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In vitro seed germination and micropropagation of a critically endangered terrestrial orchid *Erythrodes humilis* (Blume) J. J. Smith

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Abstract
Asymbiotic cultures were raised from seeds obtained from 65-70 days old green capsules of critically endangered orchid *Erythrodes humilis* (Blume) J. J. Smith. MS basal medium was best for seeds germination than PM, KC, MVW media respectively. Sucrose was enhanced for germination as source of carbohydrate than glucose and lactose. Maximum elongation of germinated seedlings was recorded on agar solidified MS medium with 1.0 mg/l NAA + 1.0 mg/l BAP (3.50 ± 0.23cm) followed by liquid PM + 1.0 mg/l IAA + 1.0 mg/l BAP (3.34 ± 0.19cm) medium. Rhizome segments directly produced highest average number of multiple shoot buds *via* organogenesis sprouted on MS + 1.0 mg/l Pic + 2.0 mg/l BAP (6.55 ± 0.43/segment) medium followed by MS + 1.0 mg/l IAA + 2.0 mg/l BAP (6.23 ± 0.38/segment) medium. Highest mean increased of individual shoot bud length was achieved on agar solidified MS with 1.0 mg/l NAA + 1.0 mg/l BAP (3.27 ± 0.21cm) followed by liquid PM with 1.0 mg/l IAA + 1.0 mg/l BAP (3.16 ± 0.18cm) medium. MS media supplemented with 0.5 mg/l IBA was best for rooting followed by MS + 1.0 mg/l IAA. The highest number of SPSs development in liquid MS + 0.5 mg/l NAA + 1.0 mg/l BAP followed by agar solidified MS + 1.0 mg/l Pic + 1.0 mg/l BAP medium. Rooted plantlets were transferred to *ex vitro* condition after a short period of acclimatization.

Keywords: endangered; *Erythrodes humilis*; *in vitro* germination; MSBs; SPSs

1. Introduction
The Orchidaceae is one of the largest families of flowering plants represented by about 25,000-35,000 species in 600-800 genera in the world [1]. Orchids are known for the complexity and captivating beauty of their highly colourful and long-lived flowers of diverse shapes and sizes, and is considered as doyens among the ornamentally significant plants [2]. Application of tissue culture techniques has added new possibilities in plant breeding and propagation. Mass propagation of endangered species, cut flowers and medicinally important orchid in a compressed time frame are now within the realms of reality [3, 4]. Orchids represent the first horticultural crop propagated through tissue culture technique [5]. Critically endangered species immediately require *ex situ* conservation [6].
Erythrodes humilis (Blume) J. J. Smith. is a terrestrial herb with a stout, erect, leafy, glabrous stem arising from a creeping rootstock [7] and flowered in January to March [8]. It has moderate horticultural value and distributed in India, Sri Lanka and Indonesia [7].
The germination rate of orchids in nature is very slow due to lack of endosperms. *In vitro* germination of orchid seeds of many species and hybrids has been successfully established as an asymbiotic germination system [9]. More efficient approach is *in vitro* seed culture and different media compositions has significant role to initiate protocorms that develop into plantlets. During the last few years, tissue culture technique has been extensively exploited for the large scale propagation as well as *ex situ* conservation of many orchids [10-12].
Micropropagation of orchid could be done with the use of aseptically grown *in vitro* seedlings. In fact, the discovery of tissue culture techniques added a new dimension to the production of quality plants in large quantities and propagation of exquisite and rare orchids [13-16]. However, very little work has been done of this species. Therefore, in the present study was undertaken to develop effective *in vitro* germination, micropropagation based rhizome culture technique and establishment of SPSs development protocol for the conservation of *E. humilis* orchid.

2. Materials and Methods
The green mature 65-70 days old capsules of *Erythrodes humilis* were collected from Sylhet, Bangladesh and washed under tap water to remove dust particles. After that, added few drops of teepol solution for few min and washed under running tap water for 10 min. The capsule was surface sterilized by immersing 0.1 % (w/v) $HgCl_2$ for 10 min, 70% (v/v) ethanol for 30 sec and finally by washed three times with sterile distilled water.
Full strength KC [17], MS [18], MVW [19] and PM [20] basal media with different carbohydrates sources sucrose, lactose, glucose were used for *in vitro* seed germination. MS and PM based eighteen types of (solid and liquid condition) elongation media with different concentrations and combinations of PGRs were prepared. In solid media 0.8% (w/v) agar was used as gelling agent but no agar was added in liquid media. MS based sixteen types of micropropagation media were prepared using with different concentrations and combinations of PGRs. Half strength MS and nine types of full strength auxin supplemented MS media were used for well-developed rooting. Plant Growth Regulators (PGRs) *viz*. Kn, BAP, Pic, NAA, IAA and IBA are freshly prepared. The pH of all media was adjusted to 5.8 with 0.1N NaOH or HCl before autoclaving. Agar was dissolved by boiling the mixture and about 50-100 ml media was dispensed into each culture vessel and autoclaved at 121ºC for 20 min/15 lb. Culture was maintained in the

growth room at 14/10h continuous light and dark conditions at 25±2 ºC. The cultures were monitored regularly and subcultures every 4 wks interval.

For the inoculation of seeds, 65-70 days old green capsule was cut opened longitudinally and the seeds were scooped out with the help of forceps and spread over the surface of the germination media. The seeds were allowed to germinate and differentiate into protocorms and seedling development. Rhizome explant were excised from *in vitro* grown seedlings and cultured on the PGRs supplemented agar solidified MS medium. Rhizome segments produced directly multiple shoot buds (MSBs) *via* organogenesis and subcultured on the elongation media; and thereafter in rooting media for induction of well-developed root system. SPSs were produced at the base of some culture vessels and subcultured on elongation and rooting media respectively. 0.8% (w/v) agar solidified half strength MS0 and auxin supplemented (IAA, IBA, NAA) nine different MS media were used for induction of strong and stout root system. The efficiency of the media in terms of enhancing the development of root system was assessed based on the increase in number and length of roots that developed within 30d of culture in rooting media.

Rooted *in vitro* developed seedlings were taken out of the culture vessel and transferred to outside the culture room by successive phases of acclimatization. The seedlings of *E. humulis* were transferred to pots containing soil, sand, pit moss, saw dust and charcoal. Transplanted seedlings were watered regularly for about 2-3 months.

3. Results and Discussions

The seeds of *Erythrodes humilis* were aseptically grown on 0.8% (w/v) agar solidified KC, MS, MVW and PM media with three different sources of carbohydrates *viz.* sucrose, glucose and lactose. Overall results proved that MS (Fig. 1a) was superior to PM (Fig. 1b), KC, MVW media in respect of required time and the percentage of germination. The germination was significantly poor on lactose containing MVW medium. Each plant species has specific nutritional requirements, for its seed germination and plant regeneration. Similar result was also found in *Calanthe densiflora* [16]; *Cymbidium* [21]; *Satyrium nepalense* [22] and *Gastrochilus calceolaris* [23] orchid species. MS media is enriched with macronutrients, micronutrients and vitamins which are enhanced for seed germination and seedling development of many orchids [24-25]. Carbon source has also great role for *in vitro* orchid seed germination. Sugar is an important component used in tissue culture studies. Among three carbon sources, the percentage of seed germination was higher in sucrose containing medium than glucose and lactose containing media respectively.

Germinated seedlings were transferred to 0.8% (w/v) agar solidified & liquid MS and PM elongation media supplemented with diverse concentrations and combinations of PGRs (BAP, Kn, NAA, IAA, IBA and Pic) elongation media for continued growth (Table-1). The efficiency of a medium in terms of enhancing shoot elongation was determined based on the increase in length of shoot system within 30d of culture. The highest rate of elongation, mean increased seedlings length developed in 0.8% (w/v) agar solidified MS medium with 1.0 mg/l NAA + 1.0 mg/l BAP (3.50 ± 0.23 cm; Fig. 1c) followed by liquid PM medium supplemented with 1.0 mg/l IAA + 1.0 mg/l BAP (3.34 ± 0.19 cm; Fig. 1d). Comparison of the results of solid and liquid media revealed that agar solidified culture was better than liquid condition and MS better than PM for enhancing elongation of shoot system of the seedlings [26, 28].

In vitro derived rhizome explant [29, 30] was cultured on 0.8% (w/v) agar solidified MS media supplemented with different combinations and concentrations of PGRs and produced multiple shoot buds *via* direct organogenesis (Table-2). The rhizome segments of *E. humilis* underwent direct organogenesis and maximum number of shoot buds were sprouted on 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l Pic + 2.0 mg/l BAP (6.55 ± 0.43/segment; Fig. 1e) followed by that on MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 2.0 mg/l BAP (6.23 ± 0.38/segment). Similarly, different explants have been investigated for the clonal mass propagation of many orchid species [10, 31, 32].

Table 1: Mean increased in length (cm) and SPSs development per seed originated and shoot bud originated seedlings of *Erythrodes humilis* on 0.8% (w/v) agar solidified and liquid media with different kinds of PGRs.

Culture medium with different combinations and concentrations of PGRs	Solid media			Liquid media		
	Mean increased seedlings length (cm) after 30d of culture ± SE	Time required (days) for SPSs production and SPSs colour	Mean increased individual shoot bud length (cm) after 30d of culture ± SE	Mean increased seedlings length (cm) after 30d of culture ± SE	Time required (days) for SPSs production and SPSs colour	Mean increased individual shoot bud length (cm) after 30d of culture ± SE
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	2.45 ± 0.21	—	1.89 ± 0.24	2.84 ± 0.19	—	1.86 ± 0.20
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	2.98 ± 0.18	Yellowish SPSs	2.73 ± 0.21	2.89 ± 0.22	Yellowish SPSs	2.61 ± 0.22
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	3.32 ± 0.25	—	3.06 ± 0.17	3.13 ± 0.15	—	3.01 ± 0.18
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	2.91 ± 0.13	—	2.09 ± 0.14	3.04 ± 0.21	—	2.03 ± 0.23
MS + 3% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	3.05 ± 0.19	Greenish SPSs	2.94 ± 0.19	3.01 ± 0.26	Greenish SPSs	2.87 ± 0.22
MS + 3% (w/v) sucrose	3.50 ± 0.23	—	3.27 ± 0.21	3.27 ± 0.24	Greenish	3.11 ± 0.19

+ 1.0 mg/l NAA + 1.0 mg/l BAP					SPSs	
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	2.84 ± 0.27	—	2.11 ± 0.27	3.12 ± 0.27	—	1.96 ± 0.25
MS + 3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	3.02 ± 0.24	Greenish SPSs	2.67 ± 0.24	3.22 ± 0.21	Greenish SPSs	2.42 ± 0.23
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	3.27 ± 0.19	Greenish SPSs	3.18 ± 0.25	3.24 ± 0.18	Greenish SPSs	2.98 ± 0.21
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	2.87 ± 0.23	—	2.13 ± 0.23	3.19 ± 0.16	—	2.06 ± 0.18
PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	2.93 ± 0.19	Yellowish SPSs	2.73 ± 0.18	3.28 ± 0.13	Yellowish SPSs	2.78 ± 0.17
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	3.27 ± 0.21	—	3.09 ± 0.14	3.34 ± 0.19	Greenish SPSs	3.16 ± 0.18
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	3.06 ± 0.17	—	2.07 ± 0.13	2.95 ± 0.22	—	1.95 ± 0.23
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	3.23 ± 0.15	Greenish SPSs	2.58 ± 0.20	3.08 ± 0.21	Greenish SPSs	2.73 ± 0.22
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	3.46 ± 0.21	—	3.15 ± 0.19	3.21 ± 0.23	Greenish SPSs	3.12 ± 0.26
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	3.13 ± 0.25	—	2.14 ± 0.23	3.06 ± 0.25	—	2.06 ± 0.28
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	3.28 ± 0.22	Greenish SPSs	2.72 ± 0.21	3.14 ± 0.23	Greenish SPSs	2.71 ± 0.21
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	3.34 ± 0.20	—	3.21 ± 0.17	3.25 ± 0.19	Greenish SPSs	3.05 ± 0.22

Shoot length recorded from 50 seedlings/shoot bud taking 5 at random from each of 10 culture vessels; '—' Indicates no response.

Fig 1: *In vitro* seed germination, elongation, micropropagation. SPSs development, rooting and acclimatization of *Erythrodes humilis*

Auxins and cytokinins supplemented elongation media were effective for SPSs development at the base of the seedlings and liquid media were more effective than agar solidified condition (Table-1). The highest number of SPSs developed in liquid MS + 0.5 mg/l NAA + 1.0 mg/l BAP (Fig. 1f) followed by agar solidified MS + 1.0 mg/l Pic + 1.0 mg/l BAP. Most of the SPSs were greenish and few were yellowish in colour. MS based media was better than PM for induction of SPSs [33-34]. The MSBs further grown individually on elongation media (Table-1) and highest mean increased individual shoot bud length was achieved on agar solidified MS + 1.0 mg/l NAA + 1.0 mg/l BAP (3.27 ± 0.21 cm; Fig. 1g) followed by liquid PM + 1.0 mg/l IAA + 1.0 mg/l BAP medium (3.16 ± 0.18 cm).

The elongated multiple hoot buds and seed originated seedlings produced roots in elongation media but those were weak and few in number. Half strength MS0 and nine different types of PGRs (IAA, IBA, NAA) supplemented MS media were used for induction of strong and stout root system (Table-3). Increased in length as well as the number of roots developed seed originated and shoot bud derived seedlings were more on MS medium supplemented with 0.5 mg/l IBA containing rooting medium (Fig. 1h) followed by MS medium with 1.0 mg/l IAA. Similar result was found in *Dendrobium* orchid, *Cymbidium finlaysonianum* and *Ilex khasiana* respectively [35, 37]. IAA was effective for rooting in *Dendrobium* hybrid and *Dendrobium thyrsiflorum* respectively [38, 39]. The opposite result was also noted that NAA was most appropriate in inducing roots in *Coelogyne fuscescens* and *Vanda tessellata* [40, 41]. Combine effect of IAA, IBA or NAA induced excellent rooting response in *Vanda tessellata* and *Aerides ringens* [42, 43] orchid species.

Well rooted *in vitro* derived seedlings and micropropagated plantlets were acclimatized at room temperature. The seedlings of *E. humulis* were transferred to pots containing soil, sand, pit moss, saw dust and charcoal (Fig. 1i). Rooted plantlets were able to grow into normal plantlets in shade house and transplanted seedlings were watered regularly for about 2-3 months.

Table 2: Development of multiple shoot buds from rhizome explant of *E. humilis* when grown on 0.8% (w/v) agar solidified MS media supplemented with different PGRs.

Combinations and concentrations of PGRs	% of induced multiple shoot buds/ segment	Time (d) required for sprouting of multiple shoot buds	Number of multiple shoot buds/ segment (Mean ± S.E.)
0.5 mg/l IAA + 1.0 mg/l BAP	60	32 – 36	5.14 ± 0.30
0.5 mg/l IAA + 1.0 mg/l Kn	45	36 - 40	3.25 ± 0.23
1.0 mg/l IAA + 2.0 mg/l BAP	70	28 - 32	6.23 ± 0.38
1.0 mg/l IAA + 2.0 mg/l Kn	55	34 - 38	4.56 ± 0.30

0.5 mg/l IBA + 1.0 mg/l BAP	50	34 - 38	4.22 ± 0.34
0.5 mg/l IBA + 1.0 mg/l Kn	45	35 - 39	3.36 ± 0.19
1.0 mg/l IBA + 2.0 mg/l BAP	60	30 - 34	5.09 ± 0.39
1.0 mg/l IBA + 2.0 mg/l Kn	55	35 - 39	4.48 ± 0.24
0.5 mg/l NAA + 1.0 mg/l BAP	60	32 - 36	5.23 ± 0.37
0.5 mg/l NAA + 1.0 mg/l Kn	55	34 - 38	4.57 ± 0.28
1.0 mg/l NAA + 2.0 mg/l BAP	65	30 - 34	5.79 ± 0.41
1.0 mg/l NAA + 2.0 mg/l Kn	55	34 - 38	4.65 ± 0.27
0.5 mg/l Pic + 1.0 mg/l BAP	50	36 - 40	4.28 ± 0.32
0.5 mg/l Pic + 1.0 mg/l Kn	45	36 - 40	3.31 ± 0.24
1.0 mg/l Pic + 2.0 mg/l BAP	75	30 - 34	6.55 ± 0.43
1.0 mg/l Pic + 2.0 mg/l Kn	55	34 - 38	4.62 ± 0.28

Based on observations recorded from 10 cultured segments in each medium.

Table 3: Mean increased in length (cm) and number of roots per seed originated and shoot bud originated seedlings of *E. humilis* in ½ MS0 and auxin supplemented MS rooting media.

Culture medium			Average increased length and number of roots per seed derived seedling		Average increased length and number of roots per shoot bud derived seedling	
			Mean length (cm) ± S.E.	Mean no. of roots/ seedling ± S.E.	Mean length (cm) ± S.E.	Mean no. of roots/ shoot bud ± S.E.
½ MS0			3.57 ± 0.27	2.27 ± 0.12	3.28 ± 0.25	2.21 ± 0.12
Auxin (mg/l)	IAA	0.5	3.66 ± 0.22	2.28 ± 0.14	3.22 ± 0.18	2.15 ± 0.15
		1.0	3.71 ± 0.24	2.34 ± 0.15	3.45 ± 0.21	2.27 ± 0.14
		1.5	3.52 ± 0.25	1.96 ± 0.11	3.14 ± 0.22	1.85 ± 0.10
	IBA	0.5	3.86 ± 0.22	2.53 ± 0.18	3.52 ± 0.20	2.42 ± 0.16
		1.0	3.62 ± 0.20	2.41 ± 0.18	3.41 ± 0.18	2.27 ± 0.17
		1.5	3.45 ± 0.21	2.36 ± 0.16	3.26 ± 0.19	1.94 ± 0.11
	NAA	0.5	3.41 ± 0.23	2.22 ± 0.14	3.25 ± 0.20	2.16 ± 0.13
		1.0	3.63 ± 0.27	2.03 ± 0.11	3.38 ± 0.22	2.01 ± 0.14
		1.5	2.87 ± 0.20	1.89 ± 0.13	3.14 ± 0.22	1.65 ± 0.12

Based on observations from 50 seedlings/ shoot buds taking five at random from each of ten culture vessels.

4. Conclusions

MS media was found superior than PM, KC, MVW media respectively and sucrose supplemented media was better than glucose & lactose for promoting germination of this orchid seeds. 2% lactose supplemented MVW medium was found to be ineffective than others for germination. For comparing the efficiency in terms of enhancing seedling, solid culture was better in terms of promoting shoot elongation than liquid condition. Increased in root length and number of roots is higher in IBA supplemented MS medium than IAA, NAA respectively for induction of well-developed root system. *Ex situ* conservation by tissue culture technique of this species is highly suggested. However, the techniques offer tremendous potential in germination, micropropagation and germplasm conservation in their natural habitat.

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