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In vitro propagation of an epiphytic and rare orchid *Eria bambusifolia* Lindl.

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Orchids seeds are minute, unique lacking storage tissues and they are marveled for their mystic shapes and colour for centuries. Asymbiotic seed germination opened up new avenues in rescuing of orchids from their wild population. In the present study, an epiphytic and rare orchid *Eria bambusifolia* seeds were germinated in two different Knudson (KC) and Murashige and Skoog's (MS) medium. Higher germination percentage was noted in MS medium so it was selected for further studies. The MS medium was nourished with different phytohormones and NAA at 2 mg/l was found to enhance shoot and root length. The rooted seedlings were acclimatized successfully.

Keywords : culture media, *Eria bambusifolia*, phytohormones, protocorms, seed germination

Orchidaceae form one of the world's largest families of flowering plants of angiosperms and have been marveled over centuries. The seeds of orchids are unique, minute lacking storage tissues, which are required for the germination of seeds and development of seedlings. Unsuitable substrate and adverse physical conditions are challenges to any viable seed, but orchid seeds have the additional problem of locating a compatible mycobiont. The great seed production in orchids suggests that the mortality of seeds and seedlings is exceedingly large (Rasmussen, 2002). The major cause of its decline is being the uncontrolled collection in the wild sites. The reduction of natural habitats for orchid growth requires special method for species conservation. Tissue culture techniques can be tremendous promise in improving their quality and yield. Inherently they are slow growing plants, the traditional propagation methods hardly fulfill the need of the market. The legendary introduction of Knudson C medium (1922) for the development of asymbiotic seed cultures has opened new possibilities for analyzing germination behaviour, fungal availability and natural substrates as they affect orchid requirements.

The genus *Eria* is a large and comprises 550 species of which 53 species are reported in India. *Eria bambusifolia* is an epiphytic orchid distributed in Orissa, Tropical Sikkim Himalaya, Meghalaya, Khasi Hills at an altitude of 1000-1300 m. The species has undergone a drastic decline in its natural population and categorized as rare species in Orissa. However, no report on *in vitro* studies of this orchid has been emphazised. The present study examines the suitability for the seed germination and seedling development of the epiphytic orchid *Eria bambusifolia in vitro*.

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Materials and Methods

Undehised capsules of *Eria bambusifolia* were washed thoroughly with running tap water and then with the detergent teepol (0.1%). They were surface sterilized with mercuric chloride solution (0.1%) for 3 minutes and were subsequently rinsed in sterilized double distilled water. The capsules were dipped in 80% ethyl alcohol for a minute and flamed. The sterilized capsules were cut longitudinally with the help of a sharp sterilized surgical blade for extracting seeds and were inoculated on two different basal media under aseptic conditions.

Knudson C (1946) and Murashige and Skoog (1962) media were tested initially to find out the suitable medium for seed germination. Among the two media tried, the best medium for germination was selected and supplemented with different phytohormones like auxins (IAA and NAA), cytokinins (BA and KN), gibberellin (GA_3) individually at various concentrations (0.5, 1.0 and 2.0 mg/l). The pH of the medium was adjusted to 5.0 -5.8 before gelling with agar. Molten medium was dispensed into test tubes and covered with aluminum foil. The tubes were autoclaved at 121lb^2/ for 121°C for 20 minutes. Cultures were maintained at 25 + 2°C, with 12 hours photoperiod provided by cool-white fluorescent tubes (Phillips, India).

Bursting of the seed coat and emergence of the enlarged embryos, *i.e.,* the protocorm was considered as germination. The germination of seeds was recorded and percentage of seed germination was calculated. Five samples of the seeds were taken out after 8 and 13 weeks at random and the slides were prepared for observations by placing a drop of glycerin and covering it with a cover slip. The seeds were scooped out and scrutinized randomly in a petriplate and observed under the microscope. The seeds were classified as germinated / ungerminated for calculating germination percentage. The percentage of germination is calculated by counting the total number of seeds germinated with that of total number of seeds observed. The seedlings were measured and datas were recorded.

The fully developed healthy plantlets were removed from the culture flasks and the plantlets were thoroughly washed in running tap water to remove the adhering nutrient medium completely without causing damage to the roots. Then the plantlets were treated with (0.5%) fungicide (Roko) and transferred to flasks filled with various types of media like Coconut husk + charcoal + brick pieces + broken tiles + perlite in the ratio of 2:1:1:1:1.

Results and Discussion

Orchids are experiencing a steady decline in tropical countries due to destruction of natural forest areas. It is essential to take measures for the conservation and propagation of endangered orchid species (Sheelavantmath *et al*., 2000). Orchid seed germination is unique and it is remarkable when compared with other flowering plants. Arditti and Ernst (1984) reported that orchid seeds could germinate on a wide variety of inorganic salt combinations and ion concentrations, but in general a balanced mineral mixture and medium concentration is an important factor for every orchid species. In the present study, the effect of different media on seed germination are represented in table 1. The process of seed germination was initiated after 4 weeks of culture. The seeds showed swelling and gradually the embryos emerged out within 7-9 weeks. The greening and germination of the seeds commenced first in MS. The present findings shown that the germination of seeds occurred in both the medium KC and MS.

Table 1. Germination response of *E. bambusifolia* seeds on MS and KC media

S. No.	Media	Percentage of Germination (%)	Time taken (weeks) for the development of		Response
			Greening	Protocorms	
1.	MS	48	8	7	+++++
2.	KC	36	10	9	+++

+++++ Best +++ moderate

Table 2. Effect of media on the development of protocorms of *E. bambusifolia*

S. No.	Media	Weeks	Mean protocorm length (µm)	Mean rhizoid number
1.	MS	8	510 ± 94.551	3.8 ± 0.374
2.	KC	8	312 ± 35.693	6.2 ± 0.734
3.	MS	13	1536 ± 70.823	11.4 ± 0.871
4.	KC	13	1204 ± 56.082	14.4 ± 0.400

Values are means (n=5) ± S.E.

Table 3. Effect of different growth regulators on the seedling growth in *E. bambusifolia*

S. No.	**Medium + GR**	**Concentration of GR (mg/l)**	**Mean shoot length (cm)**	**Mean multiple protocorms**	**Mean root number**	**Mean root length (cm)**
1.	MS	Basal	0.48 ^{a}	-	-	-
2.	NAA	0.5	0.68 ^{ab}	-	-	-
3.	NAA	1.0	1.08 ^{de}	-	1.2 ^{a}	0.16 ^{a}
4.	NAA	2.0	2.0 ^{g}	-	1.4 ^{ab}	0.32 ^{a}
5.	IAA	0.5	0.38 ^{a}	-	-	-
6.	IAA	1.0	0.70 ^{abc}	1.8 ^{a}	2.2 ^{bc}	0.24 ^{a}
7.	IAA	2.0	1.04 $^{b-e}$	2.0 ^{a}	2.4 ^{c}	0.32 ^{a}
8.	BA	0.5	0.56 ^{a}	-	-	-
9.	BA	1.0	1.06 ^{cde}	2.6 ^{ab}	-	-
10.	BA	2.0	1.36 ^{ef}	3.0 ^{b}	-	-
11.	KN	0.5	0.58 ^{a}	-	-	-
12.	KN	1.0	0.98 ^{bcd}	-	-	-
13.	KN	2.0	1.0 $^{b-e}$	-	1.2 ^{a}	0.16 ^{a}
14.	GA_3	0.5	0.42 ^{a}	-	-	-
15.	GA_3	1.0	0.94 ^{bcd}	-	-	-
16.	GA_3	2.0	1.50 ^{f}	-	-	-

Means followed by a common letter are not significantly different at 5 % level by DMRT

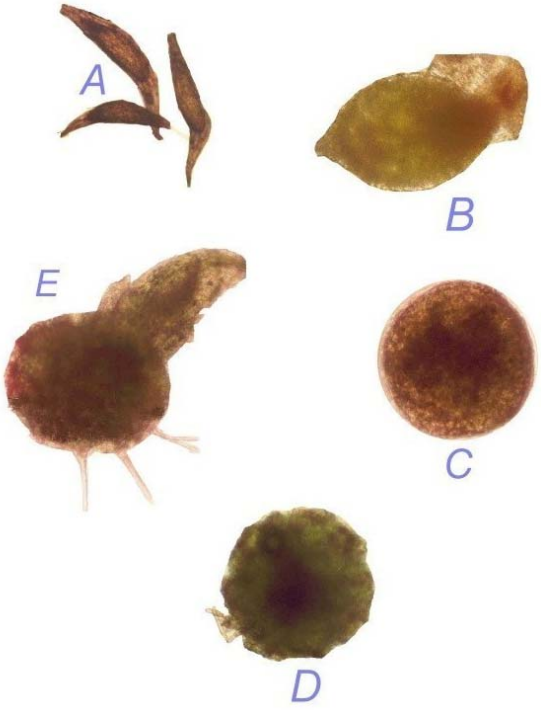

Fig. 1. A - E Different Stages in *Eria bambusifolia* seed germination

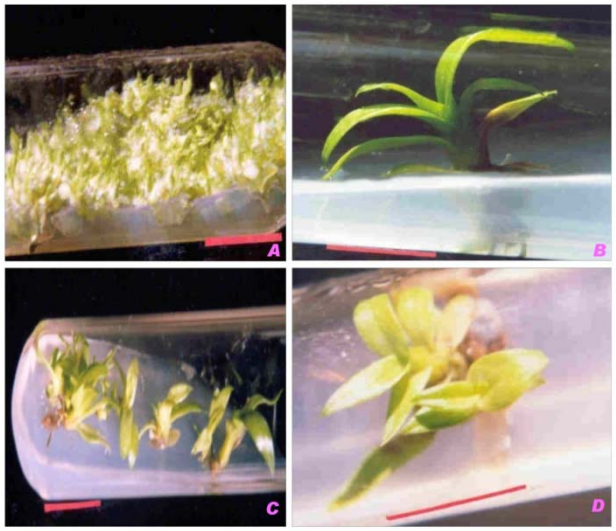

Fig. 2. A. Seedlings on MS medium; B. Seedlings developed on MS supplemented with NAA (2mg/l); C. Formation of roots in IAA incorporated MS medium; D. Multiple shoots development on NAA nourished MS medium

Initiation of germination, protocorm formation and subsequent growth and development of seedlings varied with the species and the medium employed (Reddy *et al*., 1992). A significant difference in the protocorm length and rhizoid number was noted in MS and KC media. During 8th week of culture the protocorms measured about 312 and 510 µm in KC and MS media respectively whereas, the rhizoid number was found to be higher in KC than in MS media (Table 2). On the 13th week the length of protocorms were found to be best in MS medium (fig.1). The rhizoids were short, unicellular, tubular and stout in MS, whereas, they were slender and hair like in KC medium. The shoot apex was formed first and later rhizoids were formed from the basal portion of the protocorms. However, the percentage of germination was 48 and 36% in MS and KC media respectively. Germination of seed is a prelude to protocorm formation. Some of the epidermal cells of the protocorm develop rhizoids, which may be confined only to the basal regions of the protocorm as in *Calopogon, Dendrobium, Spathoglottis* and *Laeliocattleya*, or may cover the whole protocorm except the meristem region, as found in *Vanilla* (Mitra, 1986) and *Dendrobium Lindleyi* (Kaur and Sarma, 1997). The delay in commencement of germination of seeds could be attributed to the fact that the inorganic ions present in the medium. The presence of inorganic ions in the MS media could be more effective in early germination, production of protocorms and development of seedlings. Based on these observation MS medium was selected for the further studies.

Additional use of growth adjuncts in the media selectively accentuated the seedling growth. The most important development in the culture media was the incorporation of growth regulators like auxins, cytokinins and gibberellins. Growth hormones depending on type and

concentration both inhibit and promote seed germination in orchids (Arditti *et al*., 1981). The protocorms increase in size and differentiated leaf at the shoot apex. The auxin NAA (2.0 mg/l) significantly enhanced the shoot and root length (Table 3). The beneficial effects of NAA were in line with the results of several orchids as reported by Chung and Chun (1983). NAA stimulated the seedlings in *Arundina graminifolia* (Kaur and Sarma, 1997) whereas, in *Paphiopedilum spicerianum* and *Dendrobium chrysanthamum* auxins inhibited the growth of shoots and roots (Kano, 1965) and Miyazaki and Nagamatsu (1965).

The cytokinin, BA (2.0 mg/l) proved to be beneficial for production of multiple protocorms but failed to produce roots at all concentrations. KN and GA_3 were found to be ineffective in the production of multiple shoots. Similar reports on GA_3 were obtained by Arditti and Ernst (1984). It seems that either orchid seedling synthesizes the required quantity of gibberellic acid and or the orchid seeds / seedlings have limited the ability to deactivate gibberellic acid.

In *E. bambusifolia,* in addition to the browning and death of protocorms extensive browning of the medium was also observed. The relative abundance of phenolics in *E. bambusifolia* and the negative effect of the phenolic oxidates on growth and differentiation of tissues in culture as evidenced from extensive browning of the medium and loss of cultures have been widely reported (Tanaka *et al*., 1988; Ernst, 1985). Excessive phenolic production is possibly due to increased polyphenol oxidase and catalase activity triggered by certain cultural conditions (Harvais, 1982). Phenolic exudations from the seedlings were observed in all the treatments. Browning of the medium, followed by seedling death and blackening of the leaf tips was common in all the cultures, which affected the survival rate of the plantlets. The regenerated plants of *E. bambusifolia* showed maximum survival of 60% on the potting medium containing coconut husk, charcoal, brick pieces, broken tiles and perlite (2:1:1:1:1). The above protocol can be used for large scale production of this orchid species.

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