was more effective than PM medium for SPSs induction. Most of the SPSs induced were greenish while a few were yellow. The results of SPSs development on both agar solidified and liquid media are presented in Table-1. Earlier results of *Cymbidium densiflora* [16], *Cymbidium cyperifolium* [16], *Acampe rigida* [17], *Aerides multiflora* [17], *Phalaenopsis sp.* [18] support these findings.

The SPSs were subcultured in wide range of both liquid and agar solidified nutrient media containing different PGRs. The results are shown in Table-2 and Table-3. The highest elongation (2.10 ± 0.24) was achieved in MS based liquid medium supplemented with 2.0mg/l BAP + 1.0 mg/l IAA. In case of agar solidified MS media, the maximum elongation was 1.51 ± 0.10 in 1.5 mg/l Kn + 1.0 mg/l IAA. Similar types of results were also reported in *Dendrobium chrysotoxum* [19], *Dendrobium palpebrae* [20], *Dendrobium transparens* [20], *Eria blumei* [21], *Malaxis obtuse* [22], *Rubiquitea spathulata* [23]. *Cymbidium aloifolium* [24, 25], *Dendrobium aphyllum* [26], *Dendrobium moschatum* [27] and *Cymbidium sinense* [28].

Continuous pressure on forest degradation, deforestation, shifting cultivation, tree cutting, lopping, biological invasion and indiscriminate exploitation are of the major threats to hilly regions of Bangladesh. Therefore, orchid populations have been under threat in their habitats in addition to their inherent lower germination rate due to the absence of nutritive endosperm with uneven climate change [29]. As a result, medicinally potential many orchid species have become rare or threatened along with their indigenous knowledge of therapeutic uses [30]. Plant tissue culture technique has been accepted as a potential alternative method for mass scale propagation and conservation of rare, threatened medicinal orchids and their sustainable utilization.

Various works on *in vitro* culture include propagation from seed or micropropagation, monoculture of desirable clones under uniform conditions and stopping the use of plants collected from the wild may solve problem of loss of orchid gene pool and help to conserve the gene bank of medicinal orchids [31]. *In vitro* propagation of many orchid species has been carried out by many researchers from different parts of the world to conserve their indigenous orchid population as well as to fulfill medicinal demand [32-36].

Table 1: Data* on the development of shoot primodia like structures (SPSs) in *E. tomentosa* when cultured on 0.8% (w/v) agar solidified and liquid elongation media supplemented with different kinds of PGRs.

Culture medium with different concentrations and combinations of PGRs	Solid media		Liquid media	
	Time (d) required for SPSs development	Remarks	Time (d) required for SPSs development	Remarks
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	—	—	—	—
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	50 – 60	Yellowish SPSs	35 - 45	Yellowish SPSs
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	—	—	45 - 50	Greenish SPSs
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	—	—	—	—
MS + 3% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	55 – 65	Greenish SPSs	40 - 45	Greenish SPSs
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	—	—	45 - 50	Greenish SPSs
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	—	—	40 - 50	Greenish SPSs
MS + 3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	58 – 65	Yellowish SPSs	35 - 40	Greenish SPSs
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	—	—	45 - 55	Yellowish SPSs
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	—	—	—	—
PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	50 – 60	Greenish SPSs	40 - 50	Greenish SPSs
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	—	—	50 - 55	Greenish SPSs
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	—	—	—	—
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	50 – 60	Greenish SPSs	42 - 48	Greenish SPSs
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	—	—	50 - 60	Yellowish SPSs
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	—	—	45 - 55	Yellowish SPSs
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	45 – 50	Greenish SPSs	40 - 45	Yellowish SPSs
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	—	—	50 - 60	Yellowish SPSs

*Based on observations recorded from 20 culture vessels for each medium.
'—' Indicates no response

Table 2: Data on the elongation of directly produced SPSs of *E. tomentosa* when grown on MS based liquid medium supplemented with different PGRs.

PGRs combination (mg/l)	Average* initial length (cm) of individual SPS (mean ± SE)	Average* length (cm) of SPS after 60d of culture (mean ± SE)	Average* increase in length (cm) of SPS (mean ± SE)
BAP + IAA			
1.5 + 0.5	1.25 ± 0.11	2.35 ± 0.31	1.10± 0.21
1.5 + 1.0	1.30 ± 0.21	2.00 ± 0.17	0.70 ± 0.10
2.0 + 0.5	1.00 ± 0.23	2.40 ± 0.21	1.40± 0.13
2.0 + 1.0	1.10 ± 0.14	3.20 ± 0.18	2.10 ± 0.24
BAP + NAA			
2.0 + 0.5	1.65 ± 0.29	2.60 ± 0.20	1.05 ± 0.23
3.0 + 1.0	1.00 ± 0.32	2.10 ± 0.22	1.10 ± 0.20
Kn + IAA			
1.5 + 0.5	1.90 ± 0.20	2.95 ± 0.11	1.05 ± 0.12
1.5 + 1.0	1.35 ± 0.35	2.85 ± 0.27	1.50 ± 0.13
2.0 + 0.5	1.87 ± 0.10	2.15 ± 0.31	0.28 ± 0.10
2.0 + 1.0	1.50 ± 0.18	2.75 ± 0.47	1.15 ± 0.16
Kn + NAA			
2.0 + 1.0	2.00 ± 0.26	2.80 ± 0.19	0.80 ± 0.14
3.0 + 1.0	1.67 ± 0.42	2.70 ± 0.52	1.03 ± 0.15

* Values are the mean of five replicates each with 20 explants.

Table 3: Data on the elongation of directly produced SPSs of *E. tomentosa* when grown on MS based agar solidified medium supplemented with different PGRs.

PGRs combination (mg/l)	Average* initial length (cm) of individual SPS (mean ± SE)	Average* length (cm) of SPS after 60d of culture (mean ± SE)	Average* increase in length (cm) of SPS (mean ± SE)
BAP + IAA			
1.5 + 0.5	1.35 ± 0.12	2.05 ± 0.25	0.70± 0.16
1.5 + 1.0	1.32 ± 0.20	2.25 ± 0.11	0.93 ± 0.19
2.0 + 0.5	1.21 ± 0.15	2.62 ± 0.20	1.21± 0.14
2.0 + 1.0	1.40 ± 0.15	2.40 ± 0.16	1.00 ± 0.25
BAP + NAA			
2.0 + 0.5	1.60 ± 0.25	2.50 ± 0.20	0.90 ± 0.21
3.0 + 1.0	1.05 ± 0.13	2.20 ± 0.21	1.15 ± 0.17
Kn + IAA			
1.5 + 0.5	1.91 ± 0.21	2.74 ± 0.12	0.83 ± 0.11
1.5 + 1.0	1.31 ± 0.30	2.82 ± 0.26	1.51 ± 0.10
2.0 + 0.5	1.88 ± 0.11	2.25 ± 0.20	0.37 ± 0.16
2.0 + 1.0	1.52 ± 0.14	2.65 ± 0.19	1.13 ± 0.18
Kn + NAA			
2.0 + 1.0	2.20 ± 0.17	2.90 ± 0.18	0.70 ± 0.12
3.0 + 1.0	1.55 ± 0.13	2.50 ± 0.16	0.95 ± 0.14

* Values are the mean of five replicates each with 20 explants.

Table 4: Effect of various growth regulators in liquid and agar solidified MS for elongation of shoot primordia

Media	Plant growth regulators	Combination of PGRs	Average increase in length (mean±SE) of SPSs
MS Liquid	BAP + IAA	2.0 +1.0	2.3 ±0.11^{c*}
	BAP + NAA	1.0+0.5	1.50±1.3 ^{a*}
	Kn + IAA	2.0+1.0	1.90±1.36^{a}
	Kn + NAA	1.0+0.5	1.83±1.6 ^{a}
MS (Agar solidified)	BAP + IAA	2.0 +1.0	0.98±1.6 ^{b}
	BAP + NAA	1.0+0.5	2.0±0.50^{c}
	Kn + IAA	2.0+1.0	1.75±1.6^{b}
	Kn + NAA	1.0+0.5	1.15±1.6 ^{a}

Statistical analysis was done by using Duncan's Multiple Range Test (DMRT) at 5% level of significance using SPSS software version 22.0. a, b, c indicates the level of significance where c is more significant than b. Same number indicates non significancy.

a. b. c. d. e.

Fig 1: Photographic representation of *in vitro* propagation of *Eria tomentosa.* A. *In vitro* culture of dust seeds of *E. tomentosa.* B. Germination of seeds in agar solidified medium. C. Development of shoot primordia like structures (SPS) in at the base of the shoots. D. Elongation of SPSs in liquid MS medium. E. Elongated SPSs *E. tomentosa* produced aerial roots.

4. Conclusion

Invention of *in vitro* propagation technique has saved many naturally growing orchids and their collection from the wild. Increasing popularity of orchids for cut flower and medicinal purpose has added new dimension to *in vitro* propagation technique through which a significant number of identical clones can be raised from a single protocorm or shoot tip explants [31, 32]. Thus methods for rapid multiplication of orchids are essential to meet the commercial demand. The present investigation established a protocol for mass propagation of shoot primordial like structures (SPSs) of the selected orchid species. Further studies should be initiated to acclimatize the orchid species in natural condition.

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