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Optimized *In vitro* Propagation, Acclimatization and Reintroduction of Endemic *Rhododendron inaequale* Hutch. from Northeast India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Rhododendron inaequale Hutch., an endemic plant with high economic potential and susceptibility, requires urgent conservation measures. This study aimed to develop an optimized *in vitro* propagation protocol for this species. *In vitro* seed germination was conducted using Anderson medium, Woody Plant Medium (WPM), and MS Medium. WPM supplemented with 8 mg L^{-1} 2iP [N_6-(2-Isopentenyl) adenine] yielded the highest multiple shoot induction (3.55 ± 0.14 shoots, 0.77 ±

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0.03 cm shoot length). For rooting, WPM with 0.5 mg L^{-1} IBA [Indole-3-butyric acid] produced the maximum number of roots (3.40 ± 0.43) with a mean root length of 1.84 ± 0.12 cm. Successfully acclimatized plants were reintroduced into natural habitats in various locations in Meghalaya and Nagaland. Additionally, 200 micropropagated plants were distributed for further conservation initiatives. This study provides a crucial protocol for the conservation and reintroduction of *R. inaequale*, aiding in the preservation of this vulnerable species.

Keywords: Rhododendron inaequale; endemic; micropropagation; ex situ conservation; reintroduction.

1. INTRODUCTION

"Rhododendron inaequale Hutch., an endemic species found in northeastern India, has tremendous economic potential due to its horticultural value and therapeutic properties. However, habitat destruction and other human activities are posing an increasing threat to its survival, which makes the conservation of this species a critical priority. The genus *Rhododendron* belonging to the family Ericaceae is one of the largest genera, comprising an estimated 1200 species with considerable ecological and economic significance" [1]. "The genus name *Rhododendron* is derived from two Greek words, "*rhodon*" and "*dendron*," which mean "rose" and "tree" (rose tree), respectively" [2]. "This genus is particularly valued for its attractive flowers, which provide a breathtaking view of hills and mountain slopes during the flowering season" [3]. "*Rhododendron* exhibits incredible species diversity and distribution throughout the world, ranging from Southeastern Asia, specifically the regions of Northwestern Himalaya through Nepal, Sikkim (India), Eastern Tibet, Bhutan, Northeastern India (Arunachal Pradesh, Mizoram, Manipur, Nagaland, and Meghalaya), and upper Myanmar, to Western and Central China" [4]. "These species inhabit a wide elevation gradient between 800 to 6000 meters" [1]. "Of the 135 species of *Rhododendrons* in India, 132 are found in the northeastern part of the country, making this area a hotspot for the genus's diversity" [3]. "Most *Rhododendron* species are popular for their immense horticultural value. Additionally, many species possess various therapeutic potentials, including antibacterial, anti-inflammatory, and antioxidant properties" [5].

Indian *Rhododendrons* play the role of keystone species in Himalaya regions, especially in Eastern Himalayas. It is estimated that approximately 97% of *Rhododendron* species in the Himalayas are losing their identity due to indiscriminate felling and loss of habitat. This is causing *Rhododendron* flowering plants vulnerable in their natural habitat and slowly leading to the extinction of species. In the recent few decades, the Indian Himalayan has been greatly affected by various threats imposed by nature and other anthropogenic activities. Increasing human population and associated activities through direct and indirect involvement is heavily loading pressure on forests and naturing wildlife population. The primary forests are degraded into scrub lands by rapid deforestation and desertification. *Rhododendrons* are routinely cut for firewood by local people in the summer season, thus threatening the survival of many species. According to Menon et al. [6], uncontrolled, indiscriminate, and unsustainable harvesting for firewood has resulted in several *Rhododendron* species under rare, endangered, and threatened categories. "Change in climatic variables is one of the important factors affecting the surviving population of *Rhododendrons*. Indiscriminate grazing and jhum cultivation have threatened the natural habitat of *Rhododendron* species up to a large extent" [7].

Rhododendron inaequale Hutch. is endemic to Arunachal Pradesh, Manipur, Meghalaya, and Nagaland, India [3]. It belongs to the family Ericaceae and is listed as Data Deficient by the IUCN. This species is found at higher elevations and is known for its strong fragrance. Currently, *R. inaequale* is threatened in Meghalaya due to the ongoing Shillong–Dawki bypass road construction, which is destroying its habitat. Human interference has led to the gradual decline of natural populations of *R. inaequale* in the region. The species must be mass propagated in large numbers and reintroduced to ecologically appropriate environments. More research is needed to measure threat levels, population status, and analyze survival mechanisms. Therefore, it is essential to prioritize additional research and conservation efforts aimed at safeguarding the remaining species that are highly vulnerable to extinction.

Although vegetative propagation of *Rhododendron* is practiced in many nurseries in

Europe and America, mass propagation remains a slow process for most *Rhododendron* species. Singh et al. [8] reported poor seedling regeneration in many *Rhododendron* species. Singh and Gurung [9] developed an *in vitro* propagation protocol for *Rhododendron maddeni* Hook. F., an endangered species from Sikkim Himalaya. Singh et al. [10] studied the *in vitro* propagation of *Rhododendron griffithianum* Wt., another endangered species from the same region. Sekar and Srivastava [11] conducted studies on the diversity and conservation of *Rhododendrons* in the Indian Himalayan Region. Mao et al. [3] reported *in vitro* propagation of *Rhododendron macabeanum* Watt ex Balf.f., an endangered and endemic species from Manipur and Nagaland. Mao et al. [3] also investigated the *in vitro* propagation of *Rhododendron wattii* Cowan, a critically endangered and endemic plant from India.

To date, there is no established protocol for the micropropagation of *R. inaequale*. Therefore, the main objective of this study is to develop a successful *in vitro* propagation protocol for the conservation and reintroduction of this species into its natural habitat.

2. MATERIALS AND METHODS

2.1 Collection of Seeds

Seed pods of *R. inaequale* (Fig. 1b) were collected from Laitlyngkot, East Khasi Hills District, Meghalaya. *In vitro* seed germination experiments were conducted immediately after collection.

2.2 *In Vitro* Seed Germination

Three different basal media, namely, Anderson medium (AM, Anderson) [12] Woody Plant Medium (WPM, McCown and Lloyd) [13] and MS Medium (MS, Murashige and Skoog) [14] supplemented with 100 mg L^{-1} inositol, 0.4 mg L^{-1} thiamine, 3% (w/v) sucrose, and 0.8% (w/v) agar were used for *in vitro* seed germination for a comparative study because they are commonly employed for micropropagation of *Rhododendron* [15]. "pH of the medium was adjusted to 5.8 by adding 1N HCl or 1N NaOH. The media were dispensed into 100 mL conical flasks and autoclaved at 121℃ and 1.05 kg/cm^2 for 20 minutes. Seed packets were made with sterile filter paper (Whatman™No. 1; Sigma-Aldrich®, St. Louis, MO) containing 10 seeds per pack. Seeds (Fig. 1c) were thoroughly washed with 2-3 drops of Tween®-80 per 100 mL for 10 minutes and washed with distilled water. Surface sterilization was done inside the Laminar Air Flow Cabinet using 10% (v/v) sodium hypochlorite (4% w/v solution, HiMedia Laboratories Pvt. Ltd.) solution containing 2 drops of Tween®-80 per 100 mL for 20 minutes and subsequently washed eight to ten times in sterile water. All the cultures were maintained under warm white fluorescent light with a 16h photoperiod and at 25±2℃. The initiation of seed germination and the cumulative percentage of seed germination were recorded at weekly intervals for 7 weeks" [16].

2.3 Multiple Shoot Induction

"Nodal segments from two to three months old *in vitro* raised seedlings (Fig. 1e) were used as explants to induce *in vitro* shoot proliferation. One explant containing a single node (0.3 to 0.5 cm long) was cut inside the Laminar Air Flow Cabinet and placed in test tubes (20 × 150 mm) containing 15 mL of nutrient medium. Woody Plant Medium (WPM) supplemented with vitamins, 3% (w/v) sucrose, and 0.8% (w/v) agar with different concentrations of 2iP [N^6-(2-Isopentenyl) adenine] with and without 0.2% Activated charcoal and BAP [6-Benzylaminopurine] viz., 1 mg L^{-1}, 2 mg L^{-1}, 4 mg L^{-1}, and 8 mg L^{-1} were used for setting up the experiment of multiple shoot induction following Mao et al. [16]. Basal medium was used as the control treatment. Twenty explants were set up for each treatment to compare the effect of different concentrations of Activated charcoal, 2iP and BAP. pH of the medium was adjusted to 5.8 by adding 1N HCl or 1N NaOH. The media were dispensed into culture tubes and autoclaved at 121℃ and 1.05 kg/cm^2 for 20 minutes. All the cultures were maintained under warm white fluorescent light with a 16h photoperiod and at 25±2℃. The number and length of shoots were recorded weekly and subculturing was done at regular intervals" [16]

2.4 *In Vitro* Rooting

The individual shoots of 1.5-2.0 cm length were isolated from the shoot clumps and were placed in 20 × 150 mm test tubes containing WPM solidified with 0.8% (w/v) agar (Plant culture tested, HiMedia® Laboratories, Mumbai, India) with activated charcoal (0.2%) with IBA [Indole-3-butyric acid] and NAA [1-Naphthaleneacetic acid] at 0.5 mg L^{-1} and 1 mg L^{-1} for *in vitro* rooting

experiments. Basal WPM with activated charcoal (0.2%) was also tested for root induction. Ten shoots were used for each treatment, and the experiment was repeated three times. Initiation of the roots, data for the number of roots and root length were recorded after 7 weeks of culture to enable enough time for root development to be measurable and comparable across the various treatments examined.

2.5 Data Analysis

The following formula was used in the statistical analysis to determine the final germination percentage (GP)

> Germination percentage (%) = mean number of germinated seeds / total number of seeds inoculated in each flask × 100.

Data for multiple shoot induction and rooting were analyzed using OriginPro 8SRO v8.0725 (B725) (OriginLab® Corporation, Northampton, MA) software, subjected to one-way ANOVA, and the treatment means were compared by Tukey's test at the significant level of $P = 0.05$.

2.6 Acclimatization

Two to three months old *in vitro* rooted plants with healthy shoots were transferred to the polyhouse. Before the transfer, plantlets were washed with sterile distilled water and treated with systemic fungicide [Bavistin 0.1% (w/v)] for one hour and planted in paper disposable cups containing a mixture of autoclaved compost [garden soil, soil inoculum, rotten wood, and leaf mould (3:1:1:1)]. Soil inoculum was collected by digging out soil from the rhizosphere (up to 30 cm) of *R. arboreum* trees found in the natural habitat and then mixed with garden soil and coarse sand. The plants were maintained at 25℃ in polyhouse with RH (60%) and watered on alternate days.

3. RESULTS AND DISCUSSION

3.1 *In Vitro* Seed Germination

In vitro seed germination was observed after eight days of inoculation in WPM, whereas seed germination started after twelve days in MS Medium and AM. After seven weeks of observation, it was observed that seed germination percentage was highest in WPM i.e., 75% (Table 1). The optimum seed germination was obtained within 4-6 weeks with an average number of 6.2 ± 0.25 seeds in MS, 7.6 ± 0.34 seeds in WPM, and 5.2 ± 0.25 seeds in AM (Fig. 1d). Subculturing the germinated seedlings in their respective germination media showed similar results as was observed in *R. formosum*. Therefore, further experiments were carried in WPM. The observed variance in seed germination across different media emphasizes the importance of mineral salt concentrations on *in vitro* germination. In this investigation, WPM outperformed MS Medium and AM in terms of germination percentage and onset time. These findings are consistent with prior research demonstrating that WPM, with its lower mineral nutrient concentrations, is particularly favorable for Ericaceae species that are acclimated to soils with low pH and nutrient availability [17,18].

Trivedi and Joshi [19] highlighted the importance of mineral salt concentration in determining the success of *in vitro* germination, which could explain the differences we found between WPM and MS Medium. Furthermore, the acceptability of WPM for shoot culture in *Rhododendron fortunei* has been connected to its reduced mineral nutrient content, supporting our decision to continue further experiments using WPM [18].

Table 1. Comparative study of *in vitro* seed germination of *Rhododendron inaequale* in different basal media

Media	Mean number of seeds germinated (weekly interval)						
	2 weeks Mean ± SE	3 weeks Mean ± SE	4 weeks Mean ± SE	5 weeks Mean ± SE	6 weeks Mean ± SE	7 weeks Mean ± SE	Percentage (%)
MS	1.0 ± 0.15	2.4 ± 0.16	3.8 ± 0.20	5.4 ± 0.27	6.2 ± 0.25	6.2 ± 0.25	62
WPM	1.2 ± 0.13	3.3 ± 0.15	5.0 ± 0.21	6.5 ± 0.31	7.5 ± 0.31	7.6 ± 0.34	75
AM	0.8 ± 0.13	2.0 ± 0.21	3.2 ± 0.20	4.5 ± 0.17	5.1 ± 0.23	5.2 ± 0.25	51

 # Values expressed as mean ± SE (Standard error)

Fig. 1. *In vitro* propagation of *Rhododendron inaequale* Hutch. (a) *R. inaequale* mother plant in its natural habitat (b) Seed pod (c) Seeds (d) *In vitro* seed germination on Woody Plant Medium (WPM) (e) Nodal explant from three month old *in vitro* grown seedling inoculated for shoot multiplication (f) Multiple shoot induction on WPM with 8 mg L^{-1} 2iP after four weeks in culture (g) Multiple shoots developed after repeated subculture at 25 days interval (h) *In vitro* rooting after twelve weeks in culture on WPM containing 0.2% (w/v) activated charcoal and 0.5 mg L^{-1} IBA (i) *In vitro* rooted plants ready for acclimatization (j) Six months old plants grown in polythene bags for acclimatization (k and l) Reintroduction in Mawdngong Eco park, Mawlyndiar village, East Khasi Hills, Meghalaya

3.2 Multiple Shoot Induction

The classical cytokinin used for micropropagation of evergreen *Rhododendron* spp. is 2iP [20]. In our study, 2iP proved to be a more effective cytokinin than BAP for multiple shoot induction. It was observed that WPM supplemented with 2iP (8 mg L^{-1}) showed maximum mean shoot number (3.55 ± 0.14) and highest mean shoot length (3.34 ± 0.29 cm) was observed in 2iP with 0.2% Ac (4 mg L^{-1}) (Fig. 1 f and Fig. 1 g) (Table 2). Shoot initiation was observed after 3 weeks of culture. BAP on the other hand, produced shoots that were thin, small, and showed stunted growth. This result proves the reports by McCown and Lloyd [13] and Cantos et al. [21] which showed that BAP was inferior to 2iP for induction of multiple shoots in *Rhododendron* spp. BAP has been proven to be hazardous for certain *Rhododendron* spp. [20]. A regular subculture in every 4 weeks increased the multiplication rate which became maximum after three to four subculture cycles.

In the present investigation, 2iP (8 mg L^{-1}) induced the greatest number of shoots but the longest shoots were observed in 2iP with 0.2% Ac (4 mg L^{-1}) on cultured nodal sections (Table 2). Whereas, 2iP with 0.2% Ac yielded only a single shoot but the shoots were tall in length compared to 2iP alone. Activated charcoal is able to absorb high concentrations of growth regulators in both liquid and solid media. The effects of activated charcoal may be attributed to establishing a darkened environment; adsorption of undesirable/inhibitory substances; adsorption of growth regulators and other organic compounds, or the release of growth promoting substances present in or adsorbed by activated charcoal. This can lead to a reduction in the availability of 2iP to the plant tissues, thereby inhibiting the initiation of multiple shoots [22]. Activated charcoal creates a darkened environment in tissue culture vessels, which may interfere with the photosynthetic process necessary for shoot initiation. Moreover, its ability to adsorb growth regulators and other organic compounds from the media can alter the hormonal balance required for optimal shoot induction [23].

3.3 Rooting

“NAA and IBA are highly effective in root induction studies of *Rhododendron* species plantlets, resulting in robust roots with greater root proliferation and transplants with higher survival rates” [24]. “In our study, rooting was observed on a few shoots in auxin-supplemented WPM after just 3 weeks in culture. After 8 weeks, all auxins and concentrations tested successfully induced rooting (Table 3). The medium containing 0.5 mg/L IBA produced the greatest number of roots (3.4 ± 0.43) (Fig. 1h) and also yielded the longest roots (1.84 ± 0.12 cm). This finding is consistent with earlier research indicating that IBA is ideal for root induction in *Rhododendron* spp” [25-27,9]. “The current study’s results corroborate these conclusions and further highlight the effectiveness of IBA in promoting root development in micropropagated *Rhododendron* plants, aligning with recent findings” by Wu et al. [24,28,29].

Table 2. Multiple shoot induction experiment of *Rhododendron inaequale* in WPM using different cytokinins after 12 weeks in culture

Plant growth regulator	Concentration (mg/L)	Number of shoots Mean no. of shoots ± SE	Shoot length Mean shoot length (cm) ± SE
2iP	Control	1.0	0.45 ± 0.02
	1	1.65 ± 0.11	0.40 ± 0.02
	2	1.75 ± 0.10	0.61 ± 0.02
	4	2.60 ± 0.15	0.71 ± 0.02
	8	3.55 ± 0.14	0.77 ± 0.03
2iP with 0.2% Ac	1	1.3 ± 0.15	2.17 ± 0.27
	2	1 ± 0	2.56 ± 0.17
	3	1.1 ± 0.10	2.69 ± 0.21
	4	1.1 ± 0.10	3.34 ± 0.29
BAP	1	1 ± 0	0.79 ± 0.10
	2	1.2 ± 0.13	0.64 ± 0.08
	4	1.1 ± 0.10	0.61 ± 0.07
	8	1.1 ± 0.10	0.65 ± 0.08

 # Values expressed as mean ± SE (Standard error)

Table 3. Effect of different auxins or activated charcoal on root induction after 9 weeks in culture

Plant growth regulator	Concentrations (mg L^{-1})	Number of roots Mean no. of roots ± SE	Root length Mean root length (cm) ± SE
Activated charcoal0.2 %	-	2.0 ± 0.21	1.22 ± 0.11
NAA	0.5	1.7 ± 0.37	1.12 ± 0.10
	1	2.0 ± 0.39	1.56 ± 0.24
IBA	0.5	3.4 ± 0.43	1.84 ± 0.12
	1	2.8 ± 0.25	1.35 ± 0.20

 # Values expressed as mean ± SE (Standard error)

3.4 Acclimatization

"The primary goal of undertaking *in vitro* propagation of *R. inaequale* was to increase the number of individual plantlets. Constraints included limited availability of seeds for culture initiation due to habitat destruction and geographic isolation of these materials for collection. 60% of *in vitro* raised plants transferred from lab to greenhouse successfully established in field conditions" (Fig. 1j) [16]. Near about 30 healthy plants have been reintroduced in both office garden of BSI, Shillong and the experimental Botanic Garden, Barapani, Meghalaya. Approximately 20 hardened and acclimatized plants have been reintroduced into their natural habitats in *Rhododendron* Park, Jakhama, Nagaland by the third author. 10 plants were reintroduced by the first and second author in Mawdngong Eco Park, Mawlyndiar village, East Khasi Hills, Meghalaya (Fig. 1k and Fig. 1l) and around 200 micropropagated plants were handed over to the Forest Department, Meghalaya, various organizations, institutions and stakeholders in Meghalaya for their reintroduction initiatives in different parts of Northeast India and to facilitate further research, conservation, and restoration efforts. These plants are intended to boost natural populations and facilitate further research into the species' survival and adaptation in its native environment.

4. CONCLUSION

This study represents a pioneering effort in *in vitro* propagation, *ex vitro* acclimation, and reintroduction of *R. inaequale*. The findings are crucial for advancing conservation efforts aimed at preserving this species, which faces significant threats from habitat destruction and human activities. By developing a successful *in vitro* propagation protocol, this research provides a valuable tool for the mass propagation and reintroduction of this endangered species into its natural habitat. The technique developed in this study offers a promising approach for conserving this endemic species and facilitating ecosystem restoration in the Indo-Burma biodiversity hotspot region.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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