JOURNAL OF PLANT RESEARCH AND INNOVATIONS
Jul-Dec 2025, VOL. 3, ISSUE 2, pp. 5-10
https://doi.org/10.61577/jpri.2025.100007

RESEAPRO JOURNALS

ORIGINAL ARTICLE

OPEN ACCESS

In vitro shoot regeneration and chloroplast *Maturase K* (*matK*) gene-based phylogenetic assessment in *Phalaenopsis* orchid

Rajesh Ramasandra Venkataraja[1], Athulya Surendran Nair[1], Ananya Sinha[2] , Uday Kumar Byrathanahalli Venkataswamy[2], Nagesh Narayanappa[1], and Narendra Ram[3]

[1]**Department of Plant Biotechnology, College of Agriculture, University of Agricultural Sciences, Bangalore, India**
[2]**Department of Botany, National Centre for Biological Sciences, Bangalore, India**
[3]**Green Square Technologies, Jalige, Kundana Hobli, Bengaluru, India**

ABSTRACT

Phalaenopsis orchids are globally significant ornamental plants, yet traditional propagation is inefficient for commercial demands. While micropropagation is used, standardised protocols for direct organogenesis using floral stalk nodes remain limited, and the influence of genetic lineage on these protocols is poorly understood. This study aimed to optimise a direct regeneration protocol using 6-Benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA) and to evaluate if phylogenetic relationships based on the chloroplast maturase K (*matK*) gene correlate with in vitro regeneration potential. Floral stalk nodes and in vitro subcultures were treated with MS medium supplemented with banana homogenate (100 mg/L), coconut water (100 mL/L), and varying PGR concentrations (T0–T6). Phylogenetic analysis of 14 species was conducted using Maximum Likelihood trees. Treatment T5 (2.5 mg/L BAP + 0.6 mg/L NAA) was optimal, yielding shoot initiation in 29.66 days and 18 shootlets per culture. Phylogenetic analysis grouped species into three clades; Clade I species showed superior regeneration compared to the more recalcitrant Clade III. Root development was minimal across all initial treatments, indicating that the current hormonal balance is insufficient for complete plantlet development without specialised rooting induction stages. The study provides a high-throughput protocol for specific *Phalaenopsis* clades while highlighting the need for genotype-specific modifications for evolutionarily distant species. This integrated approach offers a foundation for scalable propagation and informs future genetic improvement strategies for *Phalaenopsis* orchids.

KEY WORDS

Phalaenopsis orchids; Direct organogenesis; *matK* phylogeny

ARTICLE HISTORY

Received 21 November 2025;
Revised 12 December 2025;
Accepted 19 December 2025

Introduction

Phalaenopsis orchids are the most commercially valuable and important ornamental plants worldwide due to their vibrant colouration, long-lasting flowers, and adaptability to both indoor and outdoor environments [1]. The wide range genus *Phalaenopsis* (Aeridinae, Vandeae, Epidendroideae, Orchidaceae) comprises approximately 50–65 species. It is collectively distributed from India to China, Korea, Japan, Thailand, Indochina, Malaysia, and Indonesia, as well as the Philippines, Australia, and New Guinea [2]. The 22 species were recorded in China and India, and Burma, including the endemic species *Phalaenopsis wilsonii. Rolfe* and *P. Stobartiana Rchb.f* [3,4]. However, wild populations have declined due to habitat fragmentation and overcollection. Therefore, greater emphasis is needed on their biological conservation [4].

Their dominance in the global floriculture trade is evident, with more than 70% of tropical orchid sales attributed to the *Phalaenopsis genus* [5]. Despite their economic importance, traditional vegetative propagation through keiki (offshoot) development or clump division remains highly inefficient. These methods are slow, produce limited progeny within the genus, and there is a lack of integrated data linking molecular phylogeny to morphogenic capacity and fail to meet the demands of commercial-scale cultivation [6]. Hence, in vitro micropropagation, particularly via direct organogenesis, has emerged as a rapid and reliable alternative. Direct regeneration bypasses the callus phase, which not only shortens the culture cycle but also reduces the risk of somaclonal variation, critical for maintaining the genetic integrity of elite cultivars [7].

Among plant growth regulators (PGRs), cytokinins and auxins are key players in influencing morphogenic pathways. 6-Benzylaminopurine (BAP), a synthetic cytokinin, is especially potent in inducing axillary shoot proliferation, while Naphthalene Acetic Acid (NAA), a synthetic auxin, supports shoot elongation and adventitious rooting [8]. Previous research has highlighted the importance of optimising hormonal concentrations, as both excess and deficiency can negatively impact regeneration efficiency [9]. However, standardised protocols for direct regeneration in *Phalaenopsis* remain limited, particularly those using floral stalk node explants and subcultures, which are underexploited yet highly responsive tissues.

The maturase K (*matK*) gene, located within the chloroplast genome [10], has emerged as a powerful molecular marker for plant phylogenetics and species authentication due to its relatively high rate of nucleotide substitution and functional conservation [11]. In the genus *Phalaenopsis*, understanding phylogeny becomes critical when elite or endangered cultivars are involved in micropropagation outcomes [12]. Here, the *matK* gene serves as a molecular diagnostic tool for authenticating species identity and confirming clonal uniformity among in vitro regenerated plantlets.

The objective of this study was to establish a rapid direct organogenesis protocol and utilise *matK* gene sequencing to determine if evolutionary proximity can predict how different *Phalaenopsis* genotypes respond to identical in vitro conditions.

***Correspondence:** Mr. Rajesh RV, Department of Plant Biotechnology, College of Agriculture, University of Agricultural Sciences, Bangalore, India, e-mail: rajeshrv066@gmail.com

Materials and Methods

Plant material

Phalaenopsis amabilis (L.) Blume plants at the first blossom stage, bearing three to four open flowers, were procured from Green Square Technologies (Basavanapura, Doddaballapura, Karnataka), and floral stalk nodes with dormant buds were excised for shoot initiation experiments, while 180-day-old in vitro grown subcultures were explants for shoot multiplication trials conducted in the Department of Biotechnology, UAS Bangalore, India.

Culture medium composition

MS basal medium, enriched with autoclaved 100 mg/L banana homogenate and 100 mL/L coconut water, was used for all treatments [13,14]. Agar (8 g/L) served as the gelling agent, and the pH was adjusted to 5.8 prior to autoclaving. The complete formulation is presented in (Table 1).

Table 1. Treatment combinations of BAP and NAA used for shoot initiation and multiplication in *Phalaenopsis*.

Sl. No	Treatment	Composition
1	T0	MS + 0 mg/L BAP + 0 mg/L NAA
2	T1	MS + 0.5 mg/L BAP + 0.6 mg/L NAA
3	T2	MS + 1.0 mg/L BAP + 0.6 mg/L NAA
4	T3	MS + 1.5 mg/L BAP + 0.6 mg/L NAA
5	T4	MS + 2.0 mg/L BAP + 0.6 mg/L NAA
6	T5	MS + 2.5 mg/L BAP + 0.6 mg/L NAA
7	T6	MS + 3.0 mg/L BAP + 0.6 mg/L NAA

Surface sterilization

The floral stalk node explants were collected from healthy *Phalaenopsis* and initially washed under running tap water for 30 minutes to remove adhering dust particles. They were then soaked in a liquid detergent solution (Tween 20) for 10 minutes with vigorous shaking, followed by 4-5 rinses with running water to eliminate any detergent residues. Next, the explants were dipped in a 0.2% (w/v) Bavistin fungicide solution for 30 minutes and subsequently washed 3-4 times with distilled water.

Following Bavistin treatment, the explants were transferred to a laminar airflow cabinet for sterilisation. Surface sterilisation was performed using a 0.1% (w/v) aqueous solution of mercuric chloride ($HgCl_2$) for 30 seconds, after which the explants were thoroughly rinsed 2-3 times with sterile distilled water to remove any traces of $HgCl_2$. Subsequently, the explants were treated with 0.5% and 1.0% sodium hypochlorite (NaOCl) for varying durations as per the treatment plan. They again washed 2-3 times with sterile double-distilled water to eliminate any remaining NaOCl. All sterilisation procedures were conducted under aseptic conditions within the laminar flow cabinet. Finally, the surface-sterilised explants were air-dried on autoclaved filter paper and then placed in sterilised petri dishes containing the media.

Inoculation and incubation

Explants were trimmed into single-node segments using sterilised surgical tools and cultured on MS media containing different concentrations of BAP (0.5 to 3.0 mg/L) in combination with 0.6 mg/L NAA. Cultures were incubated at 24±2°C, 60-70% RH, and exposed to a 16 h photoperiod under 3000 lux light intensity [15].

Experimental design and data collection

Experiments followed a Completely Randomised Design (CRD) with three replicates [16]. Parameters recorded included: Time taken for shoot/shootlet initiation (days), Number of shoots/shootlets per explant or culture, Shoot/shootlet length (cm), Number of leaves per shoot/shootlet, and Number of roots per explant/culture. Statistical analysis was performed using OPSTAT software (version 1.2; CCS Haryana Agricultural University, India) available at https://www.opstat.nic.in. One-way ANOVA was used, and treatment means were compared using LSD at 5% significance level.

Phylogenetic analysis

To investigate interspecific relationships that influence in vitro regeneration potential, a phylogenetic analysis was performed using chloroplast *matK* gene sequences of *Phalaenopsis* species retrieved from the NCBI GenBank database [17]. The species included in the analysis were *Phalaenopsis* equestris (NC_017609.1), *Phalaenopsis* amabilis (EU256323.1), *Phalaenopsis* aphrodite subsp. formosana (NC_007499.1), *Phalaenopsis* mannii (KJ733592.1), *Phalaenopsis* bellina (EU251961.2), *Phalaenopsis* lamelligera (EU179845.1), *Phalaenopsis* deliciosa (NC_067938.1), *Phalaenopsis* lowii (NC_050652.1), *Phalaenopsis* chibae (AB217748.1), *Phalaenopsis* zhejiangensis (MZ326749.1), *Phalaenopsis* stobartiana (OP235488.1 and NC_059917.1), *Phalaenopsis* braceana (NC_072257.1), and *Phalaenopsis* wilsonii (MT800926.1). The sequences were aligned using ClustalW in MEGA X [18]. A Maximum Likelihood (ML) tree was constructed based on the Tamura-Nei substitution model, and robustness of branches was assessed using 1000 bootstrap replicates. The resulting tree topology was interpreted to infer evolutionary relationships among the species and explored potential correlations between phylogenetic proximity and their observed in vitro regeneration responses [19](Figure 1).

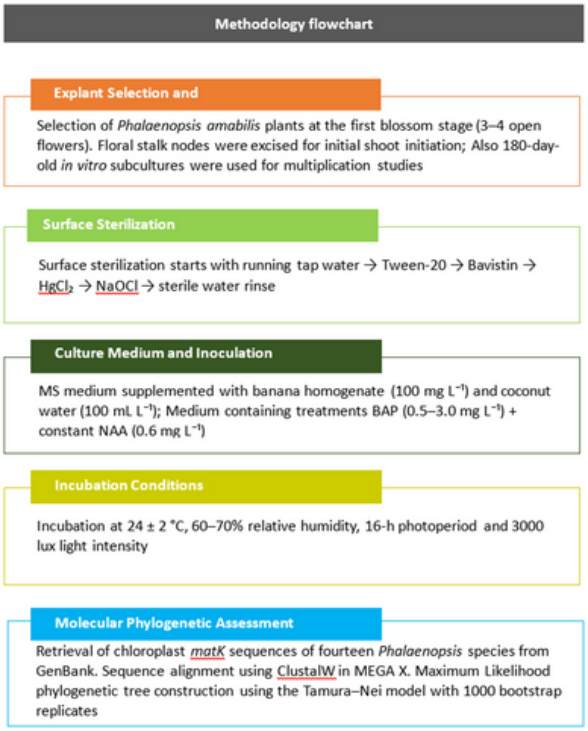

Figure 1. Flowchart showing the methodology of in vitro regeneration and phylogenetic study.

https://doi.org/10.61577/jpri.2025.100007

Results

Surface sterilisation and establishment of aseptic cultures

The explants prepared from greenhouse-grown *Phalaenopsis* were successfully surface sterilised using a multi-step protocol. Mechanical cleaning with running tap water, followed by Tween 20 treatment and bavistin immersion, removed surface contaminants effectively. Chemical sterilisation using 0.1% $HgCl_2$ for 30 seconds, followed by treatment with 0.5%–1.0% NaOCl, achieved a high success rate in eliminating bacterial and fungal contamination. Post-sterilisation, explants retained structural integrity and were visibly free from browning or necrosis.

Shoot initiation from floral stalk nodes

The combination of BAP and NAA significantly influenced shoot initiation parameters. No response was observed in the control treatment (T1), confirming the necessity of exogenous hormonal supplementation. Among all treatments, T5 (2.0 mg/L BAP + 0.6 mg/L NAA) showed the most effective response with the earliest shoot initiation (29.66 days), highest number of shoots per explant (2.0), and robust growth characterized by shoot length (2.26 cm) and leaf development (2.33 leaves/shoot) (Figure 2 and Figure 3). These differences were statistically significant ($p < 0.05$) as per ANOVA, indicating the robustness of T5 in enhancing shoot initiation over other treatments. Shoot elongation was most pronounced in T7 (3.0 mg/L BAP + 0.6 mg/L NAA) with a shoot length of 2.46 cm and 3.33 leaves per shoot, although shoot number per explant did not increase beyond T5 levels. No root development was observed in any treatment during the shoot initiation phase, suggesting the hormonal environment favoured only shoot morphogenesis.

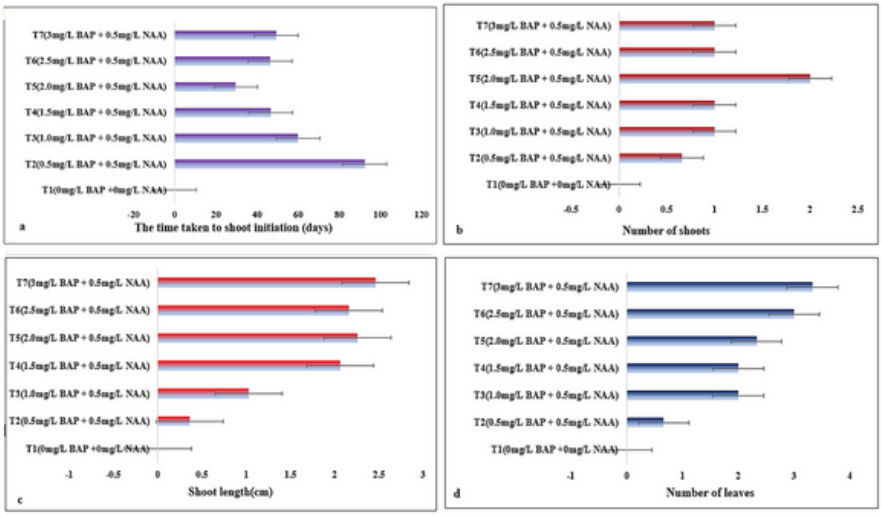

Figure 2. Summary of shoot initiation parameters in *Phalaenopsis* under various BAP and NAA treatments. (A) Days to shoot initiation; (B) Number of shoots per explant; (C) Shoot length; (D) Number of leaves per shoot.

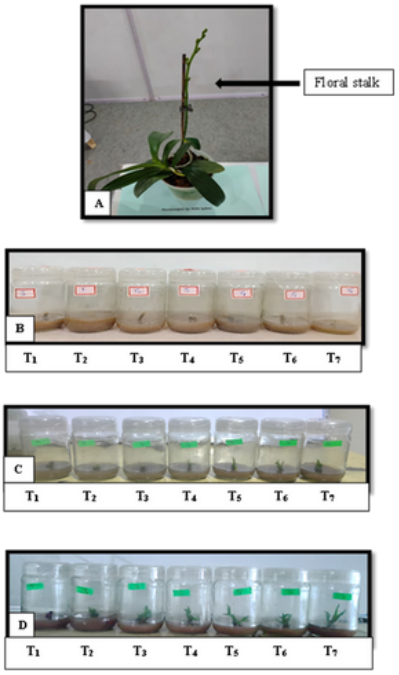

Figure 3. Shoot induction from floral stalk nodes under BAP and NAA treatments. (A) Initial explant; (B–D) Shoot emergence at successive stages.

https://doi.org/10.61577/jpri.2025.100007

Shoot multiplication from in vitro cultures

Shoot multiplication was assessed across subcultures exposed to identical BAP and NAA combinations. Once again, T1 and T2 failed to elicit any growth response, confirming the requirement of active cytokinins and auxins for regenerative activity. T5 demonstrated the most balanced and superior response with the highest number of shootlets per culture (18.0 at 120 DAS- days after subculture), short initiation period (26.66 days), and significant shootlet length (2.33 cm) (Figure 4 and Figure 5). Statistical analysis confirmed the significance of these differences ($p < 0.05$), highlighting T5 as the optimal hormonal combination for shoot multiplication. The maximum shootlet length (2.46 cm) and the highest number of leaves per shootlet (4.0) were observed in T7, while root development was consistent across T3–T7, ranging from 5.0 to 5.33 roots per culture.

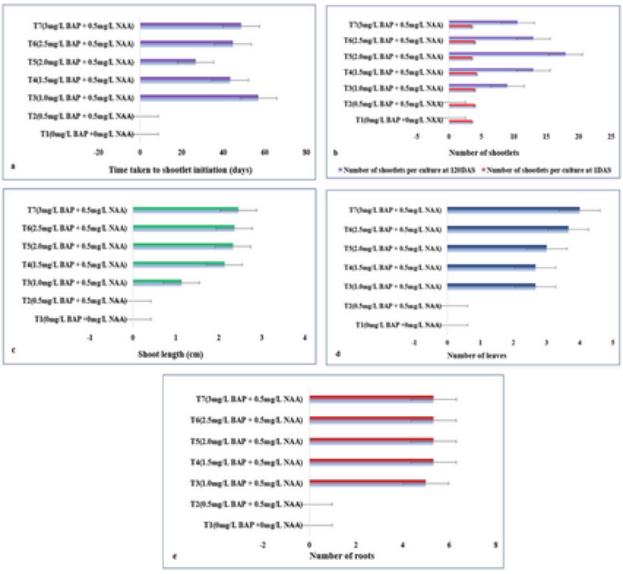

Figure 4. Shoot multiplication responses of subcultured explants under different hormonal treatments. (A) Days to shootlet emergence; (B) Number of shootlets per culture; (C) Shootlet length; (D) Number of leaves per shootlet; (E) Root number per culture.

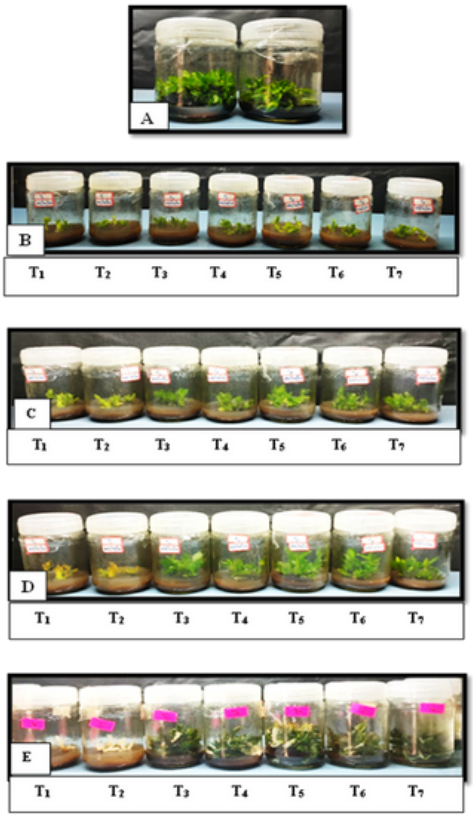

Figure 5. In vitro shoot multiplication stages in *Phalaenopsis*. (A) Subcultured explants; (B–E) Shootlet formation at different days after subculture.

https://doi.org/10.61577/jpri.2025.100007

Phylogenetic analysis of *Phalaenopsis* species

Phylogenetic analysis based on the *matK* gene provided insights into the evolutionary divergence among 14 *Phalaenopsis* species. The aligned sequences (average length: 1536 bp) were subjected to Maximum Likelihood tree construction, revealing three primary clades: Clade I included P. equestris, P. amabilis, and P. aphrodite subsp. Formosana is closely related, showing robust shoot initiation and multiplication responses in vitro. Clade II consisted of P. bellina, P. mannii, and P. chibae, moderately related species with varied shoot multiplication rates. Clade III, the most evolutionarily distant related species, such as P. stobartiana, P. wilsonii, P. zhejiangensis, and P. braceana, all of which showed delayed or reduced regeneration potential under the same hormonal conditions (Figure 6). Bootstrap values ranged from 78% to 99%, supporting the reliability of clade groupings. These clades corresponded partially with regeneration response trends, suggesting a possible link between evolutionary lineage and morphogenic capacity under specific in vitro conditions.

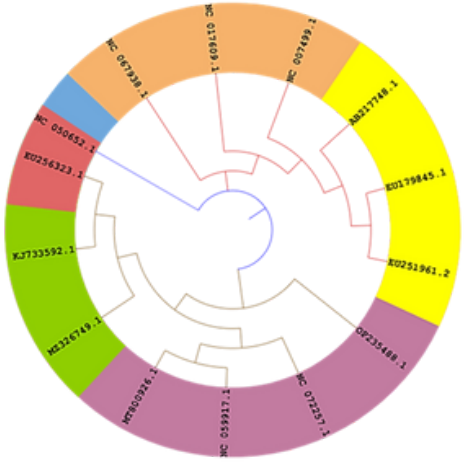

Figure 6. Circular phylogenetic tree of *Phalaenopsis* species based on *matK* sequences (Tamura-Nei model, 1000 bootstrap replicates). Clade I (P. equestris, P. aphrodite, P. deliciosa), Clade II (P. amabilis, P. mannii, P. bellina), and Clade III (P. stobartiana, P. wilsonii, P. braceana) are highlighted. Clade I species showed superior in vitro regeneration efficiency.

Discussion

The success of the established protocol is rooted in the stringent multi-step sterilization process. Mechanical cleaning combined with brief exposure to $HgCl_2$ and NaOCl ensured high survival rates by effectively decontaminating the complex, waxy tissues of the orchid stalk without inducing significant cytotoxicity. Similar effectiveness of controlled $HgCl_2$ use has been reported in other orchid genera. This balance is crucial for in vitro establishment, as excessive sterilant exposure often leads to explant necrosis. The subsequent morphogenic response confirms the potency of BAP in stimulating axillary bud proliferation. Our observation that shoot induction saturated at 2.0 mg/L BAP suggests a physiological threshold where additional cytokinin does not enhance bud primordia formation, though it may continue to drive longitudinal elongation and leaf expansion at higher concentrations like 3.0 mg/L. These findings support prior observations. where optimal cytokinin levels resulted in maximum explant survival and shoot number. The integration of *matK* phylogenetic data provides a molecular explanation for the observed variation in tissue culture success across the genus. The grouping of P. amabilis and P. equestris in Clade I, which demonstrated the highest regeneration efficiency, suggests that these species share conserved genetic pathways for hormone perception. Conversely, the delayed and reduced responses in Clade III species like P. wilsonii indicate a degree of recalcitrance that may be linked to their early evolutionary divergence. This correlation highlights that "one-size-fits-all" protocols are insufficient for the entire *Phalaenopsis* genus. Future efforts must focus on overcoming the limitation of poor rooting through the application of stronger auxins, such as IBA or IAA, to ensure the protocol's viability for commercial acclimatisation.

Conclusion

The present investigation establishes a reliable and reproducible protocol for the direct regeneration of *Phalaenopsis* using floral stalk node explants and in vitro subcultures. The combination of a stringent yet explant-safe surface sterilisation procedure (Tween 20, Bavistin, 0.1% $HgCl_2$, and NaOCl), a nutritionally enriched MS medium supplemented with banana homogenate and coconut water, and optimised levels of BAP and NAA markedly improved aseptic culture establishment, shoot induction, and subsequent shootlet multiplication. Among all treatments, T5 (2.0 mg/L BAP + 0.6 mg/L NAA) proved most effective, promoting the earliest shoot emergence, the highest number of shoots per explant, and superior shootlet proliferation. Although T7 (3.0 mg/L BAP + 0.6 mg/L NAA)enhanced shoot elongation and leaf formation, it did not exceed T5 in overall multiplication efficiency. Root induction remained modest yet consistent under higher BAP concentrations, underscoring the need for further refinement of auxin-based treatments. The incorporation of *matK*-based phylogenetic analysis revealed a discernible association between evolutionary relatedness and regeneration performance, underscoring the value of phylogenetic context in predicting species- or clade-specific responses to micropropagation.Overall, the optimised protocol offers a scalable and high-throughput system suitable for commercial multiplication, conservation of elite germplasm,and potential applications in genetic transformation and advanced biotechnological studies in *Phalaenopsis*. Future work should prioritise enhancing rooting responses using alternative auxins such as IBA or IAA, assessing antioxidant or organic supplements, and tailoring regeneration strategies for phylogenetically distant or recalcitrant species.

Acknowledgment

The authors are thankful to DST-FIST (GoI), the Department of Plant Biotechnology, GKVK, UAS Bangalore, India, for providing the facilities, and the Department of Biotechnology (DBT), New Delhi, for providing a fellowship. The authors are also thankful to Dr Nagesh, N, Associate Professor, Department of Biotechnology, UAS Bangalore, India, and Dr Narendra Ram,

Disclosure of conflict of interest

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https://doi.org/10.61577/jpri.2025.100007