

Establishment of an efficient in-vitro regeneration system from leaves of *Phalaenopsis hybrida* cv. 'Big Chili'

Fei Dong

dongfei860117@163.com

Shandong Academy of Agricultural Sciences

Yaqin Wang

Shandong Agricultural University

Mengqi Cao

Shandong Agricultural University

Jiao Zhu

Shandong Academy of Agricultural Sciences

Sek-man Wong

Shandong Academy of Agricultural Sciences

Chengpeng Wang

Shandong Academy of Agricultural Sciences

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Abstract

Phalaenopsis hybrida is one of the most commercially valuable ornamental orchids worldwide. Conventional micropropagation using pedicel axillary buds suffers from limited explant availability and low multiplication efficiency. Leaf-based in-vitro regeneration enables repeated harvesting of explants from donor plants, representing a promising approach for mass seedling production and gene-editing-assisted breeding. Nevertheless, existing regeneration protocols are hampered by three major limitations: severe explant browning, poor callus regeneration efficiency, and the lack of integrated culture media covering the complete workflow of callus induction, adventitious bud differentiation and rooting. In this study, sterile leaves of the widely cultivated cultivar 'Big Chili' were used as explants. We systematically evaluated the effects of thidiazuron (TDZ) concentration, combined supplementation of citric acid and α -naphthaleneacetic acid (NAA), as well as interactive effects between leaf age and explant inoculation orientation. Single-factor gradient experiments together with 2×2 two-way analysis of variance (ANOVA) were performed to select optimal culture media and standardized cultivation parameters for each developmental stage, and a complete leaf regeneration pipeline was established. The optimal medium for callus induction was 1/2 MS medium supplemented with $3\text{mg}\cdot\text{L}^{-1}$ TDZ, $150\text{mL}\cdot\text{L}^{-1}$ coconut water, $0.1\text{g}\cdot\text{L}^{-1}$ citric acid, $20\text{g}\cdot\text{L}^{-1}$ sucrose and $5.5\text{g}\cdot\text{L}^{-1}$ agar (pH 5.8). Two-way ANOVA revealed a significant positive interaction between leaf age and inoculation orientation. The highest callus induction rate was achieved when newly-expanded young leaves were cultured with the adaxial surface upwards under continuous darkness at 25°C for 90 days. The best medium for adventitious bud differentiation was 1/2 MS medium containing $1.0\text{mg}\cdot\text{L}^{-1}$ 6-benzylaminopurine (6-BA), $0.4\text{mg}\cdot\text{L}^{-1}$ NAA, $2.5\text{mg}\cdot\text{L}^{-1}$ adenine, $150\text{mL}\cdot\text{L}^{-1}$ coconut water, $0.5\text{g}\cdot\text{L}^{-1}$ activated carbon, $20\text{g}\cdot\text{L}^{-1}$ sucrose and $5.5\text{g}\cdot\text{L}^{-1}$ agar (pH 5.8), yielding an adventitious bud differentiation rate of 39.82%. For rooting and plantlet strengthening, the optimum medium was MS medium supplemented with $1.0\text{mg}\cdot\text{L}^{-1}$ 6-BA, $0.4\text{mg}\cdot\text{L}^{-1}$ NAA, $150\text{mL}\cdot\text{L}^{-1}$ coconut water, $1.0\text{g}\cdot\text{L}^{-1}$ activated carbon, $20\text{g}\cdot\text{L}^{-1}$ sucrose and $5.5\text{g}\cdot\text{L}^{-1}$ agar (pH 5.8), with a rooting rate of 50.81%. This work defines the critical threshold concentration of TDZ and the coordinated regulatory roles of leaf age and inoculation mode in governing leaf-explant dedifferentiation. Our findings advance the fundamental understanding of cell totipotency within Orchidaceae, and provide a standardised technical protocol for industrial-scale seedling production and genetic transformation of *P. hybrida*.

Highlights

- A complete leaf-derived in-vitro regeneration pipeline was built for *Phalaenopsis hybrida* cv. 'Big Chili'
- Young leaves placed adaxial-side-up achieved optimal callus formation under $3\text{mg}\cdot\text{L}^{-1}$ thidiazuron treatment
- Significant positive interaction exists between leaf age and explant inoculation orientation for callus induction
- Optimized multistage culture media alleviate browning for industrial seedling production and genetic transformation

1. Introduction

Phalaenopsis hybrida, commonly known as moth orchid, is an epiphytic perennial herb belonging to Orchidaceae. Praised as the "Queen of Orchids", it possesses high ornamental and commercial value owing to its elegant flower morphology, rich colour variation and long flowering period, and it constitutes a core commodity in global potted-plant and cut-flower markets (Iiyama et al., 2024). In recent years, the *Phalaenopsis* industry has expanded rapidly in China, with domestic output accounting for more than 60% of global production. Moth orchids dominate the Chinese New Year festive flower market, are increasingly consumed by the general public via e-commerce channels, and generate substantial export revenue, acting as a key driver for the transformation and upgrading of the domestic flower industry (Hong et al., 2022).

At present, large-scale propagation of *P. hybrida* mainly relies on micropropagation from pedicel axillary buds. This technique is constrained by limited explant sources and relatively low multiplication coefficients, which cannot fully satisfy growing market demand and the rapid popularisation of novel cultivars (Sarmah et al., 2024; Zhang et al., 2022). Leaves are abundant and readily accessible plant organs; therefore leaf-based in-vitro regeneration is regarded as a promising strategy for low-cost high-throughput propagation and gene-editing-mediated breeding of *Phalaenopsis* (Silva et al., 2026; Ghahremani et al., 2021; Manokari et al., 2021; Cheng et al., 2025). Leaf explants can be repeatedly harvested from donor mother plants to improve propagation coefficients, and they can serve as abundant receptor materials for genetic transformation and new cultivar development (Aker et al., 2024).

Despite the theoretical merits of leaf-derived regeneration, several bottlenecks restrict its commercial application for mass seedling production. Explant browning represents a major obstacle for establishing efficient leaf regeneration systems for *P. hybrida* (Ghahremani et al., 2021; Kang et al., 2025). Mechanical wounding and culture-environment-derived stresses trigger oxidation of phenolic compounds, producing brown by-products that impair cell viability and may cause complete explant necrosis. Most published studies have focused on screening single antioxidants or individual plant-growth-regulator concentrations (Zhang et al., 2022), whereas the combined regulatory effects of TDZ, antioxidants such as citric acid, and organic additives like coconut water remain poorly characterised, and optimal combinations for browning mitigation are yet to be defined (Yu et al., 2009). Citric acid, ascorbic acid and polyvinylpyrrolidone (PVP) can effectively alleviate browning in *Phalaenopsis* explants (Kang et al., 2025; Yu et al., 2009), and coconut water rich in natural nutrients and bioactive compounds shows good performance in orchid tissue-culture systems (Triani and Ilham, 2024; Li and Yang, 2012). Most previous investigations tested only single variables such as leaf age or inoculation orientation, while multi-factor interactive mechanisms controlling callus formation and plant regeneration remain insufficiently explored, limiting mechanistic understanding of how culture conditions jointly govern dedifferentiation and regeneration responses (Niu et al., 2013; Guan, 2013). Thidiazuron (TDZ) is a potent cytokinin, which exhibits stronger regeneration-inducing activity than conventional cytokinins in numerous plant in-vitro systems (Cui et al., 2002;

Faisal et al., 2023; Mulanda et al., 2012; Palama et al., 2010; Zavala-Hernández et al., 2025). Furthermore, integrated multi-stage medium packages for *Phalaenopsis* regeneration are scarce. Most existing media formulations are optimised exclusively for one discrete step (callus induction, adventitious-bud differentiation or rooting), and cultivar-specific whole-process optimisation strategies are rarely reported (Palama et al., 2010; Sarmah et al., 2024). Fragmented process parameters are difficult to translate into factory-scale protocols, extending culture cycles, elevating material losses and increasing commercial propagation costs.

In the present study, leaf explants derived from in-vitro-grown *P. hybrida* cv. ‘Big Chili’ plantlets were used as experimental materials. We performed sequential single-factor gradient screening of plant-growth regulators, composite medium optimisation, two-factor interaction tests for leaf age and inoculation orientation, and screening of dedicated media for adventitious-bud differentiation and rooting. One-way ANOVA and two-way ANOVA were applied to quantify treatment-related differences, so as to define complete medium recipes and standardised operational parameters across the whole leaf-regeneration workflow. Specifically, we screened suitable media for callus induction and determined the optimal TDZ concentration; assessed how leaf age (young versus mature old leaves) and inoculation orientation (adaxial-surface-up versus adaxial-surface-down placement) modify callus-induction efficiency; and selected specialised media for adventitious-bud differentiation and root development. This work aims to establish a stable and efficient leaf-based in-vitro regeneration system for *P. hybrida*. The outputs can supply technical support for large-scale production of high-quality moth-orchid plantlets, shorten culture cycles, reduce production costs, improve propagation efficiency, and support the sustainable development of the *P. hybrida* industry.

2. Materials and methods

2.1. Plant material

All experiments were carried out in the tissue-culture laboratory of the Institute of Leisure Agriculture, Shandong Academy of Agricultural Sciences, China. In-vitro-raised plantlets of *P. hybrida* cv. ‘Big Chili’ free of contamination, with intact and healthy leaves, were used as source material for explant preparation.

2.2. Explant preparation

Healthy and uniform *P. hybrida* plantlets were selected. Under laminar-flow hood conditions, leaves were cut into 0.5–0.8 cm square segments. Cutting was performed to minimise wound area so as to reduce subsequent browning risk; the resulting leaf segments were reserved for inoculation.

2.3. Screening of optimal TDZ concentration

The basal medium consisted of 1/2 MS salts supplemented with 150 mL·L⁻¹ coconut water, 0.1 g·L⁻¹ citric acid, 20 g·L⁻¹ sucrose and 5.5 g·L⁻¹ agar, adjusted to pH 5.8. Five TDZ concentration gradients (1, 2, 3, 4 and 5mg·L⁻¹) were set up, corresponding to media M1 ~ M5 (Table 1). Each treatment comprised 50 Petri dishes with three independent biological replicates. Cultures were incubated in continuous darkness at 25 ± 2°C for 90 days. The numbers of necrotic dead leaf segments, browned explants and segments producing callus were recorded. Mortality rate, browning rate and callus induction rate were calculated accordingly.

Table 1
Culture media with different TDZ concentrations

Medium Code	1/2MS(g·L ⁻¹)	TDZ(mg·L ⁻¹)	Coconut water(mL·L ⁻¹)	Citric acid(g·L ⁻¹)	Agar(g·L ⁻¹)	Sucrose(g·L ⁻¹)	pH
M1	2.3	1	150	0.1	5.5	20	5.8
M2	2.3	2	150	0.1	5.5	20	5.8
M3	2.3	3	150	0.1	5.5	20	5.8
M4	2.3	4	150	0.1	5.5	20	5.8
M5	2.3	5	150	0.1	5.5	20	5.8

2.4. Optimisation of composite media for leaf callus induction

Using the optimal TDZ-containing medium M3 as control, two variables were tested: citric acid (0.1 g·L⁻¹ and 0.2 g·L⁻¹) and exogenous NAA (0.0, 0.2 and 0.3mg·L⁻¹). Six composite media (M3, M6 ~ M10) were prepared (Table 2); concentrations of 1/2 MS basal salts, coconut water, sucrose and agar remained unchanged, and pH was adjusted to 5.8 for all formulations. Each treatment contained 50 Petri dishes with three biological replicates. Cultures were incubated in darkness for 90 days. Numbers of dead, browned and callus-forming leaf explants were counted, and mortality, browning and callus-induction percentages were computed.

Table 2
Compound media for leaf callus induction

Medium Code	1/2MS(g·L ⁻¹)	TDZ(mg·L ⁻¹)	Coconut water(mL·L ⁻¹)	Citric acid(g·L ⁻¹)	NAA (mg·L ⁻¹)	Agar(g·L ⁻¹)	Sucrose(g·L ⁻¹)	pH
M3	2.3	3	150	0.1	-	5.5	20	5.8
M6	2.3	3	150	0.2	-	5.5	20	5.8
M7	2.3	3	150	0.1	0.2	5.5	20	5.8
M8	2.3	3	150	0.2	0.2	5.5	20	5.8
M9	2.3	3	150	0.1	0.3	5.5	20	5.8
M10	2.3	3	150	0.2	0.3	5.5	20	5.8

2.5. Two-factor experiment for leaf age and inoculation orientation

This assay was performed using the optimised callus-induction medium M3. Factor A represented leaf age: A₁ = newly expanded young leaves (the first to second fully expanded apical leaves of in-vitro plantlets); A₂ = mature old leaves (basal older leaves). Factor B represented inoculation orientation: B₁ = adaxial surface facing upwards; B₂ = adaxial surface facing downwards. Four combined treatments were generated: A₁B₁, A₁B₂, A₂B₁ and A₂B₂. Each treatment included 25 Petri dishes with three biological replicates. Cultures were maintained under continuous darkness at 25 ± 2°C for 90 days. Numbers of dead, browned and callus-producing leaf segments were recorded to calculate mortality, browning and callus-induction rates. Two-way ANOVA was used to evaluate main effects of leaf age and inoculation orientation, together with their A × B interaction. Independent-samples t-tests were used for pairwise comparisons between levels within each factor.

2.6. Screening of adventitious-bud differentiation media

Table 3
Screening of shoot differentiation media

Medium Code	1/2 MS medium(g·L ⁻¹)	6-BA(mg·L ⁻¹)	NAA(mg·L ⁻¹)	Adenine(mg·L ⁻¹)	Coconut water(mL·L ⁻¹)	Sucrose(g·L ⁻¹)	Agar(g·L ⁻¹)	Activated charcoal(g·L ⁻¹)
D11	2.3	1.0	0.2	2.0	150	20	5.5	0.5
D12	2.3	1.0	0.4	2.5	150	20	5.5	0.5
D13	2.3	1.0	0.6	3.0	150	20	5.5	0.5
D14	2.3	2.0	0.2	2.5	150	20	5.5	0.5
D15	2.3	2.0	0.4	3.0	150	20	5.5	0.5
D16	2.3	2.0	0.6	2.0	150	20	5.5	0.5
D17	2.3	3.0	0.2	3.0	150	20	5.5	0.5
D18	2.3	3.0	0.4	2.0	150	20	5.5	0.5
D19	2.3	3.0	0.6	2.5	150	20	5.5	0.5

Basal differentiation medium contained 1/2 MS salts, 150 mL·L⁻¹ coconut water, 20 g·L⁻¹ sucrose, 5.5 g·L⁻¹ agar and 0.5 g·L⁻¹ activated carbon (pH 5.8). Nine media formulations (D11 ~ D19) were prepared by varying concentrations of 6-BA, NAA and adenine. Pre-induced callus tissues were sub-cultured onto these media (Table 3). Approximately 100 callus clumps were inoculated per treatment, with three biological replicates. Cultures were incubated under a 16 h light : 8 h dark photoperiod for 30 days. The number of callus clumps generating adventitious buds was counted to calculate adventitious-bud differentiation rate.

2.7. Screening of rooting media

Table 4
Screening of rooting culture media

Medium Code	MS medium(g·L ⁻¹)	6-BA(mg·L ⁻¹)	NAA(mg·L ⁻¹)	Coconut water(ml·L ⁻¹)	Sucrose(g·L ⁻¹)	Agar(g·L ⁻¹)	Activated charcoal(g·L ⁻¹)
R20	4.4	1.0	0.2	150	20	5.5	1.0
R21	4.4	1.0	0.4	150	20	5.5	1.0
R22	4.4	1.0	0.6	150	20	5.5	1.0
R23	4.4	2.0	0.2	150	20	5.5	1.0
R24	4.4	2.0	0.4	150	20	5.5	1.0
R25	4.4	2.0	0.6	150	20	5.5	1.0
R26	4.4	3.0	0.2	150	20	5.5	1.0
R27	4.4	3.0	0.4	150	20	5.5	1.0
R28	4.4	3.0	0.6	150	20	5.5	1.0

Uniform and robust adventitious buds of 2 ~ 3 cm height were selected. Basal rooting medium comprised MS salts, 150 mL·L⁻¹ coconut water, 20 g·L⁻¹ sucrose, 5.5 g·L⁻¹ agar and 1.0 g·L⁻¹ activated carbon (pH 5.8). Nine rooting media (R20 ~ R28) were prepared by adjusting 6-BA and NAA concentrations (Table 4). Around 80 individual buds were inoculated per treatment, with three biological replicates. After 30 days of culture under 16 h light : 8 h dark photoperiod, plantlets developing ≥ 2 functional roots were counted to compute rooting percentage.

2.8. General culture conditions

For callus-induction stages, cultures were incubated at 25 ± 2°C under total darkness. For adventitious-bud differentiation and rooting phases, incubation temperature was maintained at 25 ± 2°C, 16 h light : 8 h dark photoperiod and light intensity of 2000 lx.

2.9. Calculation of measured indices

Mortality rate (%) = (Number of dead leaf explants / Total inoculated leaf explants) × 100

Browning rate (%) = (Number of obviously browned explants / Total inoculated leaf explants) × 100

Callus induction rate (%) = (Number of explants forming compact callus / Total inoculated leaf explants) × 100

Adventitious bud differentiation rate (%) = (Number of callus clumps producing adventitious buds / Total inoculated callus clumps) × 100

Rooting rate (%) = (Number of adventitious buds developing ≥ 2 functional roots / Total inoculated adventitious buds) × 100

2.10. Statistical analysis

Raw experimental data were compiled in Excel 2019. Statistical analyses were implemented using DPS v14.10 software. One-way ANOVA followed by Duncan's multiple-range test was applied for single-factor experiments. Two-way ANOVA was performed for two-factor assays, and independent-sample t-tests were used for pairwise comparisons. Results are presented as mean ± standard deviation. Lower-case letters denote statistically significant differences at $P < 0.05$, uppercase letters indicate highly significant differences at $P < 0.01$, and ns represents non-significant differences.

3. Results

3.1. Effects of varying TDZ concentrations on leaf callus induction

Within the tested TDZ range of 1 ~ 5mg·L⁻¹, callus-induction rate first increased and then decreased. The highest callus-induction rate (21.15%) and the lowest browning rate were observed at 3mg·L⁻¹ TDZ, representing the optimal TDZ concentration for this cultivar. When TDZ was below 3mg·L⁻¹, insufficient cytokinin supply failed to trigger effective somatic-cell dedifferentiation, resulting in low callus yields. Concentrations exceeding 3mg·L⁻¹ caused phytotoxic effects: browning and mortality increased, while callus-induction rates declined. ANOVA showed that at 3mg·L⁻¹ TDZ (medium M3), explant mortality was highly significantly lower than for M4 and M5; browning rate was highly significantly lower than for all other four TDZ treatments; and callus-induction rate was highly significantly higher compared with M1, M2 and M5 (Fig. 1B and Table 5).

Table 5
Effects of different TDZ concentrations on leaf callus induction

Medium Code	TDZ (mg·L ⁻¹)	Number of petri dishes	Number of <i>Phalaenopsis</i> leaf explants	Number of dead leaf explants	Mortality rate(%)	Number of browning leaf explants	Browning rate(%)	Number of leaf explants with callus formation	Callus induction rate(%)
M1	1	50	1749.33 ± 6.03abcA	674.00 ± 3.61bB	38.51 ± 0.24bB	884.00 ± 6.25aA	50.51 ± 0.35aA	192.00 ± 9.54cC	10.97 ± 0.41dC
M2	2	50	1747.33 ± 2.08bcA	664.67 ± 4.51bB	38.02 ± 0.21bB	859.00 ± 5.00bB	49.14 ± 0.24bA	224.33 ± 9.50bB	12.84 ± 0.44cB
M3	3	50	1744.33 ± 4.04cA	668.33 ± 8.50bB	38.29 ± 0.15bB	707.67 ± 5.51dD	40.55 ± 0.24dC	369.00 ± 12.17aA	21.15 ± 0.26aA
M4	4	50	1751.67 ± 1.53abA	619.33 ± 5.51cC	35.33 ± 0.21cC	782.33 ± 5.86cC	44.63 ± 0.31cB	351.33 ± 11.24aA	20.04 ± 0.44bA
M5	5	50	1754.67 ± 3.06aA	714.33 ± 6.11aA	40.77 ± 0.11aA	862.33 ± 4.93bB	49.22 ± 0.44bA	175.33 ± 10.79cC	10.00 ± 0.38dC

Note: All percentage data are presented as mean ± standard deviation of three replicates (n = 3). The significance of differences among treatments was analyzed by Duncan's new multiple range test. Lowercase letters indicate significant differences at *P* < 0.05 level, and uppercase letters indicate highly significant differences at *P* < 0.01 level. The same applies hereinafter.

3.2. Performance of composite media for leaf callus induction

Using 3mg·L⁻¹ TDZ as fixed concentration, six media differing in citric-acid and NAA levels were compared. Medium M3 (0.1 g·L⁻¹ citric-acid, no exogenous NAA) delivered the best overall performance, with a callus-induction rate of 21.19% and the lowest mortality and browning percentages. Elevating citric-acid concentration to 0.2 g·L⁻¹ (M6) greatly increased browning and halved callus-induction efficiency. Within the 0.1 g·L⁻¹ citric-acid background, addition of exogenous NAA progressively suppressed callus formation with rising NAA concentration. Treatments combining 0.2 g·L⁻¹ citric-acid with NAA (M8, M10) exhibited severe stress responses and inferior regeneration performance relative to other groups. ANOVA confirmed that medium M3 had highly significantly reduced mortality and browning, and highly significantly elevated callus-induction capacity compared with the other five composite formulations; M3 was therefore selected as the optimal medium for callus induction (Table 6).

Table 6
Effects of Different Culture Medium Formulations on Leaf Callus Induction

Medium Code	Number of petri dishes	Number of <i>Phalaenopsis</i> leaf explants	Number of dead leaf explants	Mortality rate(%)	Number of browning leaf explants	Browning rate(%)	Number of leaf explants with callus formation	Callus induction rate(%)
M3	50	1751.67 ± 2.08aA	674.67 ± 4.72dD	38.53 ± 0.27eD	705.33 ± 4.16dE	40.28 ± 0.41dD	371.00 ± 8.66aA	21.19 ± 0.49aA
M6	50	1747.67 ± 1.53abABC	693.00 ± 5.29cC	39.64 ± 0.30dC	870.00 ± 4.58aA	49.77 ± 0.26aA	185.00 ± 9.54cC	10.58 ± 0.55cC
M7	50	1742.33 ± 2.52cC	636.00 ± 6.01eE	36.51 ± 0.34fE	779.33 ± 5.51cD	44.74 ± 0.32cC	326.67 ± 11.50bB	18.75 ± 0.66bB
M8	50	1744.00 ± 2.65bcBC	717.67 ± 6.11bB	41.08 ± 0.35cB	851.00 ± 8.00bC	48.71 ± 0.46bB	178.33 ± 14.05cdC	10.21 ± 0.80cdC
M9	50	1750.00 ± 3.00aAB	733.33 ± 1.53aA	41.90 ± 0.09bA	853.67 ± 5.69bBC	48.78 ± 0.33bB	163.00 ± 5.00dC	9.31 ± 0.29dCD
M10	50	1743.00 ± 3.00aA	741.33 ± 1.53aA	42.46 ± 0.09aA	865.67 ± 3.06aAB	49.58 ± 0.18aA	139.00 ± 4.36eD	7.96 ± 0.25eD

3.3. Main and interactive effects of leaf age and inoculation orientation

Table 7
Effects of different treatments on callus induction

Source of variation	Degrees of freedom	F-value (mortality rate)	P-value (mortality rate)	F-value (browning rate)	P-value (browning rate)	F-value (callus induction rate)	P-value (callus induction rate)
Leaf age(A)	1	1.27	0.268 ns	42.65	< 0.001 **	68.32	< 0.001 **
Explant orientation(B)	1	0.94	0.336 ns	18.74	< 0.01 *	31.56	< 0.01 *
A × B interaction	1	0.41	0.527 ns	3.28	0.076 ns	4.03	0.048 *

Note: Significant differences between different treatments were determined by t-test. * indicates significant difference at *P* < 0.05, and ** indicates highly significant difference at *P* < 0.01.

Two-way ANOVA indicated that leaf age and inoculation orientation exerted no significant main or interactive effects on explant mortality ($P > 0.05$), which suggests that leaf-segment death was primarily driven by cutting-derived wounding and basal phytohormone stress rather than leaf maturity or placement orientation. Leaf age exerted a highly significant effect on browning rate, and inoculation orientation produced a significant effect on browning rate, with non-significant interaction; the two factors modulated browning independently. Older leaves contained higher phenolic substrate levels, and downward-facing inoculation limited gas exchange, aggravating browning symptoms. For callus-induction rate, leaf age showed highly significant main effects, inoculation orientation showed significant main effects, and a significant positive $A \times B$ interaction was detected ($P < 0.05$). This demonstrates that young leaves together with adaxial-surface-up inoculation exerted synergistic promoting effects on callus production, and that the maximum regeneration outcome could not be obtained simply by combining the best outcome of each individual factor (Table 7).

Table 8
Effects of interactive treatments of leaf age and inoculation orientation on callus induction from leaves

Treatment combinations	Number of petri dishes	Number of <i>Phalaenopsis</i> leaf explants	Mortality rate(%)	Browning rate(%)	Callus induction rate(%)
A ₁ B ₁ : Young leaf, adaxial surface upward	25	803	35.78 ± 0.27a	44.01 ± 0.22c	20.22 ± 0.21aA
A ₁ B ₂ : Young leaf, adaxial surface downward	25	806	36.01 ± 0.23a	46.35 ± 0.19b	18.17 ± 0.17bB
A ₂ B ₁ : Mature leaf, adaxial surface upward	25	798	36.26 ± 0.15a	47.24 ± 0.26b	16.46 ± 0.08cC
A ₂ B ₂ : Mature leaf, adaxial surface downward	25	801	36.53 ± 0.20a	49.57 ± 0.23a	14.83 ± 0.12dD

Across the four combined treatments, mortality values ranged from 35.78% to 36.53% without significant inter-treatment differences. Browning rates ranked in the order A₁B₁ (young leaf, adaxial side up) < A₁B₂, A₂B₁ < A₂B₂ (old leaf, adaxial side down). Young leaves exhibited less browning than old leaves, and adaxial-surface-up placement yielded lower browning than downward placement. Callus-induction rates followed the sequence A₁B₁ (20.22%) > A₁B₂ (18.17%) > A₂B₁ (16.46%) > A₂B₂ (14.83%), with highly significant differences among all four combinations. Young leaves consistently out-performed old leaves, and adaxial-surface-up inoculation surpassed downward placement. The combination A₁B₁ achieved peak callus-induction rates and minimal browning losses (Table 8). Optimising only one of the two parameters brought only minor improvements in regeneration efficiency; maximal dedifferentiation capacity required the combined application of young leaf explants and adaxial-surface-up inoculation. Independent-samples t-test outputs agreed fully with two-way ANOVA results.

3.4. Adventitious-bud differentiation under different medium formulations

Nine media (D11 ~ D19) with varied combinations of 6-BA, NAA and adenine were tested. Adventitious-bud differentiation rates varied from 26.07% to 39.82%. Medium D12 (1.0mg·L⁻¹ 6-BA + 0.4mg·L⁻¹ NAA + 2.5mg·L⁻¹ adenine) produced the highest differentiation rate of 39.82% (Table 9 and Fig. 1C). Treatments supplied with low-concentration 6-BA (1.0mg·L⁻¹) gave superior bud-formation performance compared with 3.0mg·L⁻¹ high-6-BA groups. At constant 6-BA concentrations, excessive NAA inhibited adventitious-bud induction. Collectively, low-dose 6-BA combined with 0.2 ~ 0.4mg·L⁻¹ NAA and 2.0 ~ 2.5mg·L⁻¹ adenine represented the optimal phytohormone range for adventitious-bud differentiation.

Table 9
Effects of different culture media on differentiation rate

Medium Code	Number of calli	Number of shoots obtained	Differentiation rate (%)
D11	99.00 ± 3.61abA	28.33 ± 0.58cdBCDE	28.64 ± 0.76bcB
D12	98.33 ± 3.01abA	39.00 ± 1.73aA	39.82 ± 2.18aA
D13	100.00 ± 2.65abA	29.67 ± 1.15bcBCDE	29.67 ± 1.15bcB
D14	102.33 ± 2.52aA	26.67 ± 0.58dCE	26.07 ± 0.81cB
D15	101.00 ± 6.56abA	31.00 ± 3.00bcB	30.91 ± 5.02bcB
D16	103.67 ± 1.53aA	31.33 ± 0.58bB	30.23 ± 0.99bcB
D17	94.67 ± 4.93bA	30.67 ± 2.08bcBC	32.53 ± 3.96bB
D18	102.67 ± 2.08aA	30.67 ± 0.58bcBCD	29.89 ± 1.15bcB
D19	100.67 ± 3.51abA	28.33 ± 1.53cdBCDE	28.20 ± 2.52bcB

3.5. Rooting performance across different medium formulations

Rooting percentages ranged between 34.86% and 50.81%. Rooting efficiency decreased as 6-BA concentration increased. Treatments containing 1.0mg·L⁻¹ 6-BA (R20, R21, R22) achieved the best rooting results. Medium R21 (1.0mg·L⁻¹ 6-BA + 0.4mg·L⁻¹ NAA) produced the maximum rooting rate of 50.81%. Treatments with 2.0mg·L⁻¹ 6-BA displayed intermediate rooting capacity, whereas 3.0mg·L⁻¹ 6-BA strongly suppressed root development.

Within each fixed 6-BA level, 0.4mg·L⁻¹ NAA yielded optimal rooting; lower NAA concentrations limited root-primordium initiation, and higher NAA induced excessive basal callusing and reduced the number of functional roots. Accordingly, medium R21 was identified as the optimum rooting medium (Table 10 and Fig. 1D).

Table 10
Effects of different culture media on rooting

Medium Code	Inoculated bud number	Root number	Rooting rate (%)
R20	79.00 ± 1.00aA	34.33 ± 0.58bcBC	43.46 ± 0.91bcB
R21	81.33 ± 1.53aA	41.33 ± 1.53aA	50.81 ± 0.92aA
R22	81.00 ± 1.00aA	41.00 ± 1.00aA	50.62 ± 1.07aA
R23	79.33 ± 4.51aA	34.33 ± 0.58bcBC	43.35 ± 1.85bcB
R24	79.67 ± 5.51aA	35.67 ± 1.53bB	44.83 ± 1.22bB
R25	81.00 ± 1.00aA	30.33 ± 1.53deCDE	37.46 ± 2.07deCD
R26	80.67 ± 3.06aA	32.67 ± 1.53cdBCD	40.51 ± 1.74cdBC
R27	79.67 ± 1.53aA	33.33 ± 3.06bcBCD	41.88 ± 4.33bcBC
R28	79.33 ± 2.08aA	27.67 ± 1.53eE	34.86 ± 1.27eD

4. Discussion

4.1. Regulatory effects of TDZ on callus induction from *P. hybrida* leaf explants

Thidiazuron, a phenylurea-type cytokinin, exhibits higher activity for callus and somatic-embryo induction in orchids compared with classical cytokinins such as 6-BA and kinetin (Faisal et al., 2023). In our gradient experiment covering 1 ~ 5mg·L⁻¹ TDZ, combined with follow-up combinatorial tests of citric acid and NAA, we characterised how cytokinins, antioxidants and exogenous auxins jointly govern leaf dedifferentiation and browning responses in *P. hybrida* cv. ‘Big Chili’. A distinct TDZ threshold concentration was observed: 3mg·L⁻¹ TDZ maximised callus-induction rate (21.15%) while minimising browning and mortality. Increasing citric-acid concentration to 0.2 g·L⁻¹ or adding exogenous NAA at any tested concentration significantly inhibited callus formation. Therefore, for callus induction, exogenous auxin supplementation is unnecessary when using 1/2 MS + 3mg·L⁻¹ TDZ + 0.1 g·L⁻¹ citric-acid.

Multiple orchid-tissue-culture studies have confirmed that TDZ outperforms conventional cytokinins for triggering somatic-cell dedifferentiation; low TDZ dosages activate cell-cycle-related genes and stimulate parenchymal-cell proliferation (Palama et al., 2010; Faisal et al., 2023). Low-dose citric-acid suppresses polyphenol-oxidase activity and mitigates browning, which is consistent with previous reports (Kang et al., 2025; Faisal et al., 2023; Kiaheirati et al., 2024). The observation that added NAA depressed callus yields in our system may be explained by the requirement for a high endogenous cytokinin-to-auxin ratio during leaf dedifferentiation: externally supplied NAA may prematurely trigger root-primordium differentiation and block sustained callus proliferation (Faisal et al., 2023; Mulanda et al., 2012). Furthermore, our results demonstrated that higher citric-acid concentrations do not provide stronger antioxidant protection; elevated citric-acid may disturb medium osmotic balance and aggravate oxidative stress.

4.2. Synergistic influences of leaf age and inoculation orientation on callus induction

Our two-factorial experiment based on the optimised callus-induction medium M3 quantified main effects and interaction between leaf age and inoculation orientation. Both factors significantly modified callus-induction rates and exerted a significant positive interaction ($P < 0.05$). The combination of young newly-expanded leaves inoculated adaxial-surface-up gave the highest callus-induction efficiency (20.22%). Neither leaf age nor inoculation orientation altered overall explant mortality. Earlier studies reported higher regeneration competence in juvenile leaves and improved nutrient uptake when the adaxial side contacts the medium surface (Ghahremani et al., 2021; Li et al., 2022). Nevertheless, prior publications only described these two factors separately without quantifying their synergistic gains. Juvenile leaves plus adaxial-surface-up placement cannot be regarded as simple additive effects of individual factors. Only when both conditions are simultaneously satisfied can a micro-environment characterised by low phenolic substrate levels and efficient nutrient absorption be established to boost dedifferentiation capacity. Mortality remained comparable irrespective of leaf maturity or orientation, confirming that explant death was predominantly determined by mechanical injury and basal phytohormone stress, and providing new insight into the differential regulation of browning versus necrosis in leaf-explants of Orchidaceae.

4.3. Optimisation strategies for adventitious-bud-differentiation medium

Cytokinins play a dominant role during adventitious bud induction. Experimental results demonstrate that low-concentration 6-BA (1.0 mg·L⁻¹) outperforms treatments with higher concentrations, which is consistent with findings from numerous studies. In the leaf regeneration system of

chrysanthemum 'Xiaojinhuang', the regeneration rate reached 27.1% when 1.0 mg·L⁻¹ 6-BA was applied alone, whereas the rate increased to 96.9% with the addition of an appropriate concentration of NAA. This indicates that low concentrations of cytokinins combined with suitable auxin concentrations can efficiently initiate bud primordia (Naing et al, 2016). Similarly, bud sprouting can be induced across a 6-BA concentration range of 0.5 ~ 5 mg·L⁻¹ in mint stem segment cultures, yet the optimal proliferation combination was 2 mg·L⁻¹ 6-BA + 0.1 mg·L⁻¹ NAA; excessively high concentrations may trigger physiological disorders (Wu et al., 2018). In studies on petunia leaf regeneration, MS medium supplemented with 1.0 mg·L⁻¹ 6-BA and 0.10 mg·L⁻¹ NAA was identified as the optimal medium for bud differentiation, further verifying the superiority of low-to-moderate 6-BA concentrations in bud differentiation (Ping et al., 2015).

With respect to the observation that high 6-BA concentrations inhibit adventitious bud formation, existing literature reports that supraphysiological cytokinin concentrations disrupt in planta water balance and cell wall biosynthesis, leading to the generation of vitrified plantlets. In thin cell layer culture of chrysanthemum, 2 mg·L⁻¹ BA promoted bud regeneration, though sucrose concentration required stringent regulation to mitigate vitrification (Sun and Zhang, 2007). Furthermore, in the leaf regeneration system of apple 'Fuji', a regeneration frequency of 73.3% was achieved using 5.0 mg·L⁻¹ 6-BA, while the relevant study also emphasized the necessity of optimizing explant placement to prevent excessive callus proliferation (Xing and Li, 2007). The optimal performance observed with 1.0 mg·L⁻¹ 6-BA in the present experiment conforms to the general principle of regulation by moderate cytokinin levels.

As a cytokinin precursor, adenine exhibited synergistic effects in this experiment. The D12 formulation (1.0 mg·L⁻¹ 6-BA + 0.4 mg·L⁻¹ NAA + 2.5 mg·L⁻¹ adenine) yielded the highest differentiation rate of 39.82%. Although limited literature directly addresses the synergistic effects of adenine in the available references, previous research has revealed that combinations of TDZ, adenine and NAA markedly enhance regeneration potential in petiole cultures of *Populus himalayensis* (Sharma et al., 2020). By participating in cytokinin biosynthetic pathways, adenine facilitates cell division in a mild and sustained manner, improving the robustness of adventitious buds and reducing the incidence of abnormal plantlets.

4.4. Optimisation of rooting medium

Phytohormone requirements during the rooting stage are markedly different from those for shoot differentiation, manifested mainly by inhibitory sensitivity to cytokinins and dependence on auxins. In the present experiment, rooting rate declined with increasing 6-BA concentration, and the maximum rooting rate of 50.8 % was achieved with 1.0 mg L⁻¹ 6-BA combined with 0.4 mg L⁻¹ NAA. This phenomenon has been validated in multiple plant species. In a rooting study on petunia, 1/2 MS supplemented with 0.01–0.10 mg L⁻¹ NAA was identified as the optimal rooting medium, highlighting the importance of low-salt and low-auxin conditions (Ping et al., 2015). In the alfalfa regeneration system, cytokinins need to be reduced or eliminated during shoot elongation and rooting phases to promote root development (Bai et al., 2007).

The mechanism by which high cytokinin concentrations suppress rooting lies in their competitive repression of genes associated with root-primordium differentiation and disturbance of endogenous hormone homeostasis. In a regeneration study using axillary-derived leaves of spring cabbage, plantlet performance was significantly impaired as 6-BA concentration increased from 0 to 5 mg L⁻¹, implying that high cytokinin levels are detrimental to subsequent plant development (Zhang et al., 2009). Furthermore, in cucumber cotyledon-differentiation assays, low NAA concentrations (0.05 ~ 0.10 mg L⁻¹) favoured callus induction and subsequent rooting, whereas high NAA concentrations could trigger excessive basal callusing and block the formation of functional roots (Zhao et al., 2007). The optimal NAA concentration of 0.4 mg L⁻¹ obtained in this experiment is moderately higher than values reported in some references, which may be attributed to species-specific characteristics; nevertheless, the regulatory principle of "low cytokinin plus appropriate auxin" remains fully consistent.

4.5. Integrated strategies for browning control in *P. hybrida* tissue-culture

Explant browning constitutes a pervasive challenge for orchid micropropagation and severely compromises regeneration output (Chen et al., 2012). In the present work, multiple complementary measures were integrated to mitigate browning: supplementation with 0.1 g·L⁻¹ citric-acid to inhibit polyphenol oxidase, continuous dark incubation to reduce photo-oxidative damage, selection of young leaf explants with low phenolic substrate contents, and optimisation of phytohormone concentrations to minimise cellular stress. Using this combined toolkit, average browning rates were reduced to approximately 40%, markedly lower than typical values reported in comparable published systems. Importantly, browning was aggravated when citric-acid exceeded 0.1 g·L⁻¹, highlighting that antioxidants also require careful dosage optimisation. Citric-acid, ascorbic-acid and PVP are well-documented browning-mitigating agents for *P. hybrida* (Kang et al., 2025), and coconut water provides valuable natural nutrients and growth-promoting substances for orchid cultures (Triani and Ilham, 2024; Manokari et al., 2021). By optimising TDZ dosage, leaf-explants source, inoculation orientation and browning-suppression approaches, our study delivers an integrated solution for leaf-based regeneration of *P. hybrida*, underpinning mass-propagation and new-cultivar-breeding programmes.

5. Conclusions

This study successfully established a complete and efficient in-vitro regeneration workflow starting from leaf explants of *P. hybrida* cv. 'Big Chili'. The optimal callus-induction medium was 1/2 MS supplemented with 3mg·L⁻¹ TDZ, 150 mL·L⁻¹ coconut water, 0.1 g·L⁻¹ citric-acid, 20 g·L⁻¹ sucrose and 5.5 g·L⁻¹ agar (pH 5.8). The standardised culture regime used newly expanded young leaves inoculated with adaxial surface upwards, incubated in continuous darkness at 25°C for 90 days, yielding a callus-induction rate of 21.15%. Two-way ANOVA confirmed significant positive interaction between leaf age and inoculation orientation for callus-induction response; the combination of young leaf material plus adaxial-surface-up inoculation represented the optimal operational setup, while these two factors exerted no significant effects on explant mortality. For adventitious-bud

differentiation, the best medium was 1/2 MS containing 1.0mg·L⁻¹ 6-BA, 0.4mg·L⁻¹ NAA, 2.5mg·L⁻¹ adenine, 150 mL·L⁻¹ coconut water and 0.5 g·L⁻¹ activated carbon (pH 5.8). Cultivation under a 16 h light : 8 h dark photoperiod for 30 days gave an adventitious-bud differentiation rate of 39.82%; elevated 6-BA concentrations impaired normal adventitious-bud formation. For rooting and plantlet strengthening, the optimal medium was MS supplemented with 1.0mg·L⁻¹ 6-BA, 0.4mg·L⁻¹ NAA, 150 mL·L⁻¹ coconut water and 1.0 g·L⁻¹ activated carbon (pH 5.8), achieving a rooting rate of 50.81%. Increasing 6-BA concentration significantly inhibited root formation, and 0.4mg·L⁻¹ NAA represented the suitable auxin dosage for rooting in this cultivar.

Our integrated “leaf-explants → callus induction → adventitious-bud differentiation → rooting and plantlet strengthening” technical system addresses key constraints including severe browning and low regeneration efficiency in leaf-culture of *P. hybrida*. It supplies a standardised protocol for factory-scale seedling production. Future work should test the adaptability of this protocol across different *P. hybrida* genotypes and deploy molecular approaches to dissect the regulatory mechanisms underlying callus induction, so as to lay a foundation for building generalised regeneration systems for Orchidaceae.

Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

F. D. Conceptualization, Data curation, Formal analysis. **Y. W.** Methodology, Investigation. **M. C.** Investigation, Software. **J. Z.** Visualization. **S. W.** Validation. **C. W.** Supervision, Funding acquisition. All authors reviewed the manuscript.

Ethics approval and consent to participate

The experimental materials were grown in the intelligent greenhouse of Shandong Academy of Agricultural Sciences. The authors declared the compliance with institutional, national and international guidelines. The experiment was environmentally friendly and had no negative effects on the local ecosystem.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article (and its additional files).

Competing interests

The authors declare that they have no competing interests.

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Figures

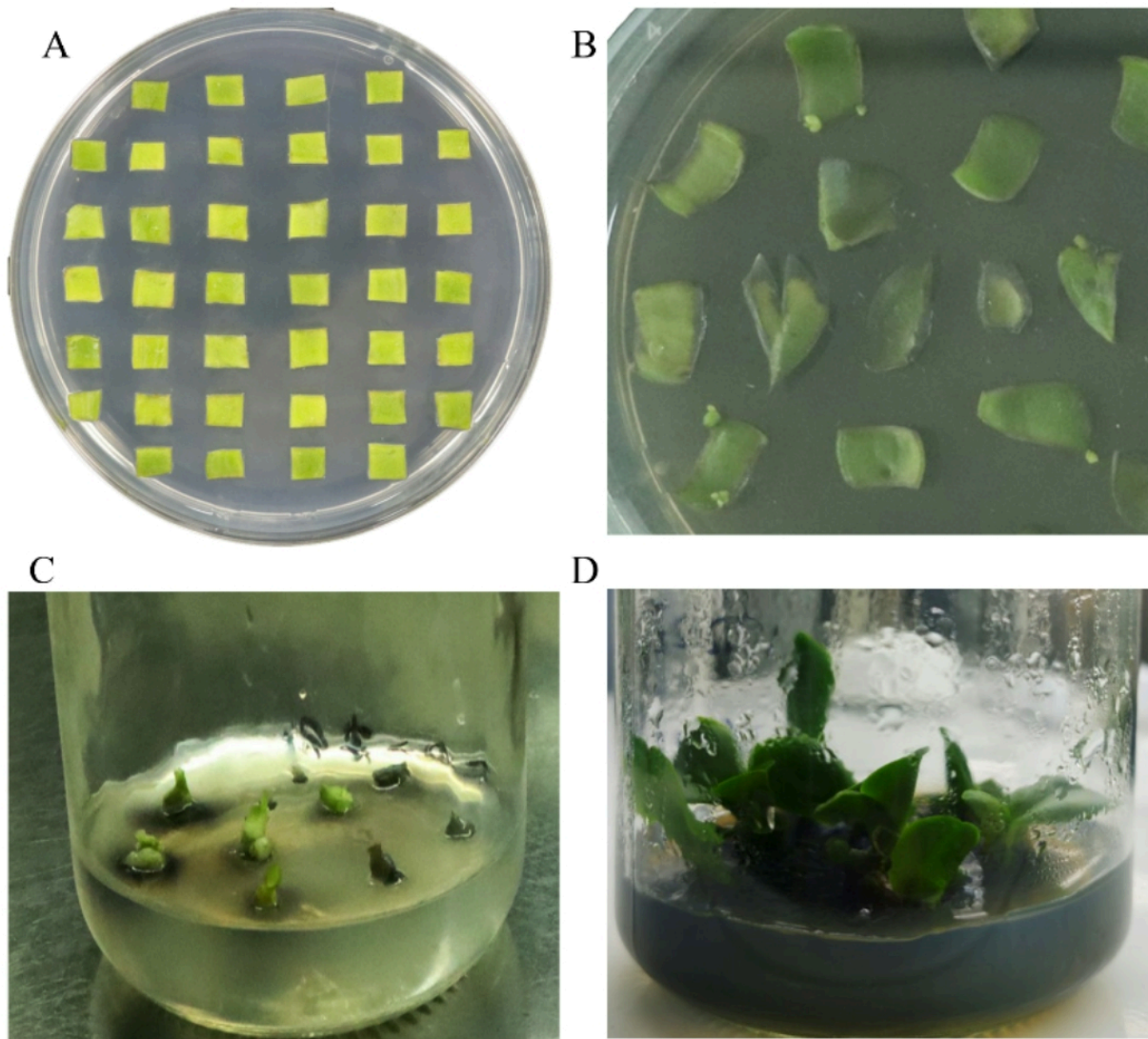


Figure 1

Plant regeneration system from leaves of *P. hybrida*. A: Cut leaves of *P. hybrida*; B: *P. hybrida* leaves with induced callus; C: Callus of *P. hybrida* differentiated into shoots; D: Complete *P. hybrida* plantlets.