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In vitro Seed Germination and Seedling Development of *Esmeralda clarkei* Rchb.f. (Orchidaceae)

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Abstract

Esmeralda clarkei Rchb.f. (Orchidaceae), an epiphytic orchid native to Nepal with high ornamental value grows from cool to intermediate climatic conditions with medium amount of light. In an attempt to preserve this important orchid by establishing an efficient *in vitro* regeneration protocol using seed culture was conducted. MS with BAP and NAA alone or in combination were used. In MS protocorms developed within 16 weeks followed by seedling development in 25 weeks of seed culture. Presence of BAP (0.5 mg/l) in MS improved seed germination by protocorm formation and seedling development in 17 and 26 weeks of seed culture, respectively. In 0.5 mg/l NAA supplemented media, development of protocorms was observed in 18 weeks and seedling growth was observed in 26 weeks of seed culture. Protocorm and seedling development were ~~was~~ delayed in other media.

Introduction

Orchid seeds are characterized by lack of storage tissues required for seed germination and seedling development. In nature, association with a specific fungal partner is a pre-requisite for orchid seed germination (Mitra 1986). However, the rate of vegetative propagation is very slow and the seed germination in nature is very poor, i.e, 0.2 - 0.5% (Vij 2002). *In vitro* germination of seeds is an important aspect in the orchid multiplication and conservation program since the dust like tiny seeds have the capability of developing into complete seedling**s** without any fungal aid. Knudson (1922) demonstrated the possibility of by-passing the fungal requirement of orchid seeds during *in vitro* germination and since then non-symbiotic seed germination has been accepted as an important tool for propagating orchids (Arditti et al. 1982).

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E. *clarkei* is an epiphytic orchid native to Nepal with a high ornamental value grows on *Rhododendron* species from cool to mild climatic conditions with medium amount of light. Plants bloom from fall to winter with three to four fragrant flowers (Fig. 1) (Ghimire 2008). The species is under threat due to removal of their natural habitat for 'Slash and Burn' cultivation, unplanned developmental activities and deforestation. No information is available on the seed germination and protocorm development of *E. clarkei*. In this paper, authors have developed an efficient seed germination protocol for *E. clarkei* that facilitates protocorm development and *in vitro* seedling development.

Materials and Methods

The green healthy capsule of ***E.*** *clarkei* (Orchidaceae) collected from the natural plant grown in primary forest of Gaurishankar Conservation Area, Lamabagar V.D.C.-9, Chhetchhet (alt. 1330, lat. $27^0$52.713', long. $86^0$13.263'), Dolakha, eastern Nepal was used.

Capsule of *E. clarkei* was dipped in water containing a few drops of teepol solution. Then, they were washed with running tap water for 30 - 40 min and thereafter rinsed with distilled water. The pod was then wrapped with cotton, dipped in 70% ethyl alcohol for 1 min and surface sterilized in 1% sodium hypochlorite solution for 10 min. Finally they were rinsed 3 - 5 times with sterile water.

MS was used as the basal medium either alone or supplemented with different concentrations and combinations of NAA and BAP for the inoculation of seeds. Medium was adjusted to pH 5.8 before autoclaving and solidified with 0.8% w/v Difco Bacto Agar. About 20 ml medium per culture tube was dispensed into culture tubes (large sized capacity of 80 ml) and each tube was tightly covered with aluminum foil. The culture tubes containing the medium were autoclaved at 121^0C and pressure of 15 lb/sq inch for 20 min. After cooling down, the tubes were taken out and kept in slanting position in the culture room.

The surface sterilized orchid capsule was then transferred into the laminar air flow cabinet and dissected longitudinally by using sterile scalpel. Immature orchid seeds in cluster were inoculated on the surface of MS alone and in combination with NAA (0.5 mg/l) and BAP (0.5 - 2.0 mg/l) using sterile forceps. The cultures were incubated at 25 ± 2ºC under the photoperiod of 16 hrs.

Results and Discussion

Immature seeds of *E. clarkei* showed varied response depending upon different plant hormones employed at varied concentrations of BAP (0.5 - 2.0 mg/l) and

NAA (0.5 mg/l) either individually and in combinations. In most of the tested media, germination of seeds was seen within 7 to 9 weeks, protocorms development within 16 to 20 weeks followed by shoots formation in 25 to 27 weeks (Table 1). In MS without plant hormones protocorms developed within 16 weeks of seed culture (Fig. 2) giving rise to seedlings after 25 weeks of culture. Similar results were reported in *Habenaria macroceratits* (Stewart and Kane 2006), *Eulophia alta* (Johnson et al. 2007). The protocorms were round, oval, elongated bodies considered as an intermediate structure between the embryos and the plants. These protocorms underwent further morphogenetic changes resulting in the development of leaf primordia and finally to the seedlings in 25 weeks in MS basal medium. Nayak et al. (1998) reported regeneration of *Cymbidium aloifolium* by using rhizomes that developed from the immature seeds when grown on MS medium supplemented with different growth regulators.

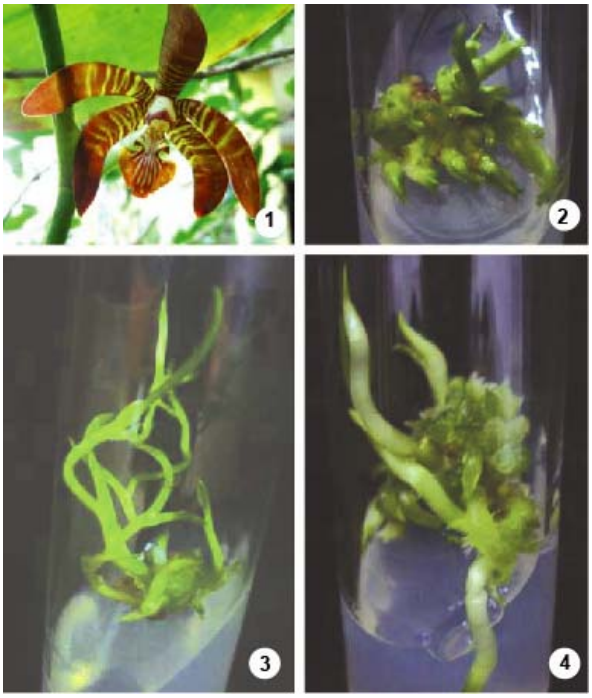
Table 1. Effect of BAP and NAA on *in vitro* seeds germination and seedling development of *E. clarkei*.

Concentrations		Germination initiation in weeks (Mean ± SE)	Protocorms formation in weeks (Mean ± SE)	Seedling development in weeks (Mean ± SE)
BAP	NAA			
0	0	7.25 ± 0.47^{a}	16.00 ± 0.40^{a}	25.50 ± 0.64^{a}
0.5	0	7.75 ± 0.47^{ab}	17.50 ± 0.65^{b}	25.75 ± 0.75^{ab}
1.0	0	8.25 ± 0.47^{b}	18.75 ± 0.47^{c}	26.50 ± 0.64^{b}
1.5	0	8.25 ± 0.47^{b}	19.51 ± 0.64^{d}	26.75 ± 0.47^{b}
2.0	0	9.00 ± 0.40^{c}	19.75 ± 0.47^{d}	26.00 ± 0.40^{b}
0	0.5	7.25 ± 0.25^{a}	18.00 ± 0.70^{bc}	26.25 ± 0.47^{b}
0.5	0.5	8.25 ± 0.47^{b}	17.75 ± 0.47^{b}	25.75 ± 0.25^{ab}
1.0	0.5	8.00 ± 0.40^{b}	19.00 ± 0.40^{d}	25.25 ± 0.47^{a}
1.5	0.5	8.25 ± 0.47^{b}	19.00 ± 0.91^{d}	25.50 ± 0.64^{a}
2.0	0.5	8.25 ± 0.47^{b}	19.75 ± 0.75^{d}	26.75 ± 0.62^{b}

Culture conditions: MS, 25 ± 2ºC, 27 weeks of culture, 16 hrs photoperiod and six replicates were used in each combination. The values with the same superscript are not significantly different at $p \leq 0.05$.

The *in vitro* sown seeds developed into protocorms within 18 weeks of culture in 0.5 mg/l NAA containing medium and are differentiated into seedlings after 26 weeks (Fig. 3). Similar findings were also reported in previous studies on seeds germination of *Aerides odorata* (Pant and Gurung 2005), seed germination of *Cymbidium* sp., *Dendrobium noblile* and *D. primulinum* (Luan et al. 2006). This result is different from that reported for germination of *Phaius tankervilliae* seeds (Luan et al. 2006).

In MS supplemented with 0.5 mg/l of BAP profuse development of protocorms was observed within the 17 weeks followed by seedling development within 26 weeks of seeds culture as compared to other tested concentrations of BAP (Fig. 4). The results reported here are in agreement with those of *Epidendrum ibaguense* (Hossain 2008) and *Phaius tancarvilleae* (Pant et al. 2011).



Figs 1-4: 1. A flower of *E. clarkei*. 2. Protocorms developed on MS.3. Seedlings developed on 0.5 mg/l NAA. 4. Seedlings developed on 0.5 mg/l BAP.

In media supplemented with 1.0 - 2.0 BAP alone or fortified with a combination of 0.5 - 2.0 mg/l of BAP and NAA 0.5 mg/l, delayed response both for the development of protocorms and seedlings from seeds was observed as compared to the other tested media.

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