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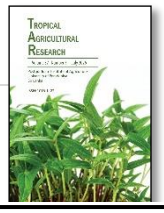

RESEARCH

Optimized Micropropagation of *Rosa indica* L. via BAP–IAA Synergy and Hormone-Free Rooting

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ABSTRACT

Conventional propagation of *Rosa indica* L. through stem cuttings is limited by low rooting efficiency and seasonal dependence, restricting large-scale production. This study aimed to develop a simplified and cost-effective micropropagation protocol by optimizing cytokinin–auxin interactions for shoot multiplication, assessing the need for exogenous auxins during rooting, quantifying multiplication efficiency and examining In vitro flowering. Single-node explants from field-grown clonal mother plants were cultured on Murashige and Skoog (MS) medium supplemented with combinations of 6-benzylaminopurine (BAP) and auxins. The experiment followed a completely randomized design (CRD) (n = 60 per treatment) and data were analyzed using ANOVA and LSD at $p < 0.05$. The combination of 3 mg L^{-1} BAP + 3 mg L^{-1} indole-3-acetic acid (IAA) produced the best results, with the highest shoot proliferation (3.55 ± 0.15 shoots per explant), longest shoots (4.72 ± 0.12 cm) and fastest initiation (17.77 ± 0.33 days). The multiplication rate was 3.55 shoots per explant per 30-day cycle, yielding approximately 12.6 shoots after two cycles. For rooting, micro shoots were transferred to half-strength MS media with or without auxins. Half-strength MS without growth regulators showed superior rooting (4.67 ± 0.09 roots per shoot; 2.60 ± 0.07 cm), significantly outperforming auxin-supplemented media. In vitro flowering occurred in 8.3% of cultures after 8 weeks on BAP–IAA medium, representing a novel observation under simplified conditions. Somaclonal variation increased with subcultures, reaching 21.7% after four cycles. The optimized low-input protocol enables year-round mass propagation of *Rosa indica*, giving nurseries a practical way to avoid seasonal dependence and lower input costs for plant growth regulators.

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INTRODUCTION

Rosa indica L. a member of the Rosaceae family, is one of the most widely cultivated ornamental species, valued globally for landscaping, essential oil extraction and its use as a vigorous rootstock for hybrid roses (Gudin, 1999; Zeng *et al*., 2013). In Pakistan and other South Asian regions, it plays a central role (claim with specific tonnage data to show exactly why Rosa indica matters economically) in local floriculture markets. With an estimated annual floricultural output of 10,000–12,000 tons, the non-traditional agricultural sector relies heavily on *Rosa indica* the region's leading cut-flower and rootstock crop making reliable mass-propagation essential for agricultural security. However, large-scale propagation of *Rosa indica* L. by conventional techniques such as stem cuttings and grafting is difficult due to low and inconsistent rooting success, vulnerability to pathogens and strong dependence on environmental seasonality (Rout *et al*., 2000; Shabbir *et al*., 2009). These limitations underscore the need for a reliable, year-round propagation system.

In vitro micropropagation offers an efficient alternative by enabling the rapid production of genetically uniform, disease-free plants independent of seasonal fluctuations (George *et al*., 2008; Tawfik *et al*., 2018). The success of micropropagation, however, depends on optimizing the balance between cytokinins and auxins, the key determinants of organogenic pathways. Cytokinins, such as 6-benzylaminopurine (BAP) stimulate axillary bud break and shoot proliferation, whereas auxins including indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) regulate adventitious rooting (Kanchanapoom *et al*., 2009; Omidi *et al*., 2016). Since hormonal requirements are highly genotype-specific, micropropagation protocols must be customized for each species or cultivar (Mahmood *et al*., 2016).

Although numerous protocols have been developed for rose micropropagation, many rely on multi-step, hormone-intensive culture systems, increasing cost, labor and risk of callus-mediated somaclonal variation (Mehbub *et al*., 2022). Despite the widespread assumptions regarding the necessity of exogenous auxins, the role of endogenous auxin levels in *Rosa indica* L. micro-shoots during rooting have not been rigorously evaluated. Furthermore, comprehensive comparisons of BAP–auxin synergies specifically tailored for *R. indica* remain limited (Pasternak & Steinmacher, 2024).

This study proposes a streamlined, two-stage micropropagation system for *Rosa indica* L., aiming to identify the optimal BAP–IAA combination for efficient shoot multiplication and to determine the necessity of exogenous auxins during rooting. The approach leverages a specific cytokinin–auxin synergy to enhance shoot induction while relying on endogenous auxins to support effective rooting, ultimately providing a cost-effective method to improve commercial propagation and enable large-scale cultivation.

MATERIALS AND METHODS

Plant Material and Surface Sterilization

Explant material of *Rosa indica* L. was collected from two-year-old clonal mother plants maintained at the experimental field of Sindh Agriculture University, Tandojam, Pakistan, established via stem cuttings to ensure genetic uniformity. The plants were grown under natural agro-climatic conditions, with average temperatures of 25–30 °C and relative humidity of 60–70%, following standard agronomic practices to maintain vigor. Monthly fertilization with a balanced NPK (20:20:20) at $2\,g\,L^{-1}$ and pruning every three months promoted actively growing lateral shoots. To reduce microbial contamination, no systemic fungicides or pesticides were applied for at least four weeks before explant collection. Healthy nodal segments (1–1.5 cm) were excised from the middle portion of semi-hardwood shoots, avoiding apical and basal regions due to strong apical dominance and reduced regenerative potential, respectively.

Explants were initially rinsed under running tap water for 10 minutes, followed by a 2 minute treatment with 0.1% (v/v) Tween 20

(Sigma Aldrich, St. Louis, MO, USA) to remove surface debris. For surface sterilization, explants were immersed in 0.1% (w/v) mercuric chloride ($HgCl_2$) (Merck, Darmstadt, Germany) for 4 minutes. After sterilization, they were rinsed three times with sterile distilled water under aseptic conditions. Finally, the cut ends were trimmed by 1–2 mm to eliminate any damaged tissue and ensure optimal regeneration.

Culture Media and Conditions

Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) was prepared using 3% (w/v) sucrose (Sigma-Aldrich, St. Louis, MO, USA) and solidified with 0.8% (w/v) plant agar (Duchefa Biochemie, Haarlem, Netherlands). The pH of the medium was adjusted to 5.8 ± 0.1 using 0.1 N NaOH or HCl prior to autoclaving at 121 °C for 15 minutes (Sanyo autoclave, Osaka, Japan). Plant growth regulators (PGRs), including 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA), were obtained from Duchefa Biochemie. Cultures were incubated in a controlled growth chamber (Percival Scientific, Perry, IA, USA) at 25 ± 2 °C under a 16-hour photoperiod with a light intensity of approximately 3000 lux provided by cool white fluorescent lamps (Philips, Amsterdam, Netherlands).

Shoot Induction and Rooting

For shoot induction, single-node explants were inoculated onto MS medium supplemented with four different combinations: BAP 2 mg L^{-1}+IAA 2 mgL^{-1}, BAP 3 mgL^{-1} + IAA 3 mgL^{-1}, BAP 3 mgL^{-1} + IBA 3 mgL^{-1} and BAP 3 mgL^{-1} + NAA 3 mgL^{-1}. A hormone-free MS medium served as the control. After 30 days of culture, number of days to shoot initiation, number of shoots per explant and shoot lengths were recorded. For rooting, elongated shoots (3–4 cm) were transferred to five media: half-strength MS without plant growth regulators (PGRs); half-strength MS with IAA, IBA or NAA and full-strength MS without PGRs and number of roots and root lengths were evaluated after 25 days.

Experimental Design and Statistical Analysis

All experiments were arranged as a completely randomized design (CRD) with three replicates per treatment, each consisting of 20 explants (n = 60 per treatment). Data were subjected to one-way Analysis of Variance (ANOVA) using SPSS Statistics version 28 (IBM, USA). Where significant differences were detected, means were compared using the Least Significant Difference (LSD) test at $p < 0.05$. Results are presented as mean ± standard error (SE).

RESULTS AND DISCUSSION

This study establishes a simplified and efficient micropropagation system for *Rosa indica* L. demonstrating that optimized cytokinin–auxin interactions enhance shoot proliferation, while hormone-free conditions improve rooting efficiency.

Cytokinin–Auxin Synergy in Shoot Multiplication

A statistically significant interaction between cytokinin (BAP) and auxin (IAA) was observed for all measured parameters of shoot regeneration (Table 1). Among the treatments evaluated, the combination of 3 mgL^{-1} BAP+3 mgL^{-1} IAA proved most effective, producing the highest shoot proliferation (3.55±0.15 shoots per explant), greatest shoot length (4.72±0.12 cm) and the shortest time to shoot initiation (17.77±0.33 days). Pairwise LSD comparisons confirmed that this treatment was significantly superior ($p < 0.05$) to all other combinations, including the hormone-free control. Moderate responses were obtained with BAP 2 mgL^{-1}+ IAA 2 mgL^{-1}, which yielded 2.67±0.09 shoots per explant with a mean shoot length of 3.56 ± 0.11 cm and initiation at 19.44±0.45 days. In contrast, replacing IAA with IBA or NAA at 3 mg L^{-1} reduced efficiency. Specifically, BAP + IBA produced 3.11±0.12 shoots per explant (4.03±0.09 cm), while BAP + NAA resulted in 2.82±0.10 shoots (3.60±0.14 cm) with comparatively delayed initiation (20.33±0.38 days).

The hormone-free MS medium performed poorest, confirming the necessity of exogenous plant growth regulators for effective shoot induction in *Rosa indica* L. The superior performance of the BAP–IAA combination indicates an optimal cytokinin–auxin balance that enhances meristematic competence. This likely occurs through coordinated activation of cytokinin response regulators (ARR genes) alongside auxin-mediated polar transport, promoting both shoot initiation and elongation.

These findings are consistent with earlier studies in rose species, including Rosa hybrida, Rosa damascena and Rosa bourboniana, where balanced hormonal interactions were critical for maximizing shoot proliferation (Rout *et al*., 2006; Aggarwal *et al*., 2020 and Chhalgri *et al*., 2020). The comparatively lower performance of IBA and NAA further supports reports of genotype-specific auxin responses and their limited effectiveness in shoot multiplication relative to IAA (Omidi *et al*., 2016).

Table 1: Shoot Induction of *Rosa indica* L. under different BAP–Auxin combinations after 30 Days (n = 20)

Treatment	Days to shoot initiation	Shoot number	Shoot length (cm)
BAP 2 + IAA 2 mg L^{-1}	19.44 ± 0.45^{b}	2.67 ± 0.09^{b}	3.56 ± 0.11^{b}
BAP 3 + IAA 3 mg L^{-1}	17.77 ± 0.33^{a}	3.55 ± 0.15^{a}	4.72 ± 0.12^{a}
BAP 3 + IBA 3 mg L^{-1}	18.55 ± 0.41^{b}	3.11 ± 0.12^{b}	4.03 ± 0.09^{b}
BAP 3 + NAA 3 mg L^{-1}	20.33 ± 0.38^{c}	2.82 ± 0.10^{b}	3.60 ± 0.14^{b}
Control (MS only)	25.50 ± 0.62^{d}	1.33 ± 0.08^{c}	2.05 ± 0.10^{c}

Values are mean ± SE. Different superscript letters within a column indicate significant differences at *p* < 0.05 (LSD test).

Figure 1: Initial Shoot initiation of *Rosa indica* L. under different BAP–Auxin treatments. (a) Shoot initiation observed in culture bottle 1; (b) Shoot initiation observed in culture bottle 2 under the same treatment conditions.

Figure 2: Shoot proliferation after subculturing of *Rosa indica* L. on different BAP–IAA medium. (a)Shoot proliferation response observed in culture bottle 1; (b) Shoot proliferation response observed in culture bottle 2

Exogenous Hormone-Free Rooting

A key outcome of this study is the superior rooting response obtained on half strength MS medium without exogenous PGRs (Table 2). This treatment produced the highest number of roots (4.67 ± 0.09 per shoot) and greatest root length (2.60 ± 0.07 cm), significantly outperforming all auxin-supplemented media (*p* < 0.05), which yielded fewer, shorter roots and frequently induced basal callus formation.

This finding demonstrates that, when shoots have been primed with IAA during multiplication (3 mg L^{-1} for 30 days), exogenous auxins in the rooting medium are unnecessary and can even be inhibitory. The enhanced rooting observed here can be explained by physiological auxin carryover from the multiplication phase. This endogenous auxin reserve, combined with the reduced ionic strength and osmotic pressure of half strength MS medium, likely created optimal conditions for root primordia differentiation. Thus, the system is more accurately described as exogenous hormone free rather than auxin independent.

Comparable improvements in rooting under reduced salt conditions have been reported in *Rosa centifolia*, where lower nutrient concentrations minimized osmotic stress and promoted root elongation (Aliabad *et al*., 2019). In contrast, the application of exogenous auxins in the present study appeared to suppress rooting efficiency, suggesting that additional auxin may be inhibitory once endogenous levels are sufficient. Similar genotype-specific responses have also been documented in Rosa hybrida (Kumar *et al*., 2013).

Importantly, the absence of callus formation in hormone-free medium indicates direct root organogenesis, which is associated with stronger root–shoot connections, improved acclimatization and reduced risk of somaclonal variation (Soumia *et al*., 2025). These results highlight the strong intrinsic rooting capacity of *Rosa indica* L. and support the development of a simplified, cost-effective rooting strategy for large-scale micropropagation.

Table 2: Rooting response of *Rosa indica* L. on Auxin-free and Auxin-supplemented Medium (n = 20)

Treatment	Root number	Root length (cm)
½ MS (no PGR)	4.67 ± 0.09^{a}	2.60 ± 0.07^{a}
½ MS + IAA 1 mg L^{-1}	3.72 ± 0.11^{b}	2.22 ± 0.08^{b}
½ MS + IBA 1 mg L^{-1}	3.55 ± 0.10^{b}	2.01 ± 0.09^{b}
½ MS + NAA 1 mg L^{-1}	3.40 ± 0.12^{b}	1.92 ± 0.08^{b}
Full MS (no PGR)	2.78 ± 0.15^{c}	1.73 ± 0.07^{c}

Values are mean ± SE. Different superscript letters within a column indicate significant differences at *p* < 0.05 (LSD test).

Figure 3: Root Induction response of *Rosa indica* L. on (a) Auxin-Free-control treatment and (b) Auxin-Supplemented - IBA, IBA & NAA Media

In vitro Flowering

In vitro flowering was observed in 8.3% of cultures (5 out of 60 shoots) after 8 weeks on MS medium supplemented with BAP 3 mg L^{-1} + IAA 3 mg L^{-1} under a 16-h photoperiod at 25 ± 2 °C. Flowers developed directly within culture vessels, exhibiting reduced size (~1.5 cm diameter) and partial reproductive structures compared to ex vitro flowers.

The induction of *In vitro* flowering under BAP–IAA treatment likely reflects a hormonally mediated transition from vegetative to reproductive development. Elevated cytokinin levels may stimulate the expression of key floral integrator genes, including FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and LEAFY (LFY), which collectively regulate floral meristem identity. Additionally, the extended photoperiod (16 h), sucrose-mediated signaling and prolonged culture duration likely acted synergistically to activate the floral pathway.

The observed flowering frequency (8.3%) under a simplified hormonal regime represents a novel finding for *Rosa indica* L. and highlights the potential application of this system in rapid breeding and reproductive biology studies.

Somaclonal Variation

Phenotypic variation increased progressively with successive subculture cycles, reaching 21.7% by the fourth cycle (Table 3). Variations were primarily observed in leaf pigmentation (pale green, dark green, variegated patterns) and flower color after acclimatization. The progressive increase in phenotypic variation with successive subcultures indicates cumulative genetic and epigenetic instability, likely driven by prolonged exposure to cytokinins. Such effects may include DNA methylation changes and activation of transposable elements, leading to heritable phenotypic alterations.

While somaclonal variation is undesirable for clonal propagation due to loss of genetic fidelity, it may provide opportunities for mutation breeding and the development of novel ornamental traits.

Integrated Protocol Offers Economic and Practical Advantages

Integrating optimized shoot multiplication (3 mgL^{-1} BAP + 3 mgL^{-1} IAA) with hormone-free rooting establishes a simplified two-stage micropropagation protocol for *Rosa indica* L. that is more economical, produces higher-quality plantlets and reduces somaclonal variation. Eliminating auxins during rooting lowers media costs (Mehbub *et al*., 2022), while direct root formation without callus enhances root thickness, attachment and acclimatization success (Soumia *et al*., 2025). Reduced exposure to synthetic PGRs preserves genetic fidelity across propagation cycles (Kumar *et al*., 2013; Pasternak & Steinmacher, 2024) and the straightforward two-stage design avoids complex hormonal regimes, enabling easy standardization and scalable adoption in commercial nurseries and conservation programs (Chandler & Werr, 2015; Shabbir *et al*., 2009).

Figure 4: Genetic diversity in flower and leaf color variation in *Rosa indica* L.

CONCLUSIONS

This study establishes a scientifically robust and economically viable two-stage micropropagation protocol for *Rosa indica* L., integrating optimized shoot multiplication (3 mg L^{-1} BAP+3 mgL^{-1} IAA) with exogenous hormone-free rooting on half strength MS medium. The approach maximizes shoot proliferation (3.55 shoots per 30 day cycle), ensures direct root organogenesis, enhances plantlet quality and limits the increase in somaclonal variation compared to protocols with continuous auxin exposure. The protocol demonstrates enhanced efficiency, reproducibility and cost- effectiveness relative to many published methods (Chandler & Werr, 2015; Kumar *et al*., 2013; Pasternak & Steinmacher, 2024; Soumia *et al*., 2025; Shabbir *et al*., 2009). The induction of *in vitro* flowering (8.3% after 8 weeks) offers additional potential for accelerated breeding and genetic studies. This protocol is particularly suitable for commercial propagation, ornamental industries, conservation programs and germplasm improvement. However, long-term genetic stability under large-scale production requires further validation using molecular markers. Future research should focus on molecular profiling of endogenous hormones during rooting, scaling through automated bioreactors and extending the method to other Rosa species to enhance biodiversity conservation and support rural horticultural economies.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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