Eco. Env. & Cons. 30 (July Suppl. Issue) : 2024; pp. (S539-S543)
Copyright@ EM International
ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2024.v30i04s.088

Non-symbiotic seed germination and *in vitro* embryogenesis of *Eulophia nuda* Lindl.

Shreya K. Kasar[1], Harichandra A. Nikule[1], Satyam D. Pawar[1], Vidya R. Kothari[2] and Sunil P. Bhagat[1]*

[1]PG Research Center, Department of Botany, Shardabai Pawar Mahila Arts, Commerce and Science College, Baramati 413 115, M.S., India
[2]PG Research Center, Department of Microbiology, Shardabai Pawar Mahila Arts, Commerce and Science College, Baramati 413 115, M.S., India

(Received 7 March, 2024; Accepted 22 May, 2024)

ABSTRACT

Eulophia nuda Lindl. (Family- Orchidaceae) is an endangered species which has vast potential medicinal applications. It is naturally propagated through its tubers, yet, they only generate very few propagules. Therefore, in the present investigation, an effective protocol has been established for the asymbiotic seed germination and embryogenesis of *E. nuda*. Seeds from the capsules were used as a source of explant and were cultured on Malmgren Modified Terrestrial Orchid Medium (MG), BM-1 Terrestrial Orchid Medium (BM-1) and ½ Murashige and Skoog medium (MS). Selected media were also supplemented with coconut water (15 % CW), IAA, Kn alone and in combination. From the results, it was observed that the seeds germinated only on BM-1 media. The germination and embryo formation were recorded during 10-25 days, seed coat splitting during 25-50 days and development of protocorm like bodies during 60-90 days. The explants grown on BM-1 supplemented with 15% CW & IAA and Kn alone and in combination showed *in vitro* germination with globular embryo development in 3-4 weeks and development of protocorm in 4-5 weeks. The first leaf primordia development was highly influenced by addition of IAA &Kn, alone and in combination with time required in range of 7-8 weeks. No leaf primordia development was observed on BM-1 media supplemented with CW only. The results clearly confirmed that BM-1 Terrestrial Orchid Medium was most favorable for the germination of seeds of *E. nuda*. The present research could be very important for the conservation and extensive propagation of this highly important and medicinal orchid, whose survival is under danger due to overexploitation.

Key words: *Eulophia nuda, Orchid, Seed germination, Asymbiotic seed germinantion, Embryogenesis.*

Introduction

Family Orchidaceae has ~32,000 species, distributed in 800+ genera (Mercado and Delgado, 2020), which are known for their exotic beauty and lengthy shelf life. Many large, widespread, and prominent genera are widely included in the IUCN Red Data Book (IUCN, 2023). They face serious issues of extinction and an uncertain future due to unethical collecting practices for commercial objectives (Fay, 2018; Wraith *et al.,* 2020). Because of their extended juvenile stage, orchids naturally require a symbiotic relationship with mycorrhiza for their seeds to germinate and continue developing (Albores *et al.,* 2005). Orchids are also known to harbour medicinal properties (Paul *et al.,* 2017). Among them, *Eulophia* is a genus containing over 220 species, the majority of which are found in Africa. Because of its unusual ecosystem and wide-ranging decorative and medicinal qualities, this genus is extremely significant

(Shriram and Kumar, 2021). About 40 of these species are spread throughout tropical Asia, up to the foothills of the Himalayas (Teoh, 2021). *Eulophia nuda* Lindl. is commonly known as 'Manya' in Sanskrit, 'Salammishri' or 'Goruma' in Hindi, and as 'Amarkand' in Marathi. Besides that, among various tribes, uses as medicines and food (rhizome) are known (Dawande and Gurav, 2015). Tubers are effective against tumors, inflammation of bronchi, diseases of the blood, as a vermifuge, etc. (Bhatt *et al.*, 2020). Orchid plants show flowering at 4-5 years, but the majority of flowers do not pollinate naturally, pollination occurs in only ~5% flowers, fertilization of ovules and formation of capsules is also rare (Roberts and Dixon, 2008).

Besides the conventional methods of vegetative propagation, the *in-vitro* culture methods can be used for large-scale multiplication (Nongdam *et al.*, 2023). Natural propagation is through its tubers, but very few propagules are produced. In situ conservation methods have had mixed results due to the poor germination and survival rates in natural settings as well as the need for particular fungal interactions (Shriram and Kumar, 2021). While several reports on *in vitro* propagation of orchids are known, *in vitro* culture of *E. nuda* seeds as explants is scantily reported. Therefore, the objective of the present study was to establish a method for its propagation through seed germination *in vitro* and embryogenesis.

Materials and Methods

Plants material

The plant material (pods) was collected from plants of *E. nuda*, planted and maintained at the Botanical Garden of the Department of Botany, Shardabai Pawar Mahila Arts, Commerce and Science College, Shardanagar, Baramati, Maharashtra, India. Seeds were harvested from the pods and used as a source of explants.

Media, Plant Growth Regulators and Organic Supplements

Various basal media like ½ MS, MG and BM-1 were used. These three media were supplemented with organic supplement, coconut water (CW) (15%), and plant growth regulators (PGRs) like, indole acetic acid (IAA) alone (0.5-1.5 mg/l), kinetin (Kn) alone (0.5-1.5 mg/l), and in combination were tested. As control, BM-1, MG, and ½ MS media without any adjuvant were used.

Sterilization of the explant and inoculation

The pod was first washed under running tap water. It was treated with 2-3 drops of Dettol in distilled water, sterilized with 70% alcohol for 30 seconds, treated with 1% Bavistin (fungicide) for 20 minutes on shaker. The pod was transferred to a clean laminar airflow chamber, 0.1%mercuric chloride ($HgCl_2$) treatment was carried out for 1 min., followed by washing with sterile distilled water three times. The pod was cut open by a sterile surgical blade, followed by inoculation and culturing of seeds on BM-1, MG, and ½ MS media.

Incubation

Sterilized media (20-25 ml) of each composition was dispensed per petri plate and inoculated with sterilized seeds as explants. The cultures were incubated at 25 ± 2 °C under photoperiod of 16h (irradiance of 40.54 µmol $m^{-2}s^{-1}$) and 50-60% relative humidity (RH). After every 7-8 days, transfers to fresh media were performed and responses were noted.

Statistical Analyses

For each experiment, three replicates were maintained. The observations were taken after four weeks of the culture. The results were measured as mean ± standard error for three replicates. Comparison of means values for significant were done using Duncan's multiple range test (DMRT) at 5 % probability level. SPSS (16.0) software was used for statistical analysis.

Results and Discussion

Asymbiotic seed germination

Non-symbiotic germination of seed is significantly affected by various factors such as seed age, composition of the media and PGRs used (Bhowmik and Rahman, 2022). Wild, capsule bearing whole plants (Fig. 1A, B) were used as a mother plant. Seeds (Fig. 1C) were taken out and inoculated on different media viz. ½ MS, BM-1 and MG. The germination was observed only on BM-1 media on 10^{th} day of culture. However, other two media could not respond for seed germination. Similarly, in previous studies, maximum germination has been reached at different stages of maturity, in *E. nuda* (Nanekar *et al.*, 2014)

and *Dendrobium crepidatum* (Gurung *et al*., 2020). The findings suggested that species-dependent particular maturity stage has a major impact on orchid seed germination *in vitro*. Furthermore, because orchid cultures have a nutritional regime distinct to each species, no single medium is suitable for all of them (Nongdam *et al*., 2023). Various explants like inflorescence, leaves, flowers, roots, and pseudo bulbs (Martin and Madassery, 2006) have been used for *in vitro* propagation of different commercial orchid species like *D. moschatum, D. palpebrae* Lindl., *Vanda coerulea* and many others (Bhowmik and Rahman, 2020).

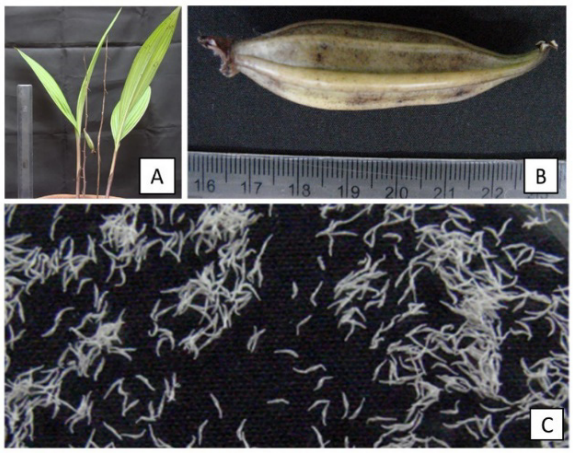

Fig. 1. *Eulophia nuda* used in the experiment: (A) whole plant, (B) pod, (C) seeds.

Effect of media on seed germination and embryo formation

BM-1 exhibited a notably greater proportion of germinating seeds and development of embryo (Table 1), e.g. seed coat splitting was observed after 25th to 50th day of culture. The protocorm development was recorded after 60th day of incubation. Positive effects of BM-1 media on a symbiotic seed germination on *E. nuda* are reported (Nanekar *et al*., 2014), which agrees with the present study. Compared to the other two media, BM1 had a noticeably higher proportion of seed germination. Similarly, in our results and in agreement with earlier reports, nutritional media is very important for germination of seeds a symbiotically and embryo formation (Pyati, 2022; Nongdam *et al*., 2023).

Table 1. Effect of different media for seed germination and embryo formation in *E. nuda*

Media	Incubation period (in days)				
	0 – 6	7 – 9	10- 25	25 - 50	60 – 90
½ MS	-	-	-	-	-
BM-1	-	-	+	++	+++
MG	-	-	-	-	-

No embryo differentiation/germination -, Embryo differentiation/germination +, Seed coat split ++, Protocorm development +++.

Effect of PGRs on embryogenesis

Different PGRs like auxin (IAA) and cytokinin (Kn), and coconut water (CW) as an organic supplement were examined separately and in combination in BM-1 media. Embryo showed swelling on each PGR combination on BM-1 medium after 15 days incubation. Embryo emerged out from seeds by rupturing test after 22 days. Globular embryo was observed after 30 days. Average time taken for development of globular embryo was 3-4 weeks. After 40 days incubation period, embryo became yellowish and after 53 days, embryo became greenish and leaf development was observed. Protocorm developed from globular embryo after 4thweek and further first leaf primordia in 7th week (Table 2).

In the present investigation, all the combinations showed globular embryo, protocorm and first leaf primordium development in *E. nuda*. However, early protocorm development were observed on BM-1 media incorporated with IAA (0.5 mg/l) alone and also on IAA (1.5 mg/l) with Kn (0.5 mg/l) in presence of 15 % CW during the 4th week of incubation. Other studied media composition and their levels showed the globular embryo, protocorm and first leaf primordium development during 3-4th week, 4-5th week and 7-8th weeks of incubation respectively. These results suggested that IAA and Kn application promotes differentiation of germinated orchid seeds. Ony globular embryo (3-4th week) and protocorm (4-5th week) were developed after the application of 15 % CW in the BM-1 media; however, first leaf primordium was not developed after this application. In this study, IAA alone showed a positive response for the embryo development; however, Kn and IAA in combination also showed the embryo development in *E. nuda*. In earlier studies, 1-Naphthaleneacetic Acid (NAA) in combination with 6-Benzyleaminopurine (BAP) was a suitable medium for the shoot development in *E. nuda* (Nanekar, 2014) and in *E. graminea* (Bhowmik and Rahman, 2022).

Table 2. *In vitro* germination and embryo formation of *E. nuda* Lindl. on BM-1 Terrestrial Orchid Media with different plant growth regulators.

Media	Plant growth regulators		Average time (in weeks) taken for development		
	IAA (mg/l)	Kin (mg/l)	Globular embryo	Protocorm	First leaf primordium
BM-1	0.0	0.0	-	-	-
BM-1 + 15 % CW	0.0	0.0	3 – 4	4 – 5	-
BM-1 + 15 % CW	0.5		3 – 4	4	7 – 8
BM-1 + 15 % CW	1.0		3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW	1.5		3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW		0.5	3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW		1.0	3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW		1.5	3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW	0.5	0.5	3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW	0.5	1.0	3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW	0.5	1.5	3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW	1.0	0.5	3 – 4	4 – 5	7 – 8
BM-1 + 15 % CW	1.5	0.5	3 – 4	4	7 – 8

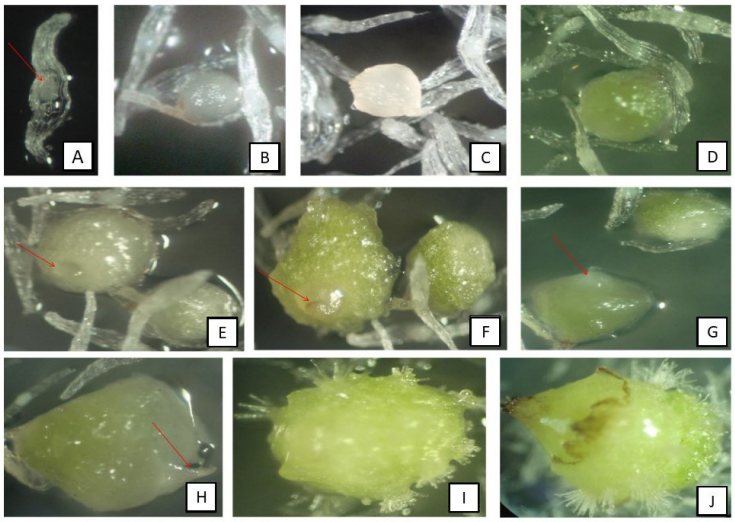

Fig. 2. Different developmental stages of *E. nuda* (A)An undifferentiated embryo inside the seed coat(52×), (B&C) Emergence of globular embryo(52×), (D) Green embryo(52×), (E&F) Globular embryo with appendicle (arrow)(52×), (G&H) Protocorm with first leaf primordium shown with arrow (52×), (I) Protocorm with first leaf primordium with rhizoid (32.5×), (J) Protocorm of pre-seedling stage with rhizoids at the base(32.5×).

Conclusion

In the present study, number of factors were optimized, including the explant selection, media composition and plant growth regulators alone and in combination with organic supplements. As a result, an effective protocol has been established for the optimum a symbiotic seed germination and embryogenesis in *E. nuda*. The results clearly demonstrated that BM-1 Terrestrial Orchid Medium was most suitable for germination of seeds. The current findings might be crucial for the conservation and widespread reproduction of this extremely significant medicinal orchid, which is being threatened by overexploitation.

Acknowledgements

All the authors gratefully acknowledge Agricultural

Development Trust's Shardabai Pawar Mahila Arts, Commerce and Science College for providing necessary facility for the research.

Conflict of interest

The authors declare no conflict of interest.

References

Albores, V., Adriano, L. and Salvador, M. 2005. Isolation of endophytic fungi and their mycorrhizal potential for the tropical epiphytic orchids *Cattleya skinneri*, *C. aurantiaca* and *Brassa vola nodosa*. *Asian J. Plant Sci.* 4: 309-315.

Bhatt, D., Jethva, K. and Zaveri, M. 2020. Phyto pharmacognostical study of tubers of *Eulophia nuda* Lindl. *Int. J. Pharm. Sci. Res.* 11: 3483-3488.

Bhowmik, T.K. and Rahman, M.M. 2022. *In vitro* Seed Germination, Seedlings and SPSs Development in a Terrestrial Medicinal Orchid *Eulophiagraminea* Lindl. of Bangladesh. *Int. Res. J. Pharm. Med. Sci.* 5(6): 27-33.

Dawande, V. and Gurav, R. 2015. *In-vitro* seed germination and effect of growth regulators on subsequent development of protocorms of *Eulophia nuda* Lindl. *Int. J. Res. Appl. Sci. Biotechnol.* 3(2): 243-247.

Fay, M.F. 2018. Orchid conservation: how can we meet the challenges in the twenty-first century?. *Botanical Studies.* 59:1-6.

Gurung, P.R., Shrestha, A., Adhikari, S., Limbu, S. and Pant, B. 2020. Asymbiotic seed germination and seedling development of a medicinally important epiphytic orchid, *Dendrobium crepidatum* Lindl. & Paxton. *J. Plant Res*. 18: 226-234.

IUCN, 2023. The IUCN Red List of Threatened Species. Version 2023-1. https://www.iucnredlist.org. Accessed on 15 May 2024.

Martin, K.P. and Madassery, J. 2006. Rapid *in vitro* propagation of *Dendrobium hybrids* through direct shoot formation from foliar explants, and protocorm-like bodies. *Sci. Hortic.* 108(1): 95-99.

Mercado, S.A.S. and Delgado, E.A.B. 2020. Effect of the medium composition on the asymbiotic germination and *in vitro* development of the *Laeliocattleya* hybrid. *S. Afr. J. Bot.* 135: 80-86.

Nanekar, V., Shriram, V., Kumar, V. and Kishor, P.K. 2014. Asymbiotic *in vitro* seed germination and seedling development of *Eulophia nuda* Lindl., an endangered medicinal orchid. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* 84: 837-846.

Nongdam, P., Beleski, D.G., Tikendra, L., Dey, A., Varte, V., El Merzougui, S., Pereira, V.M., Barros, P.R. and Vendrame, W.A. 2023. Orchid micropropagation using conventional semi-solid and temporary immersion systems: a review. *Plants.* 12(5): 1136.

Paul, P., Joshi, M., Gurjar, D., Shailajan, S. and Kumaria, S. 2017. *In vitro* organogenesis and estimation of β-sitosterol in *Dendrobium fimbriatum* Hook.: an orchid of biopharmaceutical importance. *S. Afr. J. Bot.* 113: 248-252.

Pyati, A.N. 2022. *In vitro* propagation of some important medicinal and ornamental *Dendrobiums* (Orchidaceae): A review. *J. Appl. Hortic.* 24(2): 245-253.

Roberts, D.L. and Dixon, K.W. 2008. Orchids. *Curr. Biol.* 18(8): R325-R329.

Shriram, V. and Kumar, V. 2021. *Eulophia spp.*: *In vitro* Generation, Chemical Constituents, and Pharmacological Activities. In: *Orchids Phytochemistry, Biology and Horticulture: Fundamentals and Applications*. Cham: Springer International Publishing. 1-23.

Teoh, E.S. 2021. *Eulophia* R. Br. ex Lindl. *Orchid Species from Himalaya and Southeast Asia Vol. 1 (A-E)*: 85-489.

Wraith, J., Norman, P. and Pickering, C. 2020. Orchid conservation and research: An analysis of gaps and priorities for globally Red Listed species. *Ambio*. 49(10): 1601-1611.