

AN EFFICIENT *IN VITRO* MICROPROPAGATION PROTOCOL FOR *RHYNCHOSTYLIS RETUSA* (L.) BLUME USING SHOOT MERISTEM AND LEAF EXPLANTS

Tapash Kumar Bhowmik, Somaya Emrog Nayma, Marzia Akter Sumi, and
Momena Khanam Mumu

Department of Botany, Faculty of Biological Sciences, University of Chittagong, Chattogram- 4331, Bangladesh

Abstract

Rhynchostylis retusa, popularly known as the *Foxtail Orchid*, is an epiphytic orchid of significant ornamental and medicinal value. Due to indiscriminate collection and habitat destruction, its natural populations are declining, necessitating efficient *in vitro* micropropagation protocol for conservation and commercial production. The present study established a rapid and reproducible micropropagation protocol using shoot meristem and leaf explants excised from *in vitro* raised plantlets. Murashige and Skoog (MS) medium supplemented with various concentrations of Plant Growth Regulators (PGRs) was used. shoot meristem segments exhibited direct organogenesis, yielding the highest number of multiple shoot buds (6.3 ± 0.67 shoots/explant) on MS medium fortified with BAP (2.0 mgL^{-1}) and NAA (1.0 mgL^{-1}). Conversely, leaf segments underwent somatic embryogenesis, producing the maximum percentage ($77.33 \pm 1.96\%$) of Protocorm-like bodies (PLBs) on the same hormonal regime. For shoot elongation, agar solidified MS medium supplemented with BAP (1.5 mgL^{-1}) and NAA (0.9 mgL^{-1}) proved better than liquid nutrient media, achieving a shoot length of 2.84 ± 0.04 cm. Rooting was best induced on half strength MS medium supplemented with a combination of IBA (0.5 mgL^{-1}) and NAA (0.5 mgL^{-1}), resulting in an average of 5.17 roots per plantlet. The plantlets were successfully acclimatized with a survival rate of 70% in a potting mixture of coconut coir, sawdust, and coal. This protocol offers a viable pathway for the mass propagation and *ex situ* conservation of *R. retusa*.

Introduction

ORCHIDS, BELONGING to the family Orchidaceae, represent one of the most diverse and evolutionarily advanced groups of flowering plants, comprising over 29,481 species (WFO, 2023). Amongst these, *Rhynchostylis retusa* (L.) Blume, an epiphytic monopodial orchid, is renowned for its pendulous, dense inflorescences of violet spotted white flowers, earning it the *Foxtail orchid*. Beyond its floricultural significance, *R. retusa* holds a prominent place in traditional medicine. In the Indian subcontinent, it is utilized to treat a spectrum of ailments, including rheumatism, asthma, tuberculosis, epilepsy, and menstrual disorders (Hossain, 2009; Huda *et al.*, 2006; Tiwari *et al.*, 2012). Furthermore, its root extracts are used to treat wounds and dried flowers serve as insect repellents (Subedi *et al.*, 2013).

Despite its economic and therapeutic potential, the natural propagations of *R. retusa* are declining in their habitats due to presence of non-endospermic seeds which necessitates a specific mycorrhizal association for their germination (Arditti, 1967). Furthermore, the species is categorized as vulnerable or endangered in several regions due to habitat fragmentation and over exploitation for the floral trade. This decline threatens not only the species' survival but also the availability of bioactive metabolites found in these plants (Bhattacharyya *et al.*, 2018). Conventional vegetative

propagation is slow, yielding a few progenies per year, which is insufficient to meet commercial demands or conservation goals. Consequently, biotechnological interventions, specifically plant tissue culture, offers a pragmatic solution for mass multiplication and germplasm conservation (Borah *et al.*, 2015).

In vitro propagation has emerged as a preferred method for clonal propagation in orchids, ensuring genetic fidelity and high multiplication rates (Bhowmik and Rahman, 2022, 2023; Dhillon and Pathak, 2023; Pathak *et al.*, 2001, 2023; Jaryal *et al.*, 2025a,b). While previous studies have explored seed germination in this species (Kumari and Pathak, 2021; Thakur and Pathak, 2021) efficient protocols using auxins and cytokinins for organogenesis from vegetative explants in *R. retusa* remain an area requiring refinement. The balance between cytokinins-induced shoot proliferation and auxin-mediated rooting is critical for successful regeneration (Phillips, 2004; Skoog and Miller, 1957). Recent investigations in genera such as *Dendrobium* and *Vanda* have highlighted the efficacy of BAP and NAA in breaking dormancy and inducing PLBs (Gangaprasad *et al.*, 2024; Hossen *et al.*, 2021; Roy *et al.*, 2011).

The present study aims to establish a standardized, high frequency regeneration protocol for *R. retusa* by investigating the morphogenic potential of shoot meristem and leaf explants derived from *in vitro*

plantlets. The optimization of culture conditions for shoot multiplication, elongation and rooting, providing a robust framework for the large scales production and conservation of this valuable orchid species.

Material and Methods

Plant material and Explant Preparation

Capsules of *R. retusa* were collected from Mirsarai, Chattogram, Bangladesh. The capsules were thoroughly washed under running tap water, treated with a detergent solution, and surface sterilized with 0.2% (w/v) mercuric chloride (HgCl_2) for 5 mins, followed by a one minute rinse in 70% ethanol. After repeated washings with sterile distilled water, the seeds were aseptically inoculated onto MS (Murashige and Skoog, 1962) basal medium. Eight months old *in vitro* raised plantlets, approximately 3-4 cm in length, served as the source of explants. Shoot meristem and leaf segments (1.0-1.5 cm) were excised aseptically and used as explants.

Culture Media and Conditions

The basal nutrient medium consisted of MS salts and vitamins supplemented with 3% (w/v) sucrose. The medium was gelled with 0.8% (w/v) agar (Fluka) and pH was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl prior to autoclaving at 121°C and 1.05 kg/cm² pressure for 20 mins. For micropropagation, the basal medium was supplemented with various concentrations and combinations of plant growth regulators (PGRs), specifically BAP, KN, NAA, IAA, and Picloram Pic. All cultures were maintained in a growth room at 25±2°C under a 14/10 hr light/dark photoperiod provided by cool white fluorescent tubes (2500-3000 lux).

Experimental Design

Shoot Multiplication

Shoot meristem and leaf explants were cultured on MS medium supplemented with BAP (1.0-2.0 mgL⁻¹), Kn

(1.0-2.0 mgL⁻¹) and varying combinations of auxins (NAA, IAA, Pic). Parameters recorded included the percentage of explants responding, time to initiation, and number of multiple shoot buds (MSBs) or PLBs per explant.

Shoot Elongation

Proliferated shoot clumps were transferred to both solid and liquid MS media supplemented with BAP (1.0-2.0 mgL⁻¹) and NAA (0.6-1.2 mgL⁻¹) to determine the optimal conditions for shoot elongation.

Rooting

Elongated shoots (2-3 cm) were excised and transferred to half strength MS medium supplemented with auxins (IAA, IBA, NAA) at concentrations of 0.5-1.0 mgL⁻¹ to induce rooting.

Acclimatization

Well rooted plantlets were subjected to a hardening process. The culture vessels were opened and maintained in the culture room for 24 hrs, followed by exposure to ambient laboratory conditions for 48 hrs. The plantlets were then removed, washed to remove agar, and transplanted into small pots containing a mixture of moistened coconut coir, sawdust, and coal. These were maintained in a greenhouse with controlled humidity.

Statistical Analysis

All experiments were conducted with at least five replicates per treatment. Data were recorded after 30-60 days of culture. The results are expressed as Mean ± Standard Error (SE).

Results

The morphogenetic response of *Rhynchosytilis retusa* explants varied significantly depending on the explant type and the PGRs regime used (Table 1). Shoot



Fig. 1. *In vitro* regeneration in *Rhynchosytilis retusa* using shoot meristem and leaf explants; A, Development of MSBs from *in vitro* shoot meristem segment (MS + 2.0 mgL⁻¹BAP + 1.0 mgL⁻¹ NAA); B, Development of PLBs from *in vitro* leaf segment (MS + 2.0 mgL⁻¹BAP + 1.0 mgL⁻¹ NAA); C, Plantlets elongation on agar solidified (MS + 1.5 mgL⁻¹BAP + 0.9 mgL⁻¹NAA); D, Seedling elongation in liquid (MS + 2.0 mgL⁻¹BAP + 1.2 mgL⁻¹NAA); E, Induction of root system on half strength (MS + 0.5 mgL⁻¹IAA + 0.5 mgL⁻¹NAA).

meristem segments responded *via* direct organogenesis, producing multiple shoot buds (MSBs) without an intervening callus phase. The highest proliferation efficiency was observed on MS medium supplemented with BAP (2.0 mgL⁻¹) + NAA (1.0 mgL⁻¹), yielding an average of 6.3±0.67 shoots per segment with a 93.33% response rate (Fig. 1A). The time required for bud initiation in this combination was minimal (4.34±0.19 wks). Media supplemented with KN alone or in combination with auxins generally resulted in lower shoot multiplication rates compared to BAP supplemented media.

Leaf segments differentiated *via* somatic embryogenesis, forming PLBs. The highest frequency of PLB formation (77.33±1.96%) was recorded on MS medium supplemented with BAP (2.0 mgL⁻¹) + NAA (1.0 mgL⁻¹) (Fig. 1B). The PLBs formed were vibrant green and healthy. Leaf segments failed to respond on media containing KN alone, suggesting thereby that BAP is crucial for inducing embryogenic competence in foliar tissues of this species.

To optimize shoot development, MSBs were cultured onto elongation media. A comparison between agar solid

and liquid MS media revealed that solid media generally supported better growth (Table 2). The maximum shoot length (2.84±0.04 cm) was achieved on agar solid MS medium fortified with BAP (1.5 mgL⁻¹) + NAA (0.9 mgL⁻¹) after 30 days (Fig. 1C). Liquid media with the same hormonal composition yielded a slightly lower elongation (2.38±0.03 cm), although it promoted rapid nutrient uptake (Fig. 1D). In solid MS medium with BAP (2.0 mgL⁻¹) + NAA (1.2 mgL⁻¹) also supported significant elongation (2.53±0.03 cm).

Robust root system development is prerequisite for survival during acclimatization. Elongated shoots (2-3 cm) were transferred to rooting media containing various auxins. The use of auxins proved better than that of single auxin treatments. The highest number of roots (5.17±0.52) and maximum root length (4.37±0.07 cm) was recorded on half strength MS medium supplemented with IBA (0.5 mgL⁻¹) + NAA (0.5 mgL⁻¹) (Fig. 1E). Media containing IBA alone also induced rooting but with lower frequency and vigour as compared to the medium with IBA and NAA.

Plantlets with well developed roots were hardened sequentially. The survival rate after transplantation

Table. 1. Development of MBSs/PLBs from *in vitro* raised shoot meristem and leaf explants of *Rhynchosyilis retusa* on agar solidified MS medium with different different PGRs.

PGRs (mgL ⁻¹)					Shoot meristem explants (Mean±SE)			Leaf explants (Mean±SE)	
BAP	KN	NAA	IAA	Pic	Percentage of induced MBSs/ explant	Time required (wks) for development of MSBs	Number of MBSs produced/ explant	Percentage of induced PLBs/ explant	Time required (wks) for Development of PLBs
1.0	-	-	-	-	25.33±1.28	6.38±0.11	2.4±0.57	0.00±0.00	0.00±0.00
2.0	-	-	-	-	32.00±1.56	6.24±0.14	2.6±0.34	6.67±0.00	7.32±0.09
-	1.0	-	-	-	18.67±1.44	6.56±0.17	1.8±0.53	0.00±0.00	0.00±0.00
-	2.0	-	-	-	25.33±1.28	6.31±0.12	2.2±0.38	0.00±0.00	0.00±0.00
1.0	-	0.5	-	-	88.00±1.28	4.57±0.07	5.9±0.62	52.00±1.44	5.44±0.07
2.0	-	1.0	-	-	93.33±1.72	4.34±0.19	6.3±0.67	77.33±1.96	4.65±0.15
1.0	-	-	0.5	-	76.00±1.96	4.82±0.17	5.4±0.64	37.33±1.96	6.04±0.17
2.0	-	-	1.0	-	82.67±2.61	4.67±0.18	5.8±0.58	56.00±1.96	5.29±0.08
1.0	-	-	-	0.5	64.00±1.96	5.38±0.14	4.6±0.53	44.00±1.54	5.61±0.19
2.0	-	-	-	1.0	76.00±2.31	5.14±0.12	5.2±0.65	69.33±1.96	5.08±0.15
-	1.0	0.5	-	-	69.33±1.96	5.26±0.17	4.8±0.58	20.00±1.72	6.74±0.17
-	2.0	1.0	-	-	62.67±2.61	5.47±0.08	4.4±0.42	44.00±1.96	5.77±0.19
-	1.0	-	0.5	-	56.00±1.96	5.53±0.16	3.9±0.56	13.33±1.72	7.19±0.14
-	2.0	-	1.0	-	50.67±1.96	5.64±0.17	3.8±0.47	32.00±1.44	6.30±0.16
-	1.0	-	-	0.5	45.33±1.28	5.71±0.12	3.4±0.62	13.33±1.22	7.09±0.11
-	2.0	-	-	1.0	52.00±1.28	5.82±0.19	3.6±0.47	38.67±1.44	6.14±0.18
				MS0 (Control)	13.33±1.72	6.64±0.15	1.2±0.00	0.00±0.00	0.00±0.00

Table. 2. Elongation of MSBs derived shoot bud developed from shoot meristem explant of *Rhynchostylis retusa* on 0.8% (w/v) agar solidified and liquid MS medium with different kinds of PGRs.

PGRs (mgL ⁻¹)				Solid media (Mean± SE)			Liquid media (Mean± SE)		
BAP	KN	NAA	IAA	Initial length (cm) of shoot bud	Length (cm) of shoot bud after 30 days of culture	Increase in length (cm) of shoot bud within 30 days of culture	Initial length (cm) of shoot bud	Length (cm) of shoot bud after 30 days of culture	Increase in length (cm) of shoot bud within 30 days of culture
1.0	-	-	-	1.36±0.04	3.58±0.03	2.22±0.04	1.35±0.03	3.24±0.05	1.88±0.05
1.5	-	-	-	1.36±0.05	3.77±0.05	2.41±0.03	1.38±0.02	3.47±0.04	2.09±0.04
2.0	-	-	-	1.37±0.04	3.66±0.04	2.28±0.04	1.26±0.05	3.17±0.03	1.92±0.03
-	1.0	-	-	1.29±0.03	3.31±0.03	2.02±0.05	1.34±0.02	3.09±0.04	1.75±0.02
-	1.5	-	-	1.32±0.03	3.43±0.05	2.07±0.03	1.29±0.03	3.09±0.03	1.80±0.04
-	2.0	-	-	1.36±0.04	3.43±0.03	2.07±0.04	1.32±0.04	3.09±0.03	1.77±0.03
1.0	-	0.6	-	1.36±0.05	4.06±0.04	2.69±0.03	1.34±0.02	3.56±0.05	2.22±0.05
1.5	-	0.9	-	1.34±0.03	4.18±0.03	2.84±0.04	1.35±0.03	3.73±0.03	2.38±0.03
2.0	-	1.2	-	1.36±0.04	4.10±0.04	2.74±0.02	1.29±0.04	3.59±0.04	2.30±0.04
1.0	-	-	0.6	1.32±0.02	3.75±0.03	2.43±0.05	1.33±0.05	3.44±0.04	2.10±0.03
1.5	-	-	0.9	1.31±0.04	3.99±0.05	2.68±0.04	1.28±0.04	3.54±0.03	2.28±0.05
2.0	-	-	1.2	1.34±0.03	3.87±0.04	2.53±0.03	1.34±0.03	3.53±0.05	2.19±0.04
-	1.0	0.6	-	1.39±0.05	3.67±0.02	2.28±0.04	1.35±0.03	3.40±0.03	2.05±0.03
-	1.5	0.9	-	1.36±0.03	3.97±0.06	2.61±0.05	1.34±0.04	3.56±0.04	2.22±0.03
-	2.0	1.2	-	1.36±0.04	3.87±0.04	2.51±0.04	1.31±0.02	3.44±0.05	2.13±0.03
-	1.0	-	0.6	1.35±0.02	3.62±0.05	2.27±0.03	1.28±0.05	3.27±0.04	1.99±0.04
-	1.5	-	0.9	1.35±0.04	3.90±0.04	2.53±0.02	1.34±0.02	3.51±0.03	2.17±0.05
-	2.0	-	1.2	1.34±0.03	3.79±0.03	2.45±0.03	1.36±0.05	3.40±0.02	2.04±0.04
MS0 (Control)				1.34±0.03	3.22±0.02	1.87±0.04	1.27±0.04	2.84±0.04	1.57±0.02

into the potting mixture (coconut coir, sawdust, and coal) was 70%. The surviving plantlets exhibited resumption of growth and formation of new leaves within 3-4 wks of transfer to the greenhouse conditions.

Discussion

The results of the present study underscore the pivotal role of cytokinin-auxin balance in orchid micropropagation (Griesbach, 1986; Martin and Madassery, 2006). The superiority of BAP over KN for shoot multiplication in *R. retusa* aligns with findings in other orchid species, including *Aerides multiflora* (Bhowmik and Rahman, 2020) and *Dendrobium* (Pant and Thapa, 2012), BAP is known to effectively break apical dominance and stimulate axillary bud proliferation (Sagawa, 1962; Seeni and Latha, 2000), which explains the high frequency of direct organogenesis from shoot meristem explants. Similar efficiency of BAP and NAA in shoot meristem cultures was also reported by Hossen *et al.* (2021) in *Dendrobium* sp., where a combination

of BAP (2.5 mgL⁻¹) and NAA (0.5 mgL⁻¹) yielded maximum shoot regeneration.

The explants showed differential regeneration response *i.e.* the shoot meristem segments showed organogenesis whereas the leaf segments showed somatic embryogenesis. The successful induction of PLBs using BAP (2.0 mgL⁻¹) + NAA (1.0 mgL⁻¹) in the leaf explants of the present species suggests that high cytokinin levels are promontory for dedifferentiation of somatic cells in foliar tissues. The present results are in line with the earlier studies made by Pathak *et al.* (2016) on *Dendrobium amoenum* and Kumari and Pathak (2021) on *Rhynchostylis retusa*, who reported successful regeneration of leaf explants under high cytokinin regimes in these species. Similarly, Bhattacharyya *et al.* (2018) also achieved high frequency shoot proliferation in *Dendrobium aphyllum* using cytokinins.

For shoot elongation, the preference for solid media over liquid media in the present study contradicts some earlier reports where liquid culture systems enhanced

Table. 3. Mean increase in length (cm) and number of roots per MSBs derived plantlets of *Rhynchosstylis retusa* in control and auxin supplemented half strength MS rooting nutrient medium.

PGRs (mgL ⁻¹)			Shoot meristem explants (Mean±SE)		Leaf explants (Mean±SE)	
IAA	IBA	NAA	Initial length (cm) per MSBs derived plantlet (Mean±SE)	Initial number of roots per MSBs derived plantlet (Mean±SE)	Increased length (cm) per MSBs derived plantlet (Mean±SE)	Increased number of roots per MSBs derived plantlet (Mean±SE)
0.5	-	-	1.44±0.05	1.00±0.39	3.32±0.09	4.33±0.54
1.0	-	-	1.45±0.04	0.67±0.34	3.89±0.07	3.33±0.47
-	0.5	-	1.44±0.07	1.17±0.37	3.42±0.06	4.00±0.45
-	1.0	-	1.41±0.03	0.67±0.29	3.11±0.08	3.67±0.46
-	-	0.5	1.58±0.04	1.17±0.40	3.67±0.06	4.33±0.48
-	-	1.0	1.44±0.02	0.67±0.34	3.17±0.07	3.83±0.46
0.5	0.5	-	1.48±0.06	1.17±0.33	3.84±0.05	4.50±0.48
1.0	1.0	-	1.47±0.04	0.83±0.47	3.56±0.07	4.17±0.42
-	0.5	0.5	1.42±0.03	1.00±0.39	4.18±0.04	5.00±0.42
-	1.0	1.0	1.45±0.05	1.17±0.42	3.96±0.09	4.50±0.49
0.5	-	0.5	1.48±0.07	0.67±0.29	4.37±0.07	5.17±0.52
1.0	-	1.0	1.47±0.06	0.83±0.34	4.10±0.06	4.83±0.57
MS0 (Control)			1.44±0.02	1.17±0.28	2.08±0.08	2.33±0.32

growth due to better nutrient availability. Presently, in *R. retusa*, solid media prevented vitrification, a common physiological disorder in liquid cultured orchids (Ziv, 1991). The combination of BAP and NAA during elongation suggests that a continued, albeit reduced, cytokinin supply is necessary to maintain cell division while the auxin promotes cell expansion, a finding consistent with the elongation protocols described by Nguyen *et al.* (2022) for *Dendrobium anosmum*.

Rooting efficiency was maximal with a combination of IBA and NAA. Synergistic auxin effects are well documented in tissue culture; IBA is often associated with root induction, while NAA promotes root elongation. The use of half strength MS medium for rooting is consistent with the general understanding that reduced salt concentrations facilitate rhizogenesis in epiphytic orchids, which naturally grow in nutrient poor environments (Bhowmik and Rahman, 2022). Similar synergistic effects of auxins on rooting were observed by Castillo-Pérez *et al.* (2021) in *Stanhopea tigrina* and Aktar *et al.* (2007) in *Dendrobium*, where combinations of IBA and NAA significantly enhanced root number and length as compared to the single auxin treatments.

Conclusion

The present study establishes a comprehensive and efficient protocol for the micropropagation of *Rhynchosstylis retusa*. Shoot meristem segments are

highly amenable to direct multiple shoot induction, while leaf segments can be effectively utilized for PLB production, broadening the avenues for mass multiplication. The optimized media combinations [MS + BAP (2.0 mgL⁻¹) + NAA (1.0 mgL⁻¹); half strength MS + IBA (0.5 mgL⁻¹) + NAA (0.5 mgL⁻¹)] for initiation and rooting, respectively; ensure the production of healthy, vigorous plantlets. This protocol holds immense potential for the commercial floriculture industry and for the conservation of this medicinally important orchid species.

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