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Rapid *in vitro* Multiplication of *Eulophia Cullenii* (Wight) Blume - a Rare, Endemic and Exquisite Orchid of Southern Western Ghats

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Abstract

As part of a conservation programme to multiply Western Ghats orchids, green pod culture of *Eulophia cullenii*, a rare, endemic, exquisite and therapeutically important orchid of Western Ghats was attempted. Green pod culture of *E. cullenii*, was tested in three different culture media viz. Mitra *et al*., Knudson C, and Wimber media. Mature seeds released from a three month old the green pod of *E. cullenii* were cultured in the above mentioned liquid nutrient media supplemented with different organic additives like casein acid hydrolysate (CH), peptone (P), yeast extract (YE) and coconut water (CW). Mitra nutrient medium supplemented with 0.05% CH (w/v) supported highest percentage (70%) seed germination. CW was found be inhibitory for seed germination in all the media tried. Protocorm after 60 days in liquid medium were transferred to solid Mitra medium containing same additives. Protocorm proliferation was noticed in Mitra basal and organic supplemented medium and maximum of an average of 15 daughter protocorms within 8 weeks of culture in solid basal Mitra medium. Proliferated protocorm when transferred to Mitra basal medium within 8 weeks leafy shoots with root initials and initiation of *in vitro* rhizome were obtained. These seedling again transferred to fresh basal medium healthy seedling with *in vitro* derived rhizome were formed within 8 weeks of culture. After the third subculture in solid medium, the healthy rooted seedlings with *in vitro* developed rhizome obtained were transferred to community pots containing sand and farmyard manure (1:1) and 79% survival rate was obtained after one year of establishment.

Keywords: Green capsule, Embryo culture, micropropagation, protocorm,

Orchids constitute a biologically interesting and commercially significant groups of plants represent the largest flowering plant family with nearly 30,000 species in 800 genera; about 1300 species occur in India. Of the nearly 1300 species of orchids occurring in India, at least 200 are considered horticulturally important (Lokesha and Vasudeva, 1992). Besides its horticultural values, they are used in traditional system of medicine as a remedy for number of ailments (Senthilkumar, 2004). Orchids are one of the most pampered plants and occupy top positions among all the flowering plants. Species orchids are under major threat worldwide due to over exploitation by collectors and enthusiasts. In spite of a very large number of seeds produced, only 0.2- 0.3% germinate in nature.

The Western Ghats forests are a rich repository of tropical flora and a veritable emporium of orchid plants. Altogether the Western Ghats harbour 267 species, 3 sub species and 2 varieties of orchids of which 72 species are endemic to the Western Ghats (Kumar, 1991). A cursory perusal of the horticultural merits of these orchids indicates the distribution in specific habitats of at least 15 is rare and exquisite species (Kumar, 1991) that may find a place of pride with any orchid enthusiast. In very recent times, as elsewhere in the tropics, the Western Ghats region has registered considerable environmental degradation, severe anthropogenic pressure on the vital resources and gross interference with the evolutionary processes of the plants. In the Western Ghats region, thirty species of orchids are reported to be rare and endangered of which ten are known from a single location only (Kumar, 1986).

The orchid genus *Eulophia* comprising about 210 species have distribution throughout the tropical to temperate climatic region. The genus was first described by John Lindley in 1821 and most of them are terrestrial. Seven species of the genus represented in the forest of Kerala. The underground rhizome of some the species are used for various ailments by the tribal community.

Eulophia cullenii (Wight) Blume is a rare and exquisite terrestrial orchid with beautiful golden yellow flower (Fig. 1) endemic to southern Western Ghats in habiting the forest floor (Nayar, 1997; Sasidharan, 2004). This species was originally described by Wight based on the General Cullen's collection from the Travancore area of Kerala. The loss of forest cover due to anthropogenic invasion, animal grazing and extensive collection for ornamental and medicinal purpose turned the species in the rare category (Sasidharan, 2004). The underground rhizome of this species is mixed with coconut milk and given internally against spider poison by the *kani* tribe of Kerala. The underground rhizome is fleshy and eaten by the rodents. The propagation of this species in nature is mainly by the perenating rhizome. The conventional propagation of this species is too low and unable to overcome the threat of extinction. Conservation of the shrinking plant gene pool especially of the vanishing rare and endangered taxa is a key concern of the day all over the tropics. The application of plant tissue culture in *ex situ* conservation of rare and endangered plant taxa including orchids has long been recognised. Rare and threatened orchid species are propagated by seeds rather than vegetative methods (Dutra *et al*., 2009). Rapid *in vitro* propagation of several rare and endangered orchids has been reported (Vij and Kaur 1998; Deb and Temjensangba, 2006).

The objective of the study was to develop appropriate an micropropagation methods for multiplication of *Eulophia cullenii*, a rare and exquisite orchid of Western Ghats through green pod culture.

Materials and methods

Green pod culture of *E. cullenii*, a rare, endemic and exquisite orchid of Western Ghats was tested in three different culture media viz. Mitra *et al* (1972), Knudson C (1945) and Wimber media (1963). Green capsules formed 90 days after pollination were collected from wild plants (Fig. 2). The capsules were washed in mild Labolene

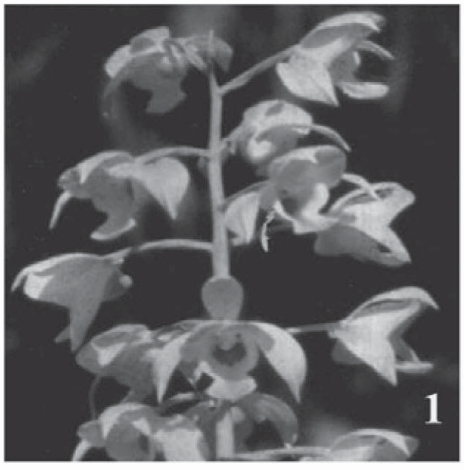

Fig. 1: *E. cullenii* : Inflorescence close up

Fig. 2: Immature capsule after 90 days of pollination

detergent and washed in running tap water. For surface decontamination, the capsules were immersed in 0.1% (w/v) $HgCl_2$ solution for 12 min. followed by rinsing three times in sterile distilled water. The disinfected capsules were split open vertically and the seeds were transferred into 10 ml sterile distilled water. Equal volume of the uniform seed suspension were transferred to 250 ml Erlenmeyer conical flasks containing 60ml pre sterilized liquid basal Mitra, Kundson C, and Wimber nutrient media with 2% sucrose alone and all the three medium were fortified with 0.05% (w/v) casein acid hydrolysate (CH), 0.05% (w/v) peptone (P), 0.05% (w/v) yeast extract (YE) and 20% (v/v) coconut water (CW). All the operations were done in a laminar air flow hood (Klenzaids, India Ltd). Aliquots of seed suspension was drawn and viewed under microscope to take viable embryo court. All the cultures were incubated at 25 ± 2^0C and 55 to 60% RH under 12 hr photoperiod using cool-white fluorescent tubes and constant agitation at 80rpm on a gyratory shaker. Observations of seed germination were made at regular intervals. After 60 days of culture, samples of developing embryos were taken for determination of size and fresh weight.

After 60 days, aliquots of the protocorms were placed on basal Mitra agar (0.8%) nutrient medium and supplemented with appropriate concentration of CH (0.05%), peptone (0.05%), YE (0.05%), CW (20%) in 250 ml Erlenmeyer conical flask (15 protocorms/flask). The cultures were incubated at 25±2^0C under 12 hr photoperiod. Since maximum germination was observed in Mitra medium, further experiments on protocorm development was done in Mitra medium only.

After 3rd subculture seedling with 2-4 leaves, well developed rhizome with 4-6 roots were recovered from the culture flasks, washed thoroughly in tap water to remove agar residue and treated with 1% (w/v) Indofilm M45 fungicide (Indofilm, Mumbai) before planting in clay pots (8cm diameter) filled with a mixture of sand and farmyard manure (1:1). The potted plants were maintained in a nursery under diffuse sunlight and watered once a day in the morning.

Statistical analyses were performed on the results of each experiment and data were compared using ANOVA and LSD multiple – range test. All the experiments were repeated thrice and identical physical conditions were maintained for the entire period of investigation and for every replicate.

Results and Discussion

Orchid seeds are unique in several aspects. They are minute and non endospermic with undifferentiated embryos and are produced in large numbers. Research on the germination of terrestrial orchid seeds has been carried out mainly by Ardtii and collaborators (Oliva and Arditti 1984., Arditti *et al*., 1985; Arditti and Ernst, 1993). The use of unripe capsules is often preferable not only for ease of sterilisation, but also for higher germination percentage (Yam and Weatherhead, 1988; Arnaud *et al*., 1992, Temjensangba and Deb, 2005, Kalimuthu *et al*., 2007; Parab and Krishnan, 2012). The use of green pods together with liquid instead of agar nutrient media might have contributed to successful germination of seeds and growth of protocorms in liquid compared to solid medium reported by many workers (Chu 1988; Chu and Mudge, 1994).

In the present study the viability of the embryo, as deduced from the presence of well developed embryos within the seed coat under microscopic examination revealed the presence of well developed embryos in 33% seeds (Fig. 3). Swelling and glistening of the embryo was noticed within 15 days of inoculation. Swelling of the embryo was closely followed by pigment synthesis. Once germination was initiated, the embryo continued to grow and develop into protocorm. After 20 days, greening of protocorm occurred in Mitra medium. Whereas in Knudson and Wimber media the protocorm appears be yellowish green or yellowish white in colour.

Fig. 3: Microscopic view of seeds

Of the three media tested for the present investigation, the Mitra basal medium supported 60% seed germination (Fig. 4). The other two media Viz. Knudson and Wimber basal media supported seed germination but the percentage of germination was inferior to Mitra medium ie 50% and 40% respectively (Table 1). The frequency of orchid seed

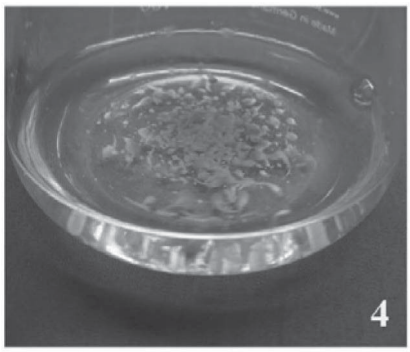

Fig. 4: Seed germination in Mitra liquid medium

germination and protocorm development and the extent of seedling growth varied with the species and culture medium (Reddy *et al*., 1992). In the present study *E. cullenii* seeds showed poor percentage (33%) viability of seeds and therefore, the poor percentage germination recorded in this species was not surprising. Asymbiotic germination of orchid seeds depends on the use of an appropriate nutrient formulation. Although no nutrient medium is universally accepted (Henrich, 1981), among the media tested, the one proposed by Mitra *et al*., (1976) favoured better seed germination. The preference of Mitra medium over others for seed culture of a number of Indian orchids is reported (Mitra 1986; Vij and Jalota 1995; Parab and Krishnan 2012). The lesser amounts of ammonium salts, nitrates of calcium and potassium and of phosphate ions and various minor salts and vitamins in Mitra medium are related to its enhanced influence on seed germination (Mitra 1986).

Supplementation of the basal nutrient medium with organic additives had marginal positive effect on the seed germination and pronounced effect on growth of the embryos (Table 1). Supplemented Mitra medium gave better value than others confirming the desirability of using this medium. Of the different organic additives used for the present investigation, 0.05% CH supported 70% seed germination followed by 65% in 0.05% peptone and 60% in 0.05% YE in Mitra medium. The promontory effect of germination and protocorm development in presence of CH followed by peptone indicated the dependence of protocorm development on exogenous supply of amino acids. The requirement for amino acids by plant tissue culture can be estimated by adding varied amount of protein hydrolysate (Seabrook, 1980). Amino acid supplements in the form of CH is an instant source of nitrogen especially beneficial for growth or morphogenesis of plant cells and is an easy way of ensuring against medium deficiency (George and Sherrington 1984). The other two medium used for the present study, the addition of organic supplements marginally increased the percentage of seed germination compared to basal medium (Table 1). In the present investigation, addition of coconut water was found to be inhibitory. The inhibitory influence of CW, the most frequently used complex additive, on the protocorm of *E. cullenni* may be related to its highly active natural cytokinins and gibberellins activities. The

Table 1: Effect of different additives on seed germination in *Eulophia cullenii in* agitated nutrient media

Media	Additives	Germination (%)	Response after 60 days	
			Fresh wt (mg) of protocorm	Diameter of protocorm
Mitra	Basal	60	2.75±0.15 e	1.65±0.40c
	0.05% CH	70	14.70±0.30 a	3.55±0.15a
	0.05% P	65	9.25±0.25 c	2.55±0.35b
	0.05% YE	40	10.50±0.5b	3.35±0.15a
	20% CW	10	0.82±0.02	0.52±0.08
Knudson-C	Basal	50	1.90±0.10f	1.00±0.00d
	0.05% CH	52	5.25±0.25d	2.45±0.30b
	0.05% P	50	1.75±0.15f	9.25±0.15cd
	0.05% YE	50	1.85±0.05f	1.35±0.05cd
	20% CW	5	0.73±0.03	0.48±0.05
Wimber	Basal	40	0.80±0.20g	1.05±0.05d
	0.05% CH	43	1.25±0.15fg	1.40±0.00cd
	0.05% P	40	1.10±0.20fg	1.20±0.10cd
	0.05% YE	45	1.00±0.10fg	1.30±0.00cd
	20% CW	8	0.70±0.05	0.46±0.03

Mean followed by same letter in a block do not differ significantly at 5% level based on Duncan's multiple range test

growth retarding influences of CW on Dendrobium (Kotomori and Murashige, 1965) and *Cattleya lawrenceana* seed germination and *Phalaenopsis* hybrid embryo cultures (Mariat, 1951; Ernst, 1967) presumably due to its gibberellins activity are reported.

Although shoot initiates were already formed in most of the individual protocorm developed from the embryos during culture initiation in liquid medium, they did not develop into leafy shoots. Transfer of the protocorms for subculture on agar nutrient medium was obligatory to induce protocorm proliferation or seedling development. The developmental pattern of the protocorm subculture in Mitra basal agar medium was more or less uniform. Maximum protocorm proliferation was noticed in Mitra basal medium. An average of 15 daughter protocorms was formed from a single protocorm within 8 weeks of culture (Fig. 5). No distinct advantage was accrued by incorporating the organic additives into the medium during protocorm proliferation and shoot emergence. The protocorm proliferation observed in this species also encountered in certain other taxa (Rubluo *et al*., 1989) is formed due to meristematic activity in specific cells of the early formed protocorms and emergence of globular areas that occurred randomly on the surface of the protocorms often clustering together at the end of development. The formation of multiple protocorms contributed to enhanced propagation rate achieved through seed culture.

Development of *in vitro* rhizome, an additional structure associated with seedling development was observed during the second subculture. During seedling development, the protocorm cluster produced rhizomatous structures 0.5 – 1.0cm length and each having nodes and internodes with root initials at the nodes (Fig.6). Gradually the rhizome turned green branching of the rhizome occurred and 1-2 leafy shoots emerged from the tip of each rhizome (Fig.7). The transformation of the protocorms into elongated, branched rhizomatous structure with definite nodes and internodes during the second subculture and eventual greening of the rhizome and development of 1-2 leafy shoots from the meristematic tip of the rhizome in this species finds its similarity in *Cymbidium virescens* and *C. faberi* (Champagnat *et al*., 1968), *Eulophia yushuiana* (Yam and Weatherhead, 1988). This kind of developmental pattern is consistent with the life cycle of the plant *in situ* where the underground perennating rhizome occasionally produced flowering scape appearing above ground in leafless saprophytes (Yamand Weatherhead, 1988) and both leafy and flowering shoots in *E. cullenii*.

The seedlings after the 3rd subculture were deflasked (Fig. 8) and established in community pots with 79% establishment rate (Fig.9). The condition of weaning away the micropropagated plants, potting and post transplantation care in a nursery shed under diffused light as described were sufficient to ensure high percentage (79%) survival without hardening. This was particularly so since the plants were quite hardy and each possessed at least three healthy roots. Thus, the present protocol provides an efficient *in vitro* multiplication method for *E. cullenii* from green capsule.

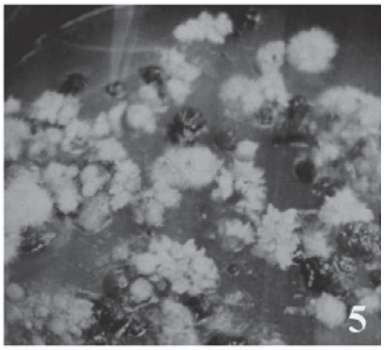

Fig. 5: Protocorm proliferation in solid Mitra medium

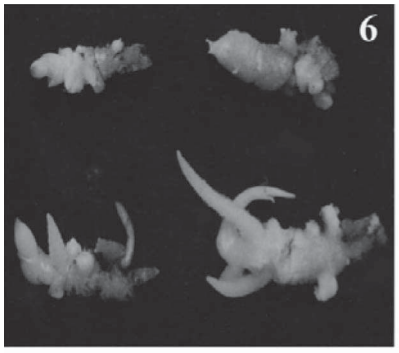

Fig. 6: *In vitro* developed rhizome with shoot and root initials

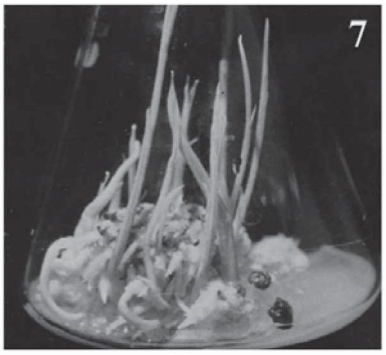

Fig. 7: Leafy shoot emergence

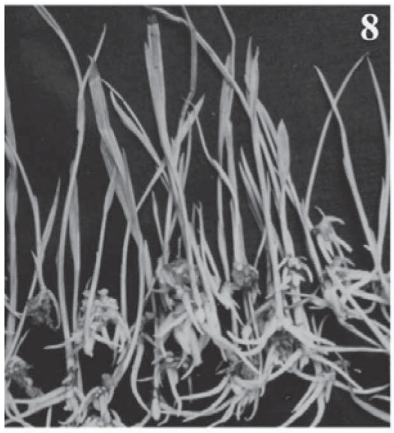

Fig. 8: Deflasked seedlings

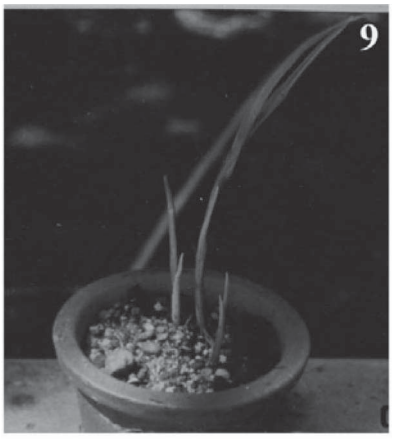

Fig. 9: Acclimatized plants in clay pot

Conclusion

The endemic and therapeutically important orchid species tested for the purpose responded positively to embryo culture. Among the different nutrient formulation tested, high percentage seed germination together with rapid development of protocorm was obtained in Mitra medium. The different organic additives used maximum germination was obtained in presence of casein acid hydrolysate. High percentage establishment rate of the seedling was achieved in appropriate potting media after transplantation in community pots.

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