J. Orchid Soc. India, 39: 193-197, 2025 *ISSN 0971-5371*
https://doi.org/10.64873/JOSI.v39.i1-2. 193-197

IN VITRO PROPAGATION AND CONSERVATION OF *RENANTHERA IMSCHOOTIANA* ROLFE- AN ENDANGERED ORCHID SPECIES OF NORTHEAST INDIA

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Abstract

Renanthera imschootiana is an endangered orchid species listed under CITES Appendix I, indicating that it is threatened with extinction and highly restricted from trade; it is found only in the NorthEastern states of India. The present study deals with its *in vitro* seed germination and seedling development. Seeds procured from green and undehisced capsules were cultured on Murashige and Skoog (1962; MS) medium containing different concentrations and combinations of Kinetin and/or GA_3 representesd as T_1-T_9. The results revealed that the seed germination took only 48.47 days on MS+0.5 mgL^{-1} KN+ 1.0 mgL^{-1} GA_3 medium (T_8), while MS+1.0 mgL^{-1} +KN+1.0 mgL^{-1} (T_9) promoted early protocorm formation (78.40 days), seedling development (96.60 days) and maximum plant height (6.90 cm). The maximum number of shoots per plant (5.20) with the maximum number of leaves (6.33) was found in T_7 (MS+1.0 mgL^{-1} KN+1.0 mgL^{-1} GA_3). However, the longest leaves (1.96 cm) were found in T_8 treatment and the maximum leaf breadth (1.11 cm) was observed in T_9 treatment. The T_9 treatment also favoured the maximum number of plantlets (4.93) with the thick (1.063 mm) and maximum roots; while T_7 treatment showed comparatively longer roots in the plantlets (4.67 mm). *In vitro* conservation of Red *Vanda* can be achieved through the use of immature seeds from green capsules (*pods*) at a rapid rate in a short period of time on MS+KN (0.5-1.0 mgL^{-1}) + GA_3 (0.5-1.0 mgL^{-1}). The seedlings were later hardened in in pots with vermiculite potting mixture and then replanted containing potting mixture comprising broken brick pieces and charcoal; these showed survivability rate of 90.10%.

Introduction

RED *VANDA* (*Renanthera imschootiana* Rolfe), an epiphyte belonging to the plant family Orchidaceae has been classified as an ally to the *Vanda* group. It grows well on shrubs, tree trunks, and branches of tall trees at an elevation of 300-500 m amsl and blooms during spring. It is the only species belonging to the genus *Renanthera* and found only in the NorthEastern states of India endemic to three states Manipur, Mizoram, and Nagaland, which come under the Indo-Burma mega bio-diversity hot spot. It has become an endangered tropical epiphytic orchid due to its over-collections as ornamental plant or as one of the breeding parents. As a protective measure for conserving our rich orchid diversity, this species was listed in Appendix-I, CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna). *In vitro* propagation has become the preferred method for clonal propagation in orchids, offering advantages such as genetic fidelity and high multiplication rates (Arora *et al*., 2004; Bhowmik and Rahman, 2022, 2023; Dhillon and Pathak, 2023; Gangaprasad *et al*., 2024; Pathak *et al*., 2001, 2016, 2023; Jaryal *et al*., 2025a, b; Piri *et al*., 2013; Sembi *et al*., 2011; Verma *et al*., 2013). The technique of *in vitro* propogation using seed culture has been successfully used presently in *Renanthera imschootiana*, so as to meet its growing demand for commercial importance. The objective of the present experiment was to conserve this endangered species and to develop disease-free plantlets through *in vitro* asymbiotic seed germination and seedling development.

Material and Methods

Green and undehisced capsules were collected from the Manipur state Orchidarium, Khonghampat, Imphal, West, Manipur. After collection, the capsules were washed under running water with a few drops of Tween-20 for a few min to remove the debris present on the surface of the capsules. Later, these were treated with 1% Dhanustin (fungicide) for 10-15 min and then washed properly with running tap water. Inside the laminar air flow chamber, sterilised with UV rays and High-Efficiency Particulate Air (HEPA), the work surface was wiped with 75% ethanol. Surface sterilisation of the capsules was performed by flaming these with 75% alcohol, subsequently dipping these in the sterilised distilled water, and allowing these to dry. The sterilized capsules were excised for the extraction of immature seeds (Fig. 1A); these seeds were scooped out with the help of a spatula and inoculated on Murashige and

Received: November 10, 2025; Accepted: December 22, 2025

Table 1. Effect of different treatments of PGRs on *in vitro* asymbiotic seed germination and seedling development in *Renanthera imschootiana.*

Treatments	Time taken in days for			Plant height (cm)	Leaf length (cm)	Leaf breadth (cm)	Number of leaves/ seedling	Number of roots/ seedling	Length of roots/ seedling(cm)	Diameter of roots (mm)
	Seed germination	Protocorm formation	Seedling development							
T_1- Basal medium	121.67	193.73	262.40	2.22	0.64	0.47	3.67	1.27	1.11	0.545
T_2–MS+ 0.5 mgL^{-1} KN	92.80	138.20	172.00	4.03	0.66	0.56	5.47	3.07	2.10	0.923
T_3–MS+ 1.0 mgL^{-1} KN	90.13	112.27	145.47	4.21	1.17	0.61	5.87	3.13	2.14	0.929
T_4–MS+ 0.5 mgL^{-1} GA_3	71.73	94.87	131.47	4.94	1.31	0.91	4.87	2.80	1.94	0.643
T_5–MS+ 1.0 mgL^{-1} GA_3	76.00	92.87	129.07	5.55	1.65	0.98	5.00	2.87	2.17	0.700
T_6–MS+ 0.5 mgL^{-1} KN + 0.5 mgL^{-1} GA_3	62.13	81.27	110.27	5.05	1.90	1.00	6.13	3.40	3.73	1.038
T_7–MS+ 1.0 mgL^{-1} KN + 0.5 mgL^{-1} GA_3	59.00	82.33	106.00	5.13	1.95	1.02	6.33	4.20	4.67	1.074
T_8–MS+ 0.5 mgL^{-1} KN + 1.0 mgL^{-1} GA_3	48.47	80.60	106.73	5.62	1.96	1.07	5.93	3.87	3.05	1.047
T_9–MS+ 1.0 mgL^{-1} KN + 1.0 mgL^{-1} GA_3	52.93	78.40	96.60	6.90	1.93	1.11	6.00	4.93	2.75	1.063
S.E(m)±	**2.97**	**5.08**	**6.71**	**0.23**	**0.24**	**0.08**	**0.26**	**0.24**	**0.25**	**0.064**
C.D. (5%)	**8.84**	**15.09**	**19.93**	**0.68**	**0.70**	**0.25**	**0.77**	**0.71**	**0.74**	**0.190**

Skoog (1962) agar-gelled medium with different concentrations and combinations of growth regulators (KN, GA_3) represented as T_1-T_9 (Table 1). Before autoclaving at 121°C for 20 mins, the pH of the nutrient medium was adjusted at 5.8. The culture tubes were wrapped with aluminium foil and kept in the culture room at a temperature of 23±2°C. The observations were recorded at regular time intervals.

Results and Discussion

The present study reports the successful *in vitro* asymbiotic seed (immature) germination of *Renanthera imschootiana.* MS basal medium supplemented with different doses of plant growth regulators such as KN and GA_3, either alone or in combination, had a significant effect on time taken for germination, protocorms formation, and development of seedlings (Table 1). The ability of the orchid seeds to germinate prior to reaching maturity has been mentioned earlier by few authors (Arditti *et al.,* 1982a,b; Pathak *et al.,* 2001; Kirti *et al*., 2023) and such a culture of immature seeds is referred to as *Green Pod Culture*. This technique is very useful as it follows an easy sterilization procedure and helps in reduction of time lapse between flower pollination and seed sowing. Immature seeds are known to exhibit better germination because of their distended testa cells, metabolically awakened embryos, and absence of dormancy factors (Arditti *et al.,* 1981).

In the basal medium the seeds took long to germinate (121.67 days). The protocorms obtained in 193.7 days differentiated first leaf and root primordia and subsequently develop into seedlings in 262.40 days. Seeds, when inoculated on MS basal medium fortified with 0.5 mgL^{-1} KN+ 1.0 mgL^{-1} GA_3, showed early seed germination (48.47 days). The first signs of germination were evident with the swelling of the embryos in the seeds (which subsequently emerged as spherules from busted seed coats) and change in the embryo colour from white to light green (Fig. 1B). The same treatment combination also had a pronounced effect on leaf length (1.96 cm). MS medium supplemented with 1.0 mgL^{-1} KN + 1.0 mgL^{-1} GA_3 (T9) favoured early (78.40 days) protocorm formation (Fig. 1C) and seedling

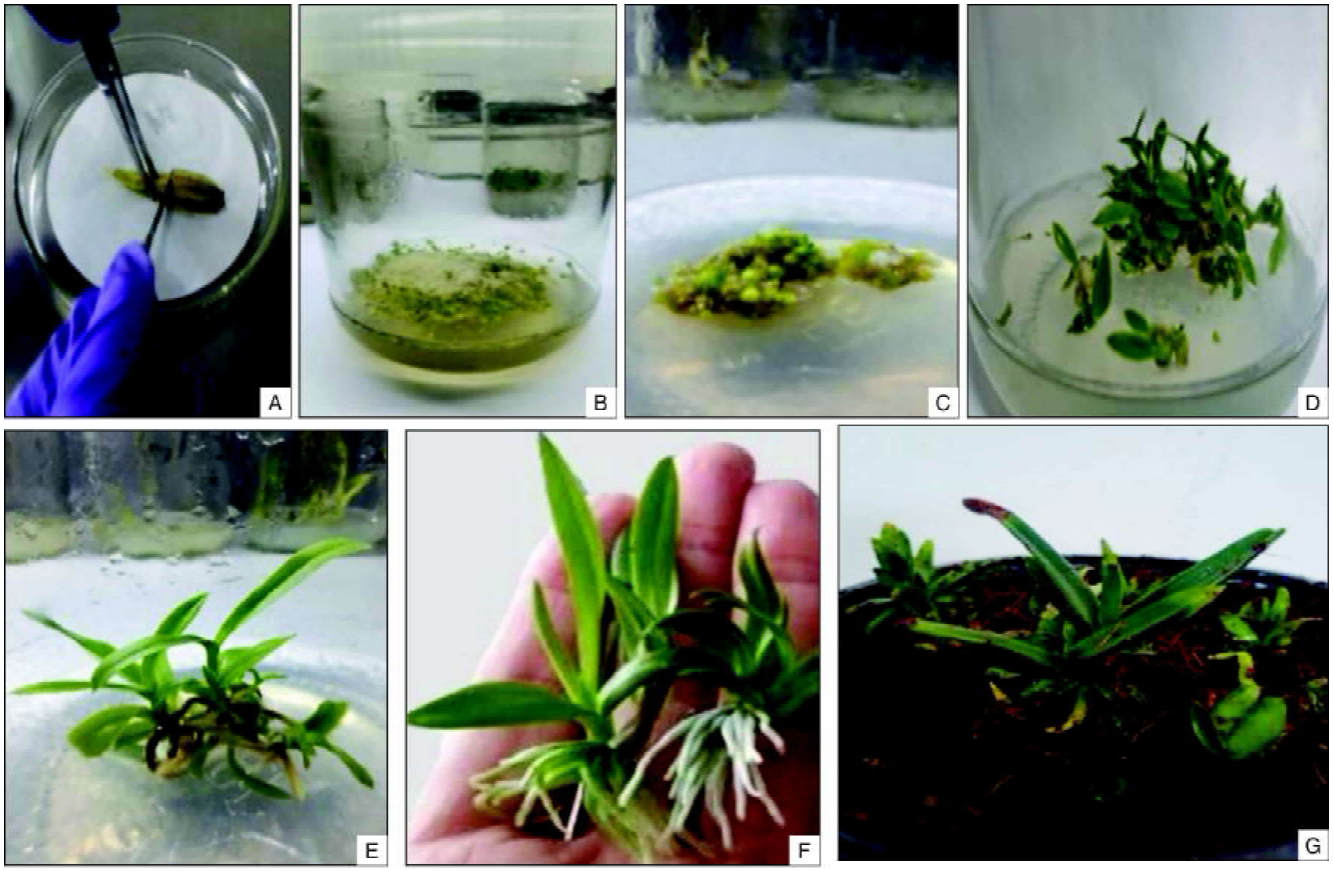

Fig. 1A-G. *In vitro* propogation and conservation of *Renanthera imschootiana* using immature seeds: A, Split open capsule for extraction of seeds; B, Onset of seed germination and formation of spherules; C, Protocorm formation; D, Leaf and root differentiation; F, Rooted seedlings subjected to hardening; E, Complete seedlings; G, Well-hardened seedlings.

development (96.60 days) (Fig. 1D); the combination also promoted maximum plant height (6.90 cm), with broader leaves (1.11 cm) and maximum number (4.93) and broader (1.063 mm) roots per seedling. The treatment T_7 (MS+ 1.0 mgl^{-1} KN + 0.5 mgl^{-1} GA3) induced the multiplication of the protocorms (Fig. 1E) and favoured the maximum number of leaves per plant (6.33) and comparatively longer roots (4.67 cm). Earlier, literature studies reveal the use of KN and/ GA_3 during *in vitro* propagation of orchids though with varied results (Pathak *et al*., 1992, 2017; Singh and Babbar, 2016; Thakur and Pathak, 2020; Vij *et al*., 1995). Udayan *et al.* (2018) indicated the combination of KN and Gibberellic acid exhibits a synergistic effect on lipid yield and increases the percentage of eicosapentaenoic acid in *Nannochloropsis oceanica* that increases in the rapid growth of the cells. MS medium is highly enriched with macro and micro elements, with different vitamins that improve the nutritional status and favour *in vitro* propagation (Arditti *et al*., 1982a,b; Bhowmik and Rahman, 2023; Gangaprasad *et al*., 2024; Pathak *et al*., 2001, 2023).

For the purpose of hardening, well-rooted *in vitro* raised *Renanthera imschootiana* seedlings (Fig. 1F) were removed from the culture vessels, washed repeatedly under running tap water to remove the traces of semisolid agar medium and then treated with fungicide (Dhanustin 50 W.P.) @ $1gL^{-1}$. Later, the seedlings were transferred to the pots having different well-sterilised potting media comprising coco-chips, cocopeat, vermiculite, perlite, broken brick pieces and charcoal pieces (Table 2). Amongst these six different hardening potting media used (Table 2), vermiculite (90.10%) was proved as the best medium for survival of *Renanthera* seedlings followed by perlite (89.20%). These findings may be due to the moisture-retaining capacity of these potting media as compared to the rest of the hardening potting media employed resulting thereby in the better establishment of the *in vitro* raised seedlings. Similar observations were made earlier in *Calanthe odora* (Gantait *et al*., 2020), *Dendrobium nobile* (Laishram *et al*., 2019) and *Phaius tankervilleae* (Thokchom *et al*. (2017). During the present hardening procedure, the seedlings were kept inside the growth chamber, maintaining 25°C temperature, 80-90 % relative humidity and 4000 lux light density for a few wks and later re potted in pots with potting mixture comprising broken brick pieces and charcoal (Fig. 1G).

Table 2. Effect of different hardening media on per cent survivability of *in vitro* raised *Renanthera imschootiana* seedlings.

Hardening media	Survivability percentage (%)
Charcoal	54.80
Cocopeat	70.50
Vermiculite	90.10
Perlite	89.20
Broken brick pieces	53.95
Coco-chips	60.25
SEm±	**2.28**
CD at 5%	**6.77**

Conclusion

The present study reports an efficient protocol for *in vitro* asymbiotic seed germination and seedling development in *Renanthera imschootiana*, an endangered orchid species of NorthEast India. The seedlings were successfully hardened in pots with vermiculite potting mixture and then replanted containing potting mixture comprising broken brick pieces and charcoal; these showed survivability rate of 90.10%. The present data may prove useful for *in vitro* propagation of other related commercially important and endangered orchid species.

Acknowledgements

The authors sincerely thank the Manipur State Orchidarium, Khonghampat, Imphal West, Manipur, for providing the explants, and Central Agricultural University, Imphal, for the encouragement and research facilities.

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