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In vitro Shoot Regeneration of the Aquatic Ornamental Plant – *Rotala rotundifolia* (Buch-Ham. Ex Roxb.) Koehne

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Abstract

Rotala rotundifolia (Buch.-Ham. Roxb.) Koehne is a popular aquatic ornamental plant possess with ecological and commercial value. The plant species are critical in position due to habitat loss and overharvesting. Thus, efficient and rapid propagation strategies are highly desirable. This study aimed to establish an efficient *in vitro* regeneration protocol for conservation and mass propagation. Nodal segment and shoot tip explants were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of cytokinins (BAP, Kinetin) and auxins (IBA, NAA). Optimal shoot proliferation was achieved from nodal segment explants on full-strength MS medium containing 1.0 µM/L BAP, with a maximum of 47.12 ± 0.86 shoots per culture and 4.97 ± 0.16 cm shoot length. The combination of 2.0 µM/L BAP and 1.0 µM/L IBA were used for further enhancement of shoot induction and elongation. Liquid MS medium was more suitable for shoot regeneration compared to solidified MS medium supplemented with 2.0 µM/L IBA, which exhibited the highest relative plant regeneration frequencies 93.33% of rooting and 15.92 ± 2.59 roots per shoot. Acclimatized plantlets grown in a pot containing garden soil and cocopeat (1:1) substrate showed 90% survival and vigorous growth. This protocol offers a reproducible platform for large-scale propagation and conservation of *R. rotundifolia* species.

Keywords: Acclimatization, Aquatic ornamental plant, Axillary shoot proliferation, *In vitro* micropropagation.

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Introduction

Aquatic ornamental plants have gained increasing prominence in aqua scaping, ecological restoration, and commercial horticulture due to their aesthetic appeal and functional roles in aquatic environments. Among them, *Rotala rotundifolia* (Buch.-Ham.Roxb.) Koehne, a small, fast-growing aquatic herb of the family Lythraceae, is widely cultivated for its attractive red-green foliage and adaptability to submerged conditions. Native to Southeast Asia, *R. rotundifolia* is a common feature in freshwater aquaria and constructed wetlands, where it contributes to nutrient cycling, habitat structure, and biofiltration (Yadav et al. 2023, Pradhan and Nayak 2021). However, the natural populations of *R. rotundifolia* are increasingly threatened by habitat degradation, water pollution, and overexploitation for the ornamental plant trade (Kumar et al. 2022). Its conventional propagation through stem cuttings is often constrained by seasonal availability, pathogen accumulation, and low multiplication rates. Consequently, there is a pressing need to develop an efficient and sustainable propagation system to ensure year-round supply and conserve its genetic diversity.

Plant tissue culture offers a promising solution for the mass propagation of aquatic species through controlled and disease-free microenvironmental conditions. *In vitro* techniques have been successfully applied to a range of aquatic and wetland plants, such as *Bacopa monnieri* (Singh et al. 2023), *Hygrophila polysperma* (Patra et al. 2022), and *Ludwigia adscendens* (Roy et al. 2021), demonstrating the utility of micropropagation in enhancing shoot regeneration and rooting efficiency. These studies emphasize the importance of selecting appropriate explants and optimizing plant growth regulator (PGR) combinations- especially cytokinins like BAP and kinetin for shoot proliferation, and auxins like IBA and NAA for rooting (George et al. 2008, Shekhawat et al. 2020). Although *R. rotundifolia* holds considerable horticultural value, standardized tissue culture protocols for this species remain scarce in the published literature. To address this gap, the present study focuses on

developing a dependable *in vitro* regeneration method utilizing nodal and shoot tip explants of *R. rotundifolia*. We evaluated the effects of different concentrations of cytokinins and auxins on axillary shoot induction and adventitious rooting, intending to support conservation, commercial propagation, and potential genetic enhancement programs for this valuable aquatic species.

Materials and Methods

Rotala rotundifolia (Buch.-Ham. ex Roxb.) Koehne, an aquatic ornamental plant, was used in this study. Nodal and shoot tip segments were collected from *ex vitro* grown plants supplied by a commercial aquatic plant vendor in Dhaka, Bangladesh. Explants (1.0-1.5 cm) containing a single node or shoot tip were prepared for *in vitro* culture. Collected shoot segments were initially washed in 1% (v/v) Savlon solution with constant shaking for 10 minutes and then rinsed three to four times with distilled water. Surface sterilization was conducted under a laminar airflow cabinet using 0.05-0.20% (w/v) mercuric chloride ($HgCl_2$) for variable durations. Following this treatment, explants were thoroughly rinsed four to five times with sterile distilled water to remove any residual sterilant. Both liquid and solid MS media were used for culture initiation and shoot proliferation. Media were supplemented with various concentrations (0.5-6.0 µM/L) of BAP and kinetin (Kn) to determine optimal conditions for axillary shoot proliferation. Additionally, MS media with different combinations and concentrations of BAP (1.0-6.0 µM/L) and IBA and NAA (0.5-4.0 µM/L) were tested to evaluate their effects on shoot multiplication.

The influence of basal medium composition was also assessed using three different formulations: standard MS, Modified MS1 (MMS1), and Modified MS2 (MMS2), each supplemented with 1.0 µM/L BAP. Furthermore, solidified agar-gelled MS medium (Murashige and Skoog 1962) containing 1.0 µM/L BAP was used to study shoot induction efficiency from nodal and shoot tip explants. Shoots measuring 1–3 cm were excised after removing basal leaves and cultured individually in 25 mm × 150 mm culture tubes containing 15–20 ml of full-strength MS medium supplemented with different auxins (NAA, IBA, or IAA) at concentrations ranging from 2.0–6.0 µM/L. Rooted plantlets were transferred to small plastic pots containing a sterilized soil mixture of garden soil, sand, and compost (2:1:1) and acclimatized in a growth chamber for 25 days. Subsequently, plants were transferred to outdoor conditions. All culture media were prepared with 0.8% (w/v) agar and 3% (w/v) sucrose (Sigma Chemical Co., USA). Media pH was adjusted to 5.7 ± 1.0 before autoclaving at 121°C for 20 minutes under 1.2 kg/cm^2 pressure. Cultures were maintained at 25 ± 1°C under a 16-hour photoperiod with cool-white fluorescent light at an intensity of 50 µmol·m^{-2}·s^{-1}. Data were recorded after 8 weeks of culture for shoot proliferation experiments, while rooting data were collected after 4 weeks. Each treatment included 10 to 155 explants, and all experiments were repeated three times. Results were expressed as mean ± standard deviation (SD). Treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% significance level.

Results and Discussion

Standardization of $HgCl_2$ treatment for surface sterilization

Effective surface sterilization is a critical prerequisite in plant tissue culture, directly influencing contamination control and explant viability. In the present study, nodal segments and shoot tip explants of *Rotala rotundifolia* were subjected to different concentrations of mercuric chloride ($HgCl_2$) and varying exposure durations to determine the optimal sterilization protocol (Table 1). At a lower concentration of 0.05% $HgCl_2$, a 1-minute treatment exhibited the highest contamination rates, with 90% and 95% of nodal segment and shoot tip explants, respectively, being contaminated. However, cellular death was negligible (0%) for both explant types, indicating minimal cytotoxic effects at this concentration and exposure time. The survival rate was correspondingly low (10% for nodal segments and 5% for shoot tip explants) due to high contamination. Increasing the treatment duration to 2 and 3 minutes at 0.05% resulted in reduced contamination but gradually increased cellular death and slightly improved survival (up to 25% in nodal segments and 15% in shoot tips at 3 minutes). At 0.10% $HgCl_2$, contamination decreased more significantly, particularly at 2 and 3 minutes, yet the toxicity became more evident, reducing explant viability. The 0.20% concentration, particularly with 3-minute exposure, exhibited complete sterilization of nodal explants (0% contamination). However, this came at the cost of elevated cellular death (50% in nodal segments and 60% in shoot tip explants), rendering it suboptimal due to poor survival rates.

The optimal treatment was found to be 0.10% $HgCl_2$ for 2 minutes, balancing contamination control and explant survival. This treatment reduced contamination to 50% in nodal segments and 45% in shoot tip explants, with manageable cellular death rates and survival rates of 30% and 20%, respectively. While not ideal, this combination provided a suitable compromise between disinfection efficacy and explant viability. These findings align with previous reports indicating that $HgCl_2$ is highly effective for surface sterilization but must be used judiciously due to its phytotoxicity. For instance, Bhuyan et al. (2010) observed that $HgCl_2$ at 0.1% for 1–2 minutes was effective in *Rauvolfia serpentina*, while higher concentrations induced oxidative stress and reduced survival. Similarly, Devi and Thirugnanakumar (2018) reported that $HgCl_2$ treatment in *Vitex negundo* required precise optimization to minimize cellular necrosis. Throughout the treatments, nodal explants demonstrated slightly higher resilience to sterilant toxicity compared to shoot tip explants, suggesting their greater suitability for micropropagation protocols in *R. rotundifolia*. This differential response may be attributed to the relative tissue maturity and physiological robustness of nodal segments compared to shoot tips, a pattern also noted in related aquatic and semi-aquatic species (Khan et al. 2021).

The data emphasizes the critical need for tailored sterilization protocols based on explant type and plant species. For *R. rotundifolia*, a semi-aquatic medicinal plant, nodal segment explants treated with 0.10% $HgCl_2$ for 2 minutes offer a promising pathway for establishing aseptic cultures with acceptable survival rates, forming the foundational step for subsequent shoot proliferation and regeneration studies.

Table 1: Standardization of $HgCl_2$ treatment period for surface sterilization of nodal and shoot tip segments of *Rotala rotundifolia.*

$HgCl_2$ Conc. (%)	Treatment duration (min)	Nodal segment			Shoot tip		
		No. of contaminated explants	Rate of cellular death of explant	Survival explants (%)	No. of contaminated explants	Rate of cellular death of explant	Survival explants (%)
0.05	1	18	0	10.00	19	0	5.00
	2	15	2	15.00	16	2	10.00
	3	10	5	25.00	11	6	15.00
0.10	1	15	2	15.00	14	3	15.00
	2	10	4	30.00	11	5	20.00
	3	5	6	45.00	6	7	35.00
0.20	1	10	5	25.00	12	6	10.00
	2	5	6	45.00	7	8	25.00
	3	0	10	50.00	2	12	30.00

There are 20 explants used in each treatment. Data was recorded after 4 weeks of culture.

Effect of cytokinins on axillary shoot proliferation

The *in vitro* response of *Rotala rotundifolia* to varying concentrations of two cytokinins like 6-benzylaminopurine (BAP) and kinetin (Kn) was evaluated using nodal segments and shoot tip explants. Shoot formation frequency, number of shoots per culture, and shoot length were recorded after 8 weeks of culture initiation (Table 2). Among the treatments, BAP demonstrated superior shoot induction potential compared to Kn. The highest shoot formation frequency (93.33%) and shoot number per culture (47.12 ± 0.86) in nodal segment explants were observed at 1.0 µM/L BAP (Fig. 1A-C). Similarly, shoot tip explants exhibited optimal performance at the same concentration, with a shoot formation rate of 81.82% and 41.32 ± 0.81 shoots per culture. Shoot length also peaked at this concentration (4.97 ± 0.16 cm in nodal and 2.97 ± 0.11 cm in shoot tip explants) (Fig. 1D-F).

Further increase in BAP concentration resulted in a gradual decline in all measured parameters. At 6.0 µM/L, shoot formation decreased to 54.55% in nodal and 46.15% in shoot tip explants, with reduced shoot numbers and significantly shorter shoot lengths (2.17 ± 0.07 cm and 1.01 ± 0.78 cm, respectively). These findings suggest that supra-optimal levels of BAP may lead to hormonal imbalances, adversely affecting morphogenesis. Although kinetin also encouraged the growth of axillary shoots, it was generally less successful than BAP at all

concentrations. At 1.0 µM/L Kn, the best response was seen, resulting in 84.62% shoot production in nodal explants with 2.01 ± 0.32 cm shoot length and 41.34 ± 0.26 shoots per culture. The maximal shoot formation in shoot tip explants was 76.92%, with 1.78 ± 0.24 cm of shoot length and 35.32 ± 0.25 shoots per culture. A significant decrease in response was observed at higher Kn concentrations (≥ 2.0 µM), which is in line with previous findings of cytokinin toxicity at high concentrations (Kumar et al. 2016, Shekhawat et al. 2020). Nodal explants consistently performed better than shoot tip explants across all treatments. This may be due to their larger meristematic zones and stronger endogenous hormonal content, which favor multiple shoot initiation. These results corroborate findings from similar studies in other aquatic and medicinal plants such as *Hygrophila polysperma* and *Bacopa monnieri*, where nodal segments were found to be more responsive to cytokinin-mediated organogenesis (Patra et al. 2022, Singh et al. 2023b).

Based on the current data, 1.0 µM/L BAP is the optimal cytokinin concentration for inducing prolific and elongated axillary shoots in both nodal and shoot tip explants of *R. rotundifolia*. BAP has been previously reported as a highly effective cytokinin for shoot induction in various aquatic and wetland species due to its higher stability and stronger effect on cell division compared to kinetin (George et al. 2008, Roy et al. 2021b).

These findings are critical for developing a reproducible and efficient micropropagation protocol for *R. rotundifolia*. Efficient cytokinin-mediated shoot proliferation forms the foundation for downstream stages, including rooting and acclimatization. The use of nodal explants with optimized BAP concentration offers a promising strategy for mass propagation and conservation of this valuable species.

Table 2: Effects of cytokinins on axillary shoots from nodal and shoot tip explants of *Rotala rotundifolia.*

PGRs	PGRs Conc. (µM)	Nodal segment explant			Shoot tip explant		
		Shoot formation (%)	Total No. of shoots/culture	Shoot length/culture (cm)	Shoot formation (%)	Total No. of shoots/culture	Shoot length/culture (cm)
BAP	0.5	80.00	43.41 ± 0.73	2.71 ± 0.07c	75.00	35.41 ± 0.34c	2.41 ± 0.76a
	1.0	93.33	47.12 ± 0.86	4.97 ± 0.16a	81.82	41.32 ± 0.81a	2.97 ± 0.11a
	2.0	85.71	44.91 ± 0.54	4.13 ± 0.07b	78.57	37.11 ± 0.41b	3.43 ± 0.76a
	3.0	70.00	39.84 ± 0.67	3.31 ± 0.07c	64.29	30.12 ± 0.75d	2.11 ± 0.76a
	4.0	60.00	37.11 ± 0.70	3.27 ± 0.06c	53.85	28.34 ± 0.08e	2.05 ± 0.69a
	6.0	54.55	34.63 ± 0.75	2.17 ± 0.07c	46.15	24.36 ± 0.71f	1.01 ± 0.78a
Kn	0.5	76.92	35.21 ± 0.33	1.51 ± 0.21c	66.67	27.56 ± 0.21e	1.23 ± 0.12a
	1.0	84.62	41.34 ± 0.26	2.01 ± 0.32c	76.92	35.32 ± 0.25c	1.78 ± 0.24 a
	2.0	78.57	36.12 ± 0.54	1.14 ± 0.05c	69.23	31.45 ± 0.45d	1.21 ± 0.45a
	3.0	64.29	31.02 ± 0.77	1.22 ± 0.18c	58.33	25.62 ± 0.32f	1.08 ± 0.32a
	4.0	53.33	28.32 ± 0.60	1.12 ± 0.34c	46.15	23.14 ± 0.12f	1.02 ± 0.21a
	6.0	46.15	25.42 ± 0.35	1.03 ± 0.45c	36.36	17.84 ± 0.54g	1.00 ± 0.65a

Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level. There Were 10-15 explants for each treatment, and data ($\bar{X}\pm$ SE) were recorded after 8 weeks of culture initiation.

Effects of concentrations and combinations of cytokinin and auxins on the induction of axillary shoots

The present study evaluates the influence of varying concentrations and combinations of cytokinins (BAP) and auxins (IBA and NAA) on axillary shoot induction from nodal and shoot tip explants of *Rotala rotundifolia*. The results demonstrate significant variations in shoot formation percentage, shoot number per culture, and average shoot length depending on growth regulator types and concentrations (Table 3). Among the combinations tested, BAP (2.0 µM/L) + IBA (1.0 µM/L) yielded the highest shoot induction rate in both nodal (93.33%) and shoot tip (81.82%) explants. This treatment also resulted in the maximum number of shoots per culture (49.53 ± 0.31 for nodal and 36.54 ± 0.61 for shoot tip) and greatest shoot length (9.98 ± 0.31 cm for

nodal and 7.56 ± 0.24 cm for shoot tip). These findings align with earlier studies, where a moderate balance of cytokinin and auxin facilitated optimal shoot organogenesis by promoting cell division and elongation (Singh et al. 2021, Ahmad et al. 2022b). A further increase in hormone concentration (BAP 4.0-6.0 µM/L + IBA 2.0-4.0 µM/L) resulted in a decline in shoot proliferation and elongation, suggesting possible hormonal toxicity or inhibition at higher levels, as previously reported in *Bacopa monnieri* and *Phyla nodiflora* (Kumar et al. 2020).

Table 3: Effects of different concentrations and combinations of cytokinin and auxins on the induction of axillary shoots from nodal and shoot tip explants of *Rotala rotundifolia*.

Growth regulators (µM)	Nodal segment			Shoot tip		
	Shoot formation (%)	No. of total shoot/culture	Av. length of shoot/culture (cm)	Shoot formation (%)	No. of total shoot/ culture	Av. length of shoot/culture (cm)
BAP + IBA						
1.0 + 0.5	75.00	46.51 ± 0.22^{b}	5.55 ± 0.54^{b}	63.64	34.35 ± 0.12^{b}	3.98 ± 0.32^{b}
2.0 + 1.0	93.33	49.53 ± 0.31^{a}	9.98 ± 0.31^{a}	81.82	36.54 ± 0.61^{a}	7.56 ± 0.24^{a}
4.0 + 2.0	80.00	33.74 ± 0.24^{c}	4.74 ± 0.54^{d}	75.00	23.21 ± 0.34^{c}	2.89 ± 0.54^{c}
6.0 + 4.0	61.54	24.53 ± 0.52^{e}	6.53 ± 0.82^{b}	53.85	12.67 ± 0.32^{e}	2.65 ± 0.56^{c}
BAP + NAA						
1.0 + 0.5	54.55	31.76 ± 0.62^{d}	2.7 ± 0.62^{b}	46.67	21.52 ± 0.11^{d}	1.89 ± 0.54^{c}
2.0 + 1.0	72.73	34.67 ± 0.46^{c}	6.6 ± 0.57^{b}	61.54	23.65 ± 0.32^{c}	4.56 ± 0.65^{b}
4.0 + 2.0	63.64	22.72 ± 0.42^{f}	3.7 ± 0.62^{b}	53.85	12.10 ± 0.12^{e}	2.58 ± 0.35^{c}
6.0 + 4.0	30.77	13.56 ± 0.63^{g}	4.5 ± 0.43^{b}	23.08	11.83 ± 0.45^{e}	1.98 ± 0.69^{c}

Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple comparison tests at 0.05 probability level. There were 10-15 explants for each treatment, and data ($\bar{X}$± SE) were recorded after each 8 weeks of culture initiation.

Compared to IBA, NAA proved less effective in promoting shoot regeneration. The best-performing combination of BAP (2.0 µM/L) + NAA (1.0 µM/L) resulted in moderate shoot formation (72.73% in nodal and 61.54% in shoot tip explants) and fewer shoots per culture (34.67 ± 0.46 in nodal and 23.65 ± 0.32 in shoot tip). Furthermore, shoot elongation was less pronounced (6.6 ± 0.57 cm in nodal and 4.56 ± 0.65 cm in shoot tip), corroborating the differential response of auxins in organogenesis, as noted by Thakur and Sharma (2019). Higher concentrations of NAA (4.0 µM/L) combined with BAP led to further reductions in shoot induction, which could be attributed to its relatively stronger root-inducing nature interfering with shoot morphogenesis (Patel et al. 2023).

Overall, nodal explants exhibited superior regenerative capacity compared to shoot tip explants across all treatment combinations. This is consistent with previous studies where nodal tissues possessed higher endogenous cytokinin levels and a more organized meristematic region, favoring shoot bud initiation (Ali et al. 2021). The synergistic effect of BAP and IBA at lower to moderate concentrations was evident, emphasizing the importance of auxin-cytokinin balance in shoot proliferation protocols. This study establishes an efficient *in vitro* protocol for axillary shoot induction in *Rotala rotundifolia*, identifying BAP (2.0 µM/L) + IBA (1.0 µM/L) as the optimal combination. These findings contribute to micropropagation strategies for aquatic medicinal plants and can aid conservation and commercial propagation efforts.

Effects of different strengths of MS medium on axillary shoot proliferation and elongation

This study assessed the effects of varying strengths of Murashige and Skoog (MS) medium (1962), supplemented with a constant concentration of 1.0 µM/L BAP, on axillary shoot proliferation and elongation from nodal and shoot tip explants of *Rotala rotundifolia*. The results demonstrate that full-strength MS medium significantly out performs modified half (MMS1) and quarter (MMS2) strengths in terms of shoot regeneration parameters (Table 4). The full-strength MS medium supported the highest shoot formation percentage from both nodal (93.33%) and shoot tip explants (81.82%), alongside the greatest number of shoots per explant (47.85 ± 0.86 and 40.32 ± 0.14, respectively). Shoot elongation was also maximized in full-strength MS (4.95 ± 0.51 cm for nodal and 3.01 ± 0.12 cm for shoot tip explants), showing statistically significant improvements over reduced-strength media ($p<0.05$). These results align with earlier findings by Devi et al. (2022) and Ahmed et al. (2020), who demonstrated that a full complement of macro- and micronutrients in MS medium is critical for optimal morphogenic responses in tissue cultures of aquatic and herbaceous plants.

In contrast, shoot proliferation and elongation declined significantly with decreased MS medium strength. MMS1 (half-strength of MS) resulted in moderate shoot induction (nodal: 85.71%, shoot tip: 72.73%) and shorter shoots (2.45 ± 0.24 cm and 2.51 ± 0.24 cm, respectively). MMS2 (quarter-strength) showed the poorest response, with shoot formation dropping to 71.43% in nodal and 61.54% in shoot tip explants, and significantly reduced shoot lengths (1.58 ± 0.19 cm for nodal and 1.32 ± 0.32 cm for shoot tip explants). Reduced salt concentrations may limit the availability of essential ions like nitrogen, potassium, and calcium- key elements required for shoot development and elongation (Verma et al. 2023).

As in previous studies (Thakur and Sharma 2021), nodal explants consistently outperformed shoot tip explants across all medium strengths, producing higher shoot numbers and longer shoots. This can be attributed to the greater availability of meristematic tissue and higher endogenous hormone levels in nodal segments, which make them more responsive to cytokinin-induced shoot initiation. These findings underscore the importance of optimizing basal nutrient strength for the successful micropropagation of *Rotala rotundifolia*. While reducing nutrient concentrations can be cost-effective, such modifications should be carefully balanced against the risk of suboptimal morphogenesis. The use of full-strength MS medium with 1.0 µM/L BAP is recommended for efficient shoot proliferation and elongation in this species.

Table 4: Effects of different strengths of MS medium with 1.0 µM BAP on axillary shoot proliferation and elongation from nodal and shoot tip explants of *Rotala rotundifolia.*

Medium	Nodal segment			Shoot tip		
	Shoot formation (%)	Total No. of shoot/ explant	Avg. length of shoot/ explant (cm)	Shoot formation (%)	Total No. of shoot/ explant	Avg. length of shoot/explant (cm)
MS	93.33	47.85 ± 0.86[a]	4.95 ± 0.51[a]	81.82	40.32 ± 0.14[a]	3.64 ± 0.12[a]
MMS_1	85.71	24.72 ± 0.43[b]	3.45 ± 0.24[b]	72.73	21.32 ± 0.33[b]	2.51 ± 0.24[b]
MMS_2	71.43	14.65 ± 0.27[c]	1.58 ± 0.19[c]	61.54	10.22 ± 0.24	1.32 ± 0.32[c]

Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level. There were 10-15 explants for each treatment and data ($\bar{X}\pm$ SE) were recorded after 8 weeks of culture initiation.

Effect of medium type on shoot regeneration

This study evaluated the effect of medium consistency- liquid vs. agar-gelled- on axillary shoot regeneration in *Rotala rotundifolia* using nodal and shoot tip explants cultured on MS medium supplemented with 1.0 µM/L BAP (Fig. 2A-B). Significant differences were observed in shoot formation percentage, the number of

shoots per explant, and average shoot length between the two medium types (Table 5). Liquid MS medium demonstrated superior performance across all measured parameters. In nodal explants, 100% shoot regeneration was achieved, with the highest average number of shoots per explant (55.36 ± 0.54) and shoot length (5.01 ± 0.12 cm). Shoot tip explants in liquid medium also performed well, with a 93.33% shoot formation rate, 41.36 ± 0.24 shoots per explant, and an average shoot length of 3.24 ± 0.12 cm. In contrast, agar-solidified MS medium resulted in significantly lower shoot induction (86.67% for nodal and 71.43% for shoot tip explants), with reduced shoot numbers (35.23 ± 0.23 and 15.0 ± 0.43, respectively) and shorter shoot lengths (3.78 ± 0.14 cm and 2.51 ± 0.34 cm, respectively).

The superior response in liquid medium may be attributed to better nutrient availability and increased contact between explants and the medium, facilitating more efficient uptake of water, nutrients, and growth regulators. These findings are consistent with prior reports by Bhagat et al. (2021) and Gantait et al. (2019), who demonstrated that liquid media significantly enhance shoot proliferation rates in various plant species due to reduced mechanical resistance and improved medium-explant interaction. Moreover, liquid culture systems also favor uniform exposure of plant tissues to exogenous phytohormones like BAP, which promotes rapid cell division and shoot bud differentiation. However, the use of liquid media must be optimized to avoid issues such as hyperhydricity, a condition not observed in this study but reported in other species such as *Mentha arvensis* and *Oryza sativa* under similar conditions (Sharma et al. 2020). Nodal explants again demonstrated better regenerative capacity compared to shoot tip explants in both media types, reaffirming their higher morphogenic potential. These observations support previous work by Devi and Borthakur (2022), which highlighted nodal explants as a superior source for shoot multiplication due to their active meristematic regions and favorable endogenous hormone profiles. The findings highlight the potential of liquid MS medium supplemented with 1.0 µM BAP for efficient and high-frequency micropropagation of *Rotala rotundifolia*. Such optimizations are essential for large-scale propagation and conservation of this important aquatic medicinal species.

Table 5: Effects of different types of MS medium with 1.0 µM/L BAP on shoot regeneration from nodal segments and shoot tip explants of *Rotala rotundifolia.*

Types of medium	Nodal segment			Shoot tip		
	Shoot formation (%)	Total No. of shoot/ explants	Avg. length of shoot/ explant (cm)	Shoot formation (%)	Total No. of shoot/ explants	Avg. length of shoot/ explant (cm)
Liquid	100.00	55.36 ± 0.54[a]	5.01 ± 0.12[a]	93.33	41.36 ± 0.24[a]	3.98 ± 0.12[a]
Agar gelled	86.67	35.23 ± 0.23[b]	3.78 ± 0.14[b]	71.43	15.0 ± 0.43[b]	2.51 ± 0.34[b]

Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level. There were 10-15 explants for each treatment, and data ($\bar{X}$±SE) were recorded after 6 weeks of culture initiation.

Effect of auxins on adventitious root formation

The influence of different types and concentrations of auxins (IBA, NAA, and IAA) on adventitious root formation from *in vitro* grown shoots of *Rotala rotundifolia* was assessed after 8 weeks of culture on MS medium. Parameters evaluated included rooting percentage, number of roots per culture, average root length, days to root initiation, and callus formation (Table 6). Among all treatments, IBA at 2.0 µM/L demonstrated the most effective rooting response, with the highest root formation percentage (93.33%) and root number per culture (15.92 ± 2.59). This was significantly higher than the control (11.12 ± 1.55 roots) and other auxin treatments. These findings align with earlier studies, which identified IBA as superior for root induction due to its stability and slow degradation in culture media (Hassan et al. 2021, Ahmad et al. 2022a).

The longest average root length (6.95 ± 1.57 cm) was observed in the hormone-free control (HO), followed closely by IBA treatments (6.85 ± 1.49 cm for 2.0 µM and 6.20 ± 0.85 cm for 4.0 µM). Though the number of roots increased with auxin treatment, root elongation was not significantly enhanced in most cases,

with lengths often decreasing at higher auxin concentrations. For instance, IAA at 6.0 µM/L produced significantly shorter roots (2.81 ± 0.81 cm). This suggests a possible inhibitory effect of supra-optimal auxin levels on cell elongation (Patel et al. 2021).

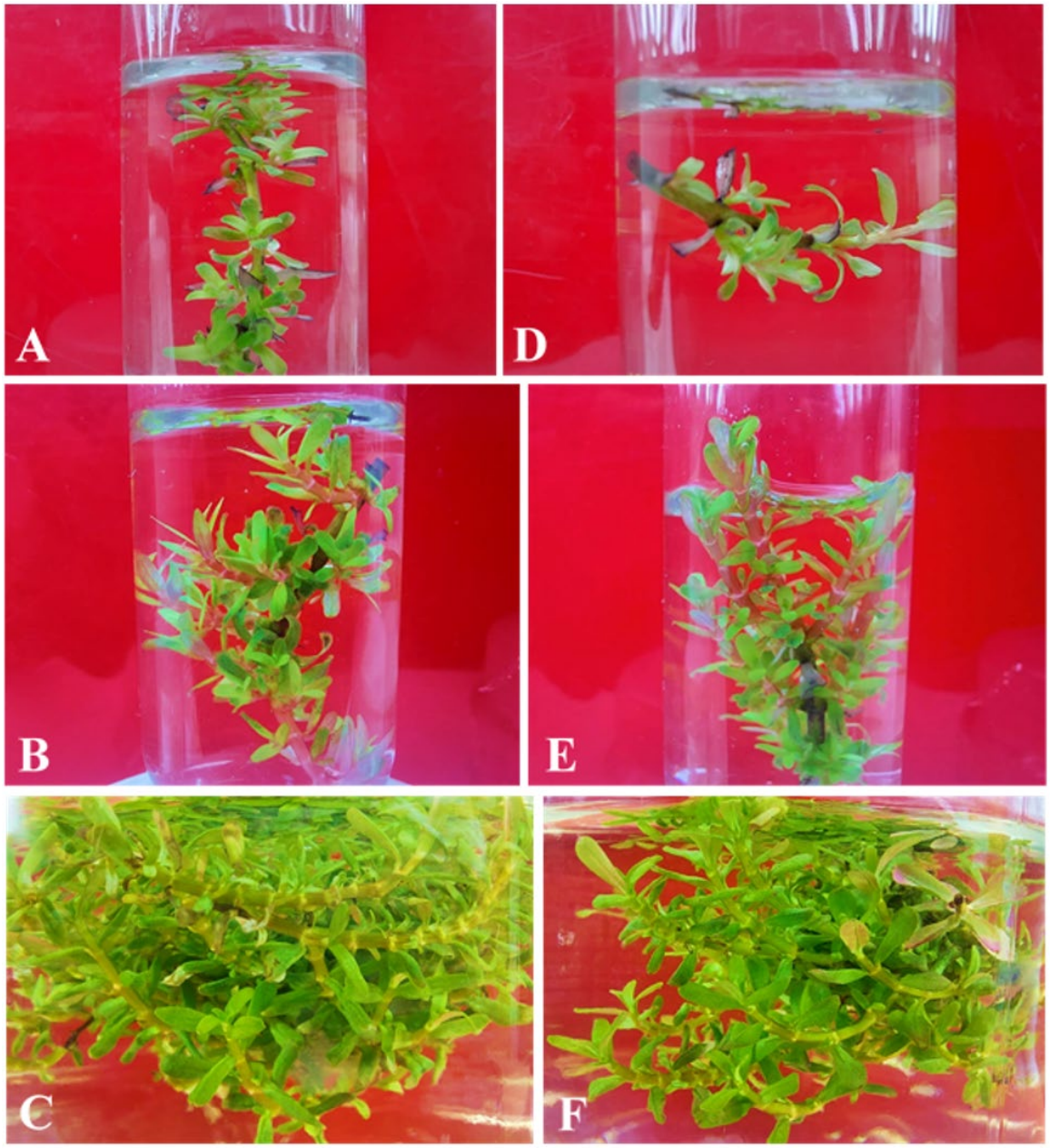

Fig. 1(A-F): Photographs showing the axillary shoots proliferation of *Rotala rotundifolia.* A-C: Axillary shoots proliferation from the nodal explant on MS medium containing 1.0 µM BAP after 4 weeks (A), 6 weeks (B) and 8 weeks (C) of culture initiation. D-F**:** Axillary shoots proliferation from the shoot tip explant on MS medium containing 1.0 µM BAP after 4 weeks (A), 6 weeks (B) and 8 weeks (C) of culture initiation.

A clear concentration-dependent trend was evident across all auxins. At higher concentrations (6.0 µM/L), IBA and NAA resulted in decreased root number and length, possibly due to auxin-induced feedback inhibition or cytotoxic effects. For example, IBA at 6.0 µM/L yielded only 8.44 ± 2.68 roots per culture, a significant drop compared to 2.0 µM. Similar inhibitory patterns were reported in *Centella asiatica* and *Bacopa monnieri* under high auxin concentrations (Kumar et al. 2022, Singh et al. 2023a). Callus formation was minimal or absent in IBA and IAA treatments but was prominently observed in NAA treatments, especially at 6.0 µM

(+++). While NAA supported moderate rooting (e.g., 12.55 ± 2.60 roots at 2.0 µM), its association with excessive callusing can interfere with root function and reduce plantlet survival during acclimatization (Roy et al. 2021b).

Considering all parameters- root number, length, rooting percentage, and minimal callus formation- 2.0 µM/L IBA emerged as the most effective treatment for rooting of *R. rotundifolia*. The treatment promoted the highest root number and satisfactory root length without undesirable callus induction. This study demonstrates that IBA, particularly at 2.0 µM/L, is optimal for *in vitro* root induction in *Rotala rotundifolia*, offering a practical rooting protocol for efficient micropropagation. These findings provide a valuable foundation for large-scale conservation and commercial production of aquatic ornamental plants.

Table 6: Effects of different concentrations of auxins on adventitious root formation from *in vitro* grown shoots in MS medium.

PGRs	PGRs Conc. (µM/L)	Root formation (%)	No. of total root/culture	Avg. length of root/culture (cm)	Days to root formation	Callus formation
HO	0.0	86.67	11.12 ± 1.55b	6.95 ± 1.57a	13	-
	2.0	93.33	15.92 ± 2.59a	6.85 ± 1.49a	16	-_
IBA	4.0	90.91	13.33 ± 2.30b	6.20 ± 0.85a	17	-
	6.0	83.33	8.44 ± 2.68c	5.55 ± 0.59a	20	-
	2.0	58.33	12.55 ± 2.60b	5.54 ± 0.64a	12	+
NAA	4.0	46.15	10.63 ± 2.46b	4.86 ± 0.48a	14	++
	6.0	38.46	7.70 ± 2.10c	4.36 ± 0.52a	18	+++
	2.0	53.85	6.55 ± 1.20c	3.51 ± 0.46a	15	_
IAA	4.0	45.45	7.55 ± 2.90c	3.75 ± 0.35a	12	-
	6.0	38.46	5.25 ± 1.28c	2.81 ± 0.81a	17	_

Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level. Each treatment consisted of 10-15 shoot-cuttings, and data ($\bar{X}$± SE) were recorded after 8 weeks of culture.

Acclimatization of *in vitro* regenerated shoots

Successful acclimatization of *in vitro* regenerated *Rotala rotundifolia* plantlets is a crucial step toward their effective establishment under *ex vitro* conditions. The survival rate and average shoot length were evaluated under three different substrate treatments over a period of 10 weeks (Table 7). Among the tested substrates, the combination of garden soil and cocopeat (1:1) significantly enhanced both survival and growth of plantlets (Fig. 2D). This treatment resulted in the highest survival rate (90% *in vivo*, 70% *ex vitro*) and maximum average shoot length (7.65 ± 0.42 cm), indicating an optimal balance of aeration, moisture retention, and nutrient availability. Cocopeat alone also supported reasonably high survival (80% *in vivo*, 60% *ex vitro*) with moderate shoot growth (5.43 ± 0.21 cm), possibly due to its excellent water-holding capacity and porosity, which facilitate root development and reduce transplant shock (Nandwani et al. 2020). In contrast, the substrate composed of garden soil, sand, and compost (2:1:1) showed the lowest performance, with survival rates of only 60% (*in vivo*) and 40% (*ex vitro*), and the shortest average shoot length (4.12 ± 0.14 cm). The presence of sand may have contributed to rapid drainage and reduced moisture retention, thereby impeding plantlet establishment.

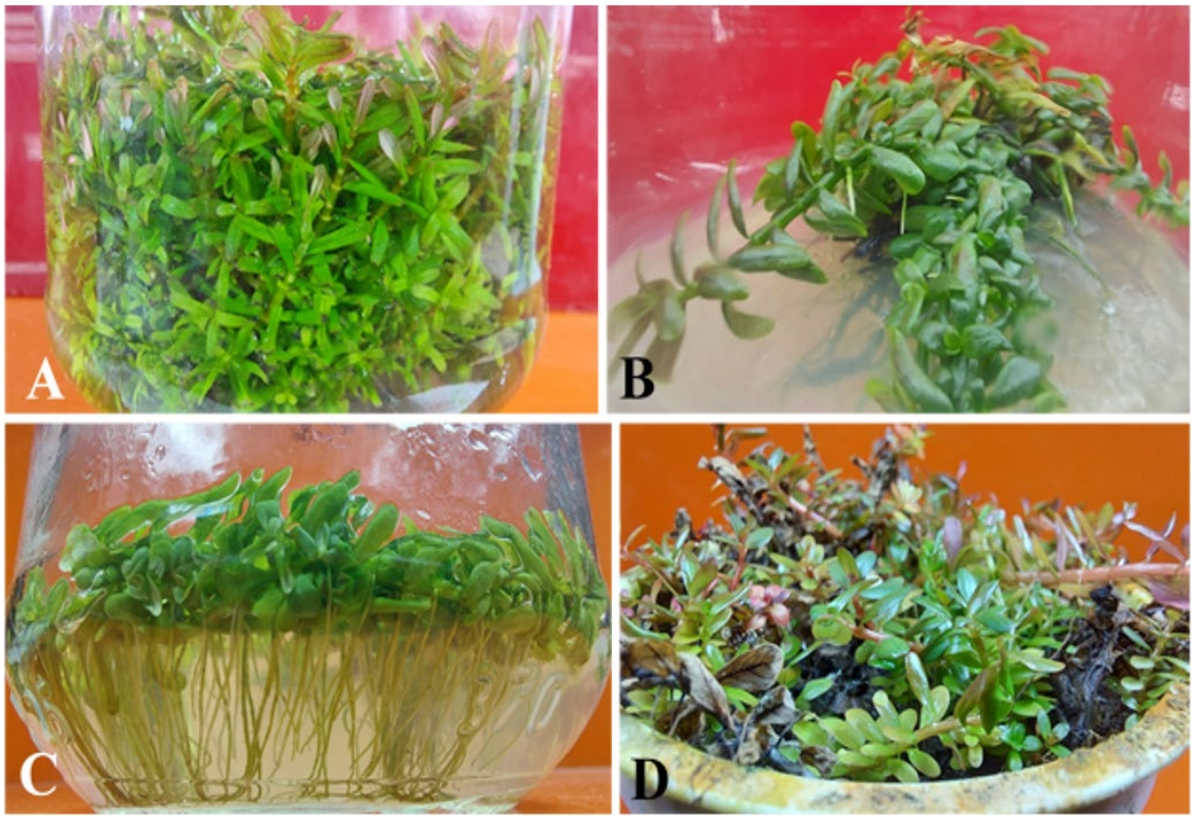

Fig. 2 (A-D): Photographs showing shoots multiplication and acclimatization of *Rotala rotundifolia.* A-B: Axillary shoots multiplication from the nodal explant on MS liquid (A) and agar-gelled solidified (B) medium containing 1.0 µM BAP after 8 weeks of culture initiation. C-D: Adventitious roots formation from *in vitro* grown shoots on MS medium containing 2.0 µM IBA after 4 weeks (C) of culture initiation. *In vitro* grown shoots acclimatized on Garden soil + cocopeat (1:1) after 6 weeks of transplantation (D).

Table 7: Effects of different substrates on the acclimatization of *Rotala rotundifolia.*

Substrate	Survival rate (%)		Avg. length of shoots (cm)
	In vivo	*Ex vitro*	*Ex vitro*
Coco peat	80.00	60.00	5.43 ± 0.21b
Garden soil + cocopeat (1:1)	90.00	70.00	7.65 ± 0.42a
Garden soil + sand + compost (2:1:1)	60.00	40.00	4.12 ± 0.14c

Values represent mean ± standard error of 20 replicates per treatment in 4 repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple comparison test at 0.05 probability level. Each treatment consisted of 10 plantlets, and data ($\bar{X}\pm SE$) were recorded after 10 weeks of transplantation.

These findings are consistent with previous reports that emphasized the role of substrate composition in determining acclimatization success in micropropagated aquatic and semi-aquatic plants (Chauhan et al. 2019, Kumari et al. 2021). Similar results were reported by Singh and Sharma (2022), where a soil-cocopeat mixture significantly improved acclimatization outcomes in *Bacopa monnieri*, another aquatic medicinal plant. The superior performance of the garden soil and cocopeat mixture in the current study may also be attributed to the synergistic effects of organic matter from the soil and the physical structure provided by cocopeat. Moreover,

shoot length is a critical parameter indicative of plant vigor and successful transition to autotrophic growth during acclimatization. The increased shoot elongation in the garden soil + cocopeat treatment may suggest a conducive microenvironment that facilitates nutrient uptake and reduces transplant stress, which is crucial for commercial-scale production and ornamental applications of *R. rotundifolia*. Present results demonstrate that the choice of substrate plays a pivotal role in the acclimatization of *Rotala rotundifolia*. The garden soil and cocopeat mixture (1:1) appears to be the most effective substrate, promoting both high survival rates and vigorous shoot growth under *in vivo* and *ex vitro* conditions.

Conclusion

An efficient and reproducible *in vitro* regeneration protocol for *Rotala rotundifolia* was successfully developed using nodal and shoot tip explants. The combination of 2.0 µM/L BAP with 1.0 µM/L IBA in full-strength MS liquid medium proved optimal for maximum shoot proliferation and elongation. For rooting, 2.0 µM/L IBA was most effective, while garden soil and cocopeat (1:1) provided the best acclimatization substrate. This optimized protocol offers a valuable tool for the large-scale propagation, conservation, and sustainable commercialization of this ecologically and ornamentally significant aquatic species.

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Conflict of interest: The authors declare that they have no conflicts of interest related to this study.

Author's contribution: Mohammad Rashedul Islam, Md. Mobarak Hosen, Faysal Ahmad and Ahmed Al Sadik collected samples and data, conducted experiments. Mustafa Abul Kalam Azad designed the experiment, supervised the study, and Muhammad Nurul Amin corrected the manuscript. All authors have read and approved the final manuscript.

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