Original Research Article

Rapid *in vitro* propagation, *ex vitro* acclimation and reintroduction of *Rhododendroninaequale* Hutch- an endemic plant to Northeast India

ABSTRACT

Rhododendroninaequale Hutch requires abrupt conservation measures *in situ* and *ex situ* for its high economic potential and susceptibility. An optimized *in vitro* propagation protocol was standardized for this endemic plant. *In vitro* seed germination was carried out using Anderson medium, Woody Plant Medium and MS Medium. WPM supplemented with different concentrations of 2iP [N^6-(2-Isopentenyl) adenine] and BAP [6-Benzylaminopurine] were used for multiple shoot induction. *In vitro* rooting experiment was conducted using IBA [Indole-3-butyric acid] and NAA [1-Naphthaleneacetic acid] with (0.2%) activated charcoal. The addition of 2iP at 8mg L^{-1} stimulated the highest number of multiple shoots (3.55 ± 0.14) and the highest mean shoot length (0.77 ± 0.03 cm). WPM with 0.5 mg L^{-1} IBA promoted the maximum number of roots (3.40 ± 0.43), with mean root length of 1.84 ± 0.12cm. *In vitro* raised plants transferred from lab to greenhouse successfully acclimatized in *ex vitro* conditions. Sixty hardened and acclimatized plants were reintroduced into their natural habitats in Botanical Survey of India, Shillong, Rhododendron Park, Jakhama, Nagaland and Mawdngong Eco Park, Mawlyndiar village, Meghalaya. Another 200 micropropagated plants were distributed to the Forest Department, Meghalaya, various institutions and stakeholders in Meghalaya for their reintroduction initiatives and *ex situ* conservation.

Keywords: *Rhododendroninaequale,* endemic, micropropagation, *ex situ* conservation, reintroduction

1. INTRODUCTION

The genus *Rhododendron* is one of the largest genera, comprising an estimated 1200 species with considerable ecological and economic significance (Shootha et al., 2022). It belongs to the family Ericaceae and was first described by Carl Linnaeus in Species Plantarum (1737): Irrelevant. The genus

name *Rhododendron* is derived from two Greek words, "*rhodon*" and "*dendron*," which mean "rose" and "tree" (rose tree), respectively (Wu et al., 2015). This genus is particularly valued for its attractive flowers, which provide a breathtaking view of hills and mountain slopes during the flowering season (Mao and Gogoi, 2012). *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 (Paul et al., 2016). These species inhabit a wide elevation gradient between 800 to 6000 meters (Shootha et al., 2022). 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 (Mao et al., 2017). Most *Rhododendron* species are popular for their immense horticultural value. Additionally, many species possess various therapeutic potentials, including antibacterial, anti-inflammatory, and antioxidant properties (Kumar et al., 2019; Kumar et al., 2020; Balkrishna et al., 2022).: Why 3 refe for small paragraph

Indian *Rhododendrons* plays the role of keystone species in Himalaya regions especially in Eastern Himalaya. It is estimated that approximately 97% of *Rhododendron* species in Himalaya is losing their identity due to indiscriminate felling and loss of habitat. This is causing *Rhododendron*flowering plants vulnerable in natural habitat and slowly leading to extinction of species. In the recent few decades Indian Himalayan is greatly affected by various threats imposed by the nature and other anthropogenic activities. Increasing human population and associated activities through direct and indirect involvement is heavily loading pressure on forests and naturing wild life population. The primary forests are degraded into scrub lands by rapid deforestation and desertification. *Rhododendrons* routinely cut for firewood by local people in summer season, and thus threatening the survival of many species. According to Menon et al. (2012) uncontrolled, indiscriminate and unsustainable harvesting for firewood has resulted 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. Roadways and hydel power projects are also imposing equal pressure on the natural habitat and population survival for *Rhododendron* species (Singh and Singh, 2019). Irrelevant.

Rhododendron inaequale Hutch. is endemic to Arunachal Pradesh, Manipur, Meghalaya, and Nagaland, India (Mao et al., 2017). 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. (2008) reported poor seedling regeneration in many *Rhododendron* species. Singh and Gurung (2009) developed an *in vitro* propagation protocol for *Rhododendron maddeni* Hook. F., an endangered species from the Sikkim Himalaya. Singh et al. (2016) published *in vitro* propagation of *Rhododendron griffithianum*Wt., another endangered species from the same region. Sekar and Srivastava (2010) conducted studies on the diversity and conservation of *Rhododendrons* in the Indian Himalayan Region. Mao et al. (2017) published*in vitro* propagation of *Rhododendron macabeanum* Watt ex Balf.f., an endangered and endemic species from Manipur and Nagaland. Mao et al. (2018) also investigated the *in vitro* propagation of *Rhododendron wattii* Cowan, a critically endangered and endemic plant from India.

Till date, there is no established protocol for the micropropagation of *R. inaequale*. Therefore, this study aims 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 1984), Woody Plant Medium (WPM, McCown and Lloyd 1981) and MS Medium (MS, Murashige and Skoog 1962)(Why only these media used) 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. 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.

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. Basal medium was used as the control treatment. Twenty explants were set up for each treatment for comparing 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.

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 0.5mg 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.: Encode reference or give reason

2.5 DATA ANALYSIS

The following formula was used in the statistical analysis to determine the final germination percentage (GP) (Aniat-ul-Haq and Agnihotri 2010). Irrelevant: No need ref for formula.

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

2-3: write in between text 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 1h 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 was 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

3.2 *IN VITRO* SEED GERMINATION

In vitro seed germination was observed within(Specify) 2 weeks in all the three basal media, namely, MS, WPM, and AM. It was observed that seed germination 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 in MS, 7.6 ± 0.34 in WPM, and 5.2 ± 0.25 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 mineral salt concentrations in WPM and MS medium differ significantly, which has a significant impact on *in vitro* germination (Trivedi and Joshi, 2014). In *R. fortunei,* the suitability of WPM for shoot culture could be related to low concentrations of mineral nutrients in the media (Wei et al., 2018). Plants in the family Ericaceae are indigenous to soils with low pH and low nutrient status (Wei et al., 2016). Mineral nutrient contents in this group of plants are generally low (Strik and Vance, 2015), suggesting that medium used for *in vitro* culture of this group of plants should have low ionic concentrations (Debnath, 2007; Fan et al., 2017). Irrelevant.

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 (%)(For which period)
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)

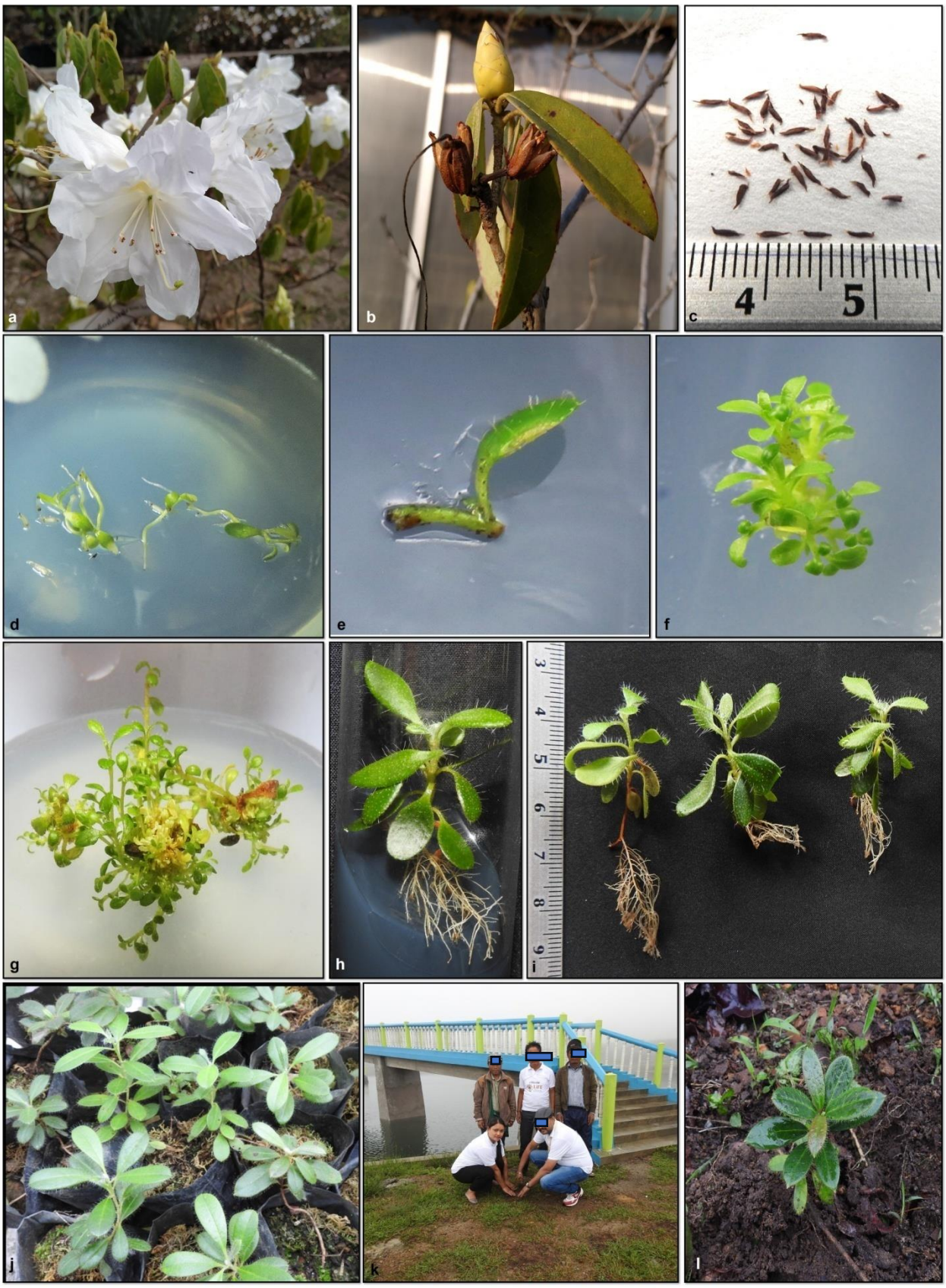
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Fig. 1.*In vitro* propagation of *Rhododendroninaequale*Hutch. (a) *R. inaequale* mother plant in its natural habitat (b) Seed pod (c) Seeds (d) *In vitro* seed germination on Woody Plant Medium (WPM) (e) Nodal explant from 3-month-old *in vitro* grown seedling inoculated for shoot multiplication (f) Multiple shoot induction on WPM with 8 mg L^{-1} 2iP after 4 weeks in culture (g) Multiple shoots developed after repeated subculture at 25-d intervals (h) *In vitro* rooting after 12 week 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
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3.3 MULTIPLE SHOOT INDUCTION

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) was observed in 2iP with 0.2% Ac (4 mg L^{-1}) (Fig. 1 f and Fig. 1g) (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 (1982) and Cantos *et al*. (2007) which showed that BAP was inferior to 2iP for induction of multiple shoots in *Rhododendron*spp. 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 (Pan and Staden, 1998). Activated charcoal can: be specific create 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 (Thomas, 2008).

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)

Data were statistically analyzed by one-way ANOVA and means were compared in each column using Tukey's test at $P = 0.05$ using Origin Pro 8SRO v8.0725 (B725).

3.4 ROOTING

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 yielded roots (Table 3). The medium containing 0.5 mg/L IBA yielded the greatest number of roots (Fig. 1h). Furthermore, WPM with 0.5 mg/L IBA also yielded the longest roots. Other researchers had previously determined that IBA was ideal for root induction in *Rhododendron* spp. (Iapichino et al. 1992; Briggs et al. 1994; Almeida et al. 2005; Singh and Gurung 2009); the present study supports their results with similar findings.

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 ±	Root length Mean root length (cm)

		SE	± SE
Activated charcoal 0.2 %	-	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)

Data were statistically analyzed by one-way ANOVA and means were compared in each column using Tukey's test at $P = 0.05$ using Origin Pro 8SRO v8.0725 (B725).

3.6 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). 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 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.

4 CONCLUSION

This study represents a pioneering effort in *in vitro* propagation, *ex vitro* acclimation and reintroduction of *R. inaequale*. A robust system for shoot proliferation, rooting, and transplanting was successfully established, which holds significant implications for species recovery program. The findings introduce a novel regeneration pathway for *R. inaequale(Repetation)*, previously undocumented, demonstrating efficient morphological uniformity????, normal leaf form????, shape???, and growth patterns????. *In*

vitro propagation technique developed in this study presents a promising approach for conserving this endemic species and facilitating ecosystem restoration in the Indo-Burma biodiversity hotspot region.

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