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Research Article

## IN-VITRO SEED GERMINATION AND EFFECT OF GROWTH REGULATORS ON SUBSEQUENT DEVELOPMENT OF PROTOCORMS OF *EULOPHIA NUDA* LINDL

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### Abstract

Asymbiotic seed germination of *Eulophia nuda* Lindl. was observed on Knudson C medium. About 90% seeds germinated within 8-10 weeks and formed green protocorms in 11-12 weeks. Effect of BA and IBA was studied on plantlet development from protocorms. BA shows the best results with respect to number and length of shoots. Maximum number ( $6.45 \pm 1.36$ ) and length ( $3.90 \pm 0.99$ ) was observed on MS medium supplemented with  $4.44 \mu\text{M}$  BA. Maximum root growth was also observed on same medium ( $4.8 \pm 0.99$  number of roots and  $1.43 \pm 0.13$  cm length). The regenerated plantlets were successfully acclimatized and transferred to earthen pots. The results presented here show that *in vitro* seed germination and plantlet development in *Eulophia nuda* Lindl., an endangered orchid, can be achieved at a higher rate by this method.

**Key words:** *Eulophia nuda* Lindl.; orchid; BA; IBA.

### Introduction

Orchids, one of the major families of angiosperms, are having immense importance as ornamental and medicinal potential. Nowadays, many orchid species are threatened and enlisted in the Red Data book of IUCN (Chugh *et al.*, 2009 and IUCN, 2011). 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 (Chugh *et al.*, 2009 and Reed *et al.*, 2011). Orchid species are under serious threat with an uncertain future due to unscrupulous collection for commercial uses (Swarts and Dixon, 2009).

Common orchid taxa may serve as models for developing reintroduction programs, which can then be applied to threatened and endangered taxa. The first step in this process is establishing efficient propagation protocols to produce plants for subsequent experimentation the most efficient method of propagating native terrestrial orchids. Seed propagation represents the straight forward method to produce large number of propagules. (Stewart and Kane 2006).

Symbiotic seed germination can be a cumbersome process; root samples must be collected from which many fungi are often isolated. Fungi must then be identified and screened for growth promoting strains. Asymbiotic seed germination can be a more straight forward process since mycobionts need not be isolated to germinate seeds of orchid taxa. Populations of orchids that are established with asymbiotic

seedlings remain dependent on naturally occurring fungal symbionts for seedling recruitment (Zettler 1997b).

*Eulophia nuda* Lindl. [Synonym: *Eulophia spectabilis* (Dennst.) Suresh] known as 'Manya' (in Sanskrit) and as 'Amarkand' (in Marathi) primarily is used as an ornamental but is also used in herbal medicines and food (rhizome) by many different tribes. An amorphous phenanthrene, named nudol, later identified as 2,7-hydroxy-3,4-dimethoxyphenanthrene has been isolated from *Eulophia nuda*, *E. ohreata*, *Erica carinata* and *Erica stricta* (Merchant *et al.*, 1962., Bhandari *et al.*, 1985, Datla *et al.*, 2010 and Kshirsagar *et al.*, 2010). *Eulophia nuda* is exploited for several of its ethno-medicinal properties like antidote for snake bite, as antihelmintic, against tumors, cases of bronchitis, Scrofulous affection of the glands of the neck and in disease of the blood (Singh and Duggal, 2009). The plant is also claimed to be useful in tuberculosis (Chopra, 1956). *Eulophia nuda* mainly propagate through the rhizomes in nature and they produce only limited number of propagules. This plant species has been over harvested because of increasing demand in the market. Threat is further exaggerated due to destruction of its natural habitat. Therefore, it is important to establish an efficient regeneration and multiplication system for the production of large number of rhizomatous plantlets. The present study describes the protocol for propagation through *in vitro* seed germination and multiplication system for its conservation and sustainable utilization.

## Materials and Methods

### *Plant material, surface sterilization and explant preparation*

Capsules of *Eulophia nuda*, maintained in Botanical garden, Shivaji University, Kolhapur, India were harvested in the month of October before dehiscence. The capsules were washed with liquid soap under tap water and further surface sterilized by dipping in 70% ethanol for 30 seconds in laminar air flow unit.

### *Culture medium and culture conditions*

The capsules were cut open and seeds sowed aseptically in petri dishes (Laxbro India 88X55mm), containing Knudson media (1946) fortified with 3% (w/v) sucrose and 0.9% agar (Himedia) as gelling agent without addition of plant growth regulators. The small protocorms obtained were transplanted on to Murashige and Skoog (1962) medium containing various concentrations of BA (6-benzyl aminopurine) and IBA (Indol-3-butyric acid), supplemented with 2% (w/v) sucrose and 0.8 % agar (Himedia) as gelling agent. The pH of the medium was adjusted to  $5.8 \pm 0.02$  using 1 N NaOH or 0.1N HCL prior to autoclaving for 15 min at 15psi, 121 °C . The cultures were maintained at  $28 \pm 2^\circ\text{C}$  under a 12 h d<sup>-1</sup> photoperiod with a white cool fluorescent light ( $40 \mu\text{Molm}^{-2}\text{s}^{-1}$ ). Subsequent transfer to fresh medium was carried out according to growth rate and stage of development, usually after each 4 to 6 weeks. The cultures were observed constantly for any response and results recorded after every 15 days.

### *Acclimatization of regenerated plantlets*

Rooted plantlets with fully expanded leaves and well developed roots were successfully transferred to pots containing sand and soil (1:1) and maintained in controlled growth chamber conditions  $25 \pm 1^\circ\text{C}$ , 16 hrs photoperiod and 80% relative humidity followed by gradual shift to greenhouse conditions.

### *Experimental design and statistical analysis*

All the experiments were conducted with the minimum of 30 replicates per treatment and the experiments were repeated three times. MS medium without growth regulator was used as control in all experiments. The results are expressed as mean  $\pm$  SD of three experiments. Data were subjected to analysis of variance (ANOVA) and means were compared by Duncan's multiple range test at  $P < 0.05$  using the SPSS ver.21.(IBM SPSS ver 21).

## Results and Discussions

Germination of orchid seeds is different from other seeds. The seeds are produced in large number within a capsule. They are very minute, with undifferentiated embryo and lack of endosperm. Due to non-endospermic nature of seed,

the germination in nature is unique phenomenon and requires fungal infection. Germination is much more successful *in vitro* on medium containing simple sugars. The production of orchid seedling from seed involves sequential phases as germination, protocorm formation and development of seedling. In present study similar sequence was observed when seeds of *Eulophia nuda* were grown on KC medium devoid of plant growth regulators (Fig 1).

Seed germination started relatively slowly within 8-9 weeks of culture where embryos swelled and broke out of the testa, and then formed green protocorms after 11-12 weeks of sowing. The germination of nongerminated seeds continued for a prolonged period of time lasting more than 10-12 months. Similar observations are reported in *Dactylorhiza futchsii*, (Jakobson 2008), where seed germination continued for almost 2.5 years under *in-vitro* conditions. The protocorms take 4 weeks to develop chlorophyll, as evident in most of the terrestrial orchids (Dressler, 1990) in contrast with *Bletilla*, *Thunia* and *Cymbidium*, where early in germination; chlorophyll appears in the protocorm cells as described by Yakovlev and Zhukova (1973).



**Fig. 1:** 1. Germinating seeds, 2. In-vitro produced rhizomes, 3. Effect of IBA and BA on development of shoots and roots, 4. Plantlet ready for hardening, 5. Acclimatized plant of *Eulophia nuda*

**Table 1:** Effect of BA and IBA on shoot and root development from PLBs of *Eulophia nuda*

BA ( $\mu\text{M}$ )	IBA ( $\mu\text{M}$ )	No. of Shoots	Length of shoots (cm)	No. of roots	Length of roots (cm)
-	-	3.19 $\pm$ 0.79d	1.45 $\pm$ 0.39e	3.8 $\pm$ 0.88b	0.69 $\pm$ 0.16cd
2.22	-	4.90 $\pm$ 1.10b	3.11 $\pm$ 0.66b	2.46 $\pm$ 0.89cd	1.29 $\pm$ 0.21b
4.44	-	<b>6.45<math>\pm</math>1.36a</b>	<b>3.90<math>\pm</math>0.99a</b>	<b>4.8<math>\pm</math>0.99a</b>	<b>1.43<math>\pm</math>0.13a</b>
-	0.98	1.45 $\pm$ 0.56c	0.90 $\pm$ 0.32c	1.6 $\pm$ 0.60c	0.77 $\pm$ 0.26cd
-	2.46	0.67 $\pm$ 0.59d	0.53 $\pm$ 0.13d	2.03 $\pm$ 0.66de	0.61 $\pm$ 0.13d
2.22	0.98	1.58 $\pm$ 0.50e	1.47 $\pm$ 0.27e	2.16 $\pm$ 0.94de	0.65 $\pm$ 0.11cd
2.22	2.46	1.45 $\pm$ 0.62e	1.34 $\pm$ 0.19e	1.9 $\pm$ 0.80ef	0.64 $\pm$ 0.55d
4.44	0.98	3.64 $\pm$ 0.60e	2.16 $\pm$ 0.72f	2.8 $\pm$ 1.06f	0.71 $\pm$ 0.10c
4.44	2.46	2.90 $\pm$ 0.83f	1.82 $\pm$ 0.30f	2.2 $\pm$ 0.76def	0.58 $\pm$ 0.10d

Basal medium: MS supplemented with 2% (w/v) sucrose and 0.8% (w/v) Agar. Means followed by the different letter within column are significantly different ( $P < 0.05$ ) using Duncan's multiple range test.

Asymbiotic germination of seeds is the most common method practiced for the propagation of both epiphytic and terrestrial orchids even though symbiotic germination succeeded in some species has been recommended for utilization in restoration program (Stewart and Kane 2006; Aggarwal and Zettler 2010). Asymbiotic seed germination is reported to be successful in some *Eulophia* species, including *E. alta*, *E. graminea*, *E. cucullata*, *E. streptopetala*, *E. petersii* and *E. nuda* (McAlister and Van Staden 1998; Johnson *et al.* 2007; Chang *et al.* 2010, Nanekar *et al.* 2014) using MS,  $\frac{1}{2}$  MS,  $\frac{1}{4}$  MS + coconut water, BM1+coconut water, or P723 (Orchid Seed Sowing Medium, PhytoTechnology Laboratories, Shawnee Mission, KS) as culture media. The present study revealed Knudson-C medium equally good for supporting seed germination without any additives.

For subsequent development, the green protocorms of ~2 mm diameter were transplanted on Murashige and Skoog (1962) medium, supplemented with BA and IBA alone and in combination, where they grew in size and differentiated to form shoots and roots (Table 1). Rhizome formation was observed after two subcultures on same medium. Their size and number increased during further subcultures. The maximum number of shoots (**6.45 $\pm$ 1.36**) and length of shoots (**3.90 $\pm$ 0.99**) were observed on medium fortified with BA 4.44 $\mu\text{M}$ . Maximum root growth was also observed on same medium (4.8 $\pm$ 0.99 number of roots and 1.43 $\pm$ 0.13cm length of roots). Similar results were observed in *Orchis coriophora* where shoot length and number and length of roots in medium supplemented with 0.25 mg/L 6-BA were longer than those grown in other medium (Bektaş 2013). Cytokinins promote rhizome formation which was best observed on media supplemented with BA alone. Development of storage organs such as root rhizome and rhizomes *in vitro*, of terrestrial orchids is important as the means to improve plant reintroduction by allowing *ex*

*vitro* translocation for the use of plant tissues that possess a greater desiccation tolerance than seedlings with photosynthetic leaves.

The type, concentrations and different combinations of PGRs plays an important role during *in vitro* propagation of many orchid species (Arditti and Ernst, 1993). The promotory effects of growth regulators in *in-vitro* culture have been worked out by a number of workers (Kano 1965, Methews and Rao 1980, Sharma and Tandon 1986, Kumaria and Tandon 1990). The enhancing effects of cytokinins on morphogenesis were previously reported (Goh & Wong, 1990). The differentiation of multiple shoots from corm and/or protocorm like bodies (PLBs) have been reported in *Renanthera imschootiana* (Seeni and Latha, 1992); *Cymbidium aloifolium* (Kaur and Sharma, 1997; Bujarbarua and Sharma, 1997); *Dendrobium aphyllum* (Talukdar, 2001) *Habenaria marginata* (Sheelavantmath and Murti, 2001) and *Eulophia nuda* (Panwar *et al.* 2012).

IBA in combination with BA has showed best results for plant development in *Cattleya walkeriana* (Maringá, *et al.* 2003). The highest rate of elongation was reported in *Dendrobium fimbriatum* in MS medium containing 2.0 mg/l BAP and 0.01 mg/l IBA. In majority of cases auxins (NAA, IAA and IBA) enhanced the seed germination and seedling growth. In present investigations IBA does not show any promotory effect in shoot, root or rhizome formation alone as well as in combination with BAP suggesting that auxins did not support differentiation in germinated seeds of *E. nuda*.

## Conclusion

The *in vitro* propagation of orchids has always been envisaged as problematic. On the other hand, such plants are economically important since they have long been used as a herbal remedy for various ailments by tribal people. As they are collected from nature, not cultivated, their

extinction is inevitable; careless collection will cause genetic and ecological erosion. The results presented here show that *in vitro* seed germination and plantlet formation of *Eulophia nuda*, an endangered orchid, can be achieved at a higher rate by this method. This data may hold the key to the mass propagation of other orchid species.

## References

- Aggarwal S and Zettler LW (2010) Reintroduction of an endangered terrestrial orchid, *Dactylorhiza hatagirea* (D. Don) Soo, assisted by symbiotic seed germination—First report from the Indian subcontinent. *Nature Science*. **8**: 139–145.
- Arditti, J and Ernst R (1993) *Micropropagation of Orchids*. John Wiley and Sons, New York
- Bektaş E, Mustafa C and Sökmen A (2013) In vitro germination, protocorm formation, and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. *Turk J. Bot.* **37**: 336-342.
- Bhandari SR and Kapadi AH (1983) A 9,10-Dihydrophenanthrene from rhizomes of *Eulophia nuda*. *Phytochemistry* **22**: 747 – 748. DOI: 10.1016/S0031-9422(00)86975-7
- Bhandari SR, Kapadi AH, Majumder PL, Joardar M and Shoolery JN (1985) Nudol, a phenanthrene of the orchids *Eulophia nuda*, *Eria carinata* and *Eria stricta*. *Phytochemistry* **24**: 801 – 804.
- Bujarbarua A and Sharma CM (1997) Micropropagation of *Cymbidium aloifolium* Sw from seeds. *J. Adv. Plant Sci.* **1**: 27–31.
- Chang C, Hu W, Chen Y, Su Y and Chiu YT (2010) In vitro flowering and mating system of *Eulophia graminea* Lindl. *Bot. Stud.* **51**: 357–362.
- Chopra RN (1956). Letters to the editor, Glossary of Indian Medicinal Plants **31**: 112.
- Chugh S, Guha S and Rao U (2009) Micropropagation of orchids: a review on the potential of different explants. *Sci. Hortic.* **122**: 507–520. DOI: 10.1016/j.scienta.2009.07.016
- Datla P, Kalluri MD, Basha, K, Bellary A, Kshirsagar R., Kanekar Y, Upadhyay S, Singh S, and Rajagopal, V (2010). 9,10-Dihydro-2,5-dimethoxyphenanthrene-1,7-diol, from *Eulophia ochreatea*, inhibits inflammatory signalling mediated by Toll like receptors. *Br. J. Pharmacol.* **160**: 1158–1170. DOI: 10.1111/j.1476-5381.2010.00780.x
- Dressler RL (1990) *The orchids, Natural history and classification*. Harvard University Press. Cambridge
- Goh CJ and Wong PF (1990) Micropropagation of the monopodial orchid hybrid *Aranda deborah* using inflorescence explants. *Scientia Horticulturae* **44**: 315–321. DOI: 10.1016/0304-4238(90)90132-x
- IUCN (2011) IUCN Red List of Threatened Species. Version 2011.1. <<http://www.iucnredlist.org>>. Downloaded on 14 September 2011
- Johnson TR, Stewart SL, Dutra D, Kane ME and Richardson L (2007) Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)—preliminary evidence for the symbiotic culture advantage. *Plant Cell Tiss. Organ Cult.* **90**: 313–323. DOI: 10.1007/s11240-007-9270-z
- Kalimuthu K, Senthilkumar R and Vijayakumar S (2007) In vitro micropropagation of orchid, *Oncidium* sp. (dancing dolls) *Afr. J. Biotechnol.* **6**: 1171–1174. DOI: 10.5897/AJB06.325
- Kano K (1965) Studies on the media for orchid seed germination, *Mem. Fac. Agric. Kagava. Univ.* **20**: 1-68.
- Kaur S, Sharma CM (1997) Application of Rhizontonia for establishment of plantlets of *Cymbidium aloifolium* Sw under natural conditions. *J. Adv. Plant Sci.* **1**: 17–20.
- Kshirsagar RD, Kanekar YB, Jagtap SD, Upadhyay S, Rao R., Bhujbal SP and Kedia JN (2010) Phenanthrenes of *Eulophia ochreatea* Lindl. *Int. J. Green Pharm.* **4**: 147–152. DOI: 10.4103/0973-8258.69/64
- Kumaria S, Chrungoo N and Tandon P (1990) Activities of some oxidative enzymes in axenic cultures of protocorms of *Cymbidium giganteum* Wall as influenced by different growth regulators. *J. Orchid Soc. India.* **4**: 34-44.
- McAlister BG and Van Staden J (1998) In vitro culture of *Eulophia* species. *S. Afr. J. Bot.* **64**: 264–266.
- Merchant JR, Shah RS and Hirwe SN (1962) Chemical investigation of *Eulophia nuda* Lindl. *Curr. Sci.* **35**: 95.
- Methews VH and Rao PS (1980) In vitro culture of Vanda hybrid (vanda TMA X Vanda miss Joaquim): studies on seedling explants. *Proc. Indian Natl. Acad.* **51**: 496-504.
- Nanekar V, Shriram V, Kumar V and Kavikishor PB (2014) Asymbiotic In vitro seed germination and seedling development of *Eulophia nuda* Lindl., an endangered medicinal orchid. *Proc. Natl. Aca. Sci. India, sec. B Biol Sci.* **84** (3): 837-846. DOI: 10.1007/s40011-014-0353-4
- Panwar D, Kheta Ram H and Shekhawat NS (2012) In vitro propagation of *Eulophia nuda* Lindl., an endangered orchid. *Scientia Horticulturae.* **139**: 46–52. DOI: 10.1016/j.scienta.2012.01.011
- Reed BM, Sarasan V, Kane M, Bunn E and Pence VC (2011) Biodiversity conservation and conservation biotechnology tools. *In Vitro Cell. Dev. Biol. Plant* **47**: 1–4. DOI 10.1007/s11627-010-9337-0
- Seeni, S and Latha, PG (1992) Foliar regeneration of endangered red Vanda – *Renanthera imschootiana* Rolfe (Orchidaceae). *Plant Cell Tissue Organ Cult.* **29**: 167–172. DOI: 10.1007/BF00034349
- Sharma SK and Tandon P (1986) Influence of growth regulators on asymbiotic germination and early seedling development of *Coelogyne punctulata* Lindl. In: Vij SP (eds) *Biology, conservation and culture of orchids*. Affiliated East West Press, New Delhi, 441-451.
- Sheelavantmath SS and Murthy HN (2001) In vitro propagation of a terrestrial orchid *Habenaria marginata* Coleb. *J. Orchid Soc. India* **15**: 85–88.

Singh A and Duggal S (2009) Medicinal Orchids – An Overview. *Ethno botanical Leaflets* **13**: 351-363.

Stewart SL and Kane ME (2006) Asymbiotic germination and in vitro seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell Tiss. Org. Cult.* **86**:147–158. DOI: 10.1007/s11240-006-9098-y

Swarts ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. *Ann. Bot.* **104**: 543–556. DOI: 10.1093/aob/mcp025

Talukdar A (2001). Multiple shoots induction in *Dendrobium aphyllum* Roxb. *J. Orchid Soc. India* **15**: 35–38.

Yakovlev MS and Zhukova GYA (1973) Angiosperms with green and colourless embryo (chloro- and leucoembryophytes). *Nauka, Leningrad* (in Russian).