

Article

Optimization of In Vitro Rapid Propagation for *Rhododendron decorum* Winter-Flowering Mutant Stem Segments and Leaf Explant Regeneration Induction

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Abstract

Rhododendron decorum Franch. has high ornamental value and emerging edible potential, but conventional propagation cannot meet the demand for high-quality, genetically uniform planting stock. This study optimized explant disinfection, adventitious bud induction, shoot proliferation, and rooting culture using stem segments and leaves from a winter-flowering naturally mutated individual of *Rhododendron decorum*, aiming to minimize contamination and browning. Results demonstrated that combined disinfection with 1% KMnO₄ (10 min) and 10% H₂O₂ (4 min) outperformed single-treatment with 0.1% HgCl₂. For primary culture, the optimal medium for adventitious bud induction was WPM + 30 g·L⁻¹ sucrose + 7.5 g·L⁻¹ agar + 0.56 g·L⁻¹ Ca(NO₃)₂ + 100 mg·L⁻¹ VC + 3 mg·L⁻¹ TDZ + 0.1 mg·L⁻¹ NAA, achieving an induction rate of 66.67%. During subculture, when the concentrations of TDZ and NAA were adjusted to 0.6 mg·L⁻¹ and 0.05 mg·L⁻¹, respectively, and 2 mg·L⁻¹ GA₃ was supplemented, stem nodes exhibited slight thickening but no significant shoot elongation. For rooting, the basal medium WPM + 20 g·L⁻¹ sucrose + 7.5 g·L⁻¹ agar + 0.56 g·L⁻¹ Ca(NO₃)₂ + 100 mg·L⁻¹ VC + 1 g·L⁻¹ activated charcoal supplemented with 0.8 mg·L⁻¹ NAA yielded superior rooting performance. Adding 2 mg·L⁻¹ 2iP tended to enhance rooting rate relative to the NAA-only treatment from an independent rooting experiment. For callus-mediated bud induction from leaves, 0.1 mg·L⁻¹ TDZ + 0.01 mg·L⁻¹ NAA was optimal. Notched fully expanded apical leaves from tissue-cultured plantlets showed highest adventitious bud induction under 0.3 mg·L⁻¹ TDZ + 0.01 mg·L⁻¹ NAA. The established micropropagation system supports bud sport variety selection, mass seedling production, and genetic transformation research.

Keywords: *Rhododendron decorum* Franch.; tissue culture; rapid propagation; leaf regeneration induction



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1. Introduction

Rhododendron decorum Franch., a member of the subgenus *Hymenanthus* within the genus *Rhododendron*, is an evergreen shrub or small tree primarily distributed in Sichuan, Guizhou, and Yunnan provinces of China, as well as northeastern Myanmar. It typically occurs in mountain gullies, open forests on slopes, shrublands, and thickets at elevations ranging from 1540 to 2900 m [1]. Characterized by large, showy flowers in pale red or white hues and a pleasant fragrance, this species serves as a premium ornamental plant suitable

for pot cultivation. Additionally, it exhibits notable medicinal properties, with documented clinical efficacy in relieving cough and resolving phlegm [2]. Its corolla and flower buds are edible and constitute a local specialty food ingredient. Owing to its combined ornamental, medicinal, and edible values, *R. decorum* holds substantial potential for industrial development. However, its large-scale production and commercialization are severely constrained by inherent limitations in conventional propagation: prolonged seedling cycles from seeds, low success rates in cutting propagation, and a generally low propagation coefficient.

Plant tissue culture is an *in vitro* asexual propagation technique that leverages the totipotency of plant cells to regenerate clonal offspring with genetic traits identical to the maternal donor, under strictly sterile conditions. This approach offers a robust strategy for the propagation and germplasm conservation of species recalcitrant to conventional propagation methods [3,4], making it a promising candidate for the efficient propagation of *R. decorum* Franch. [5]. Plant regeneration *in vitro* typically proceeds via two primary pathways: direct organogenesis, wherein adventitious buds or roots arise directly from pre-existing meristematic cells of the explant without an intervening callus phase; and indirect organogenesis, which involves an initial callus formation step followed by subsequent organ differentiation [6].

Thorough surface sterilization of woody plant explants is challenging due to the susceptibility of tender tissues to oxidative browning and the persistence of bacterial contaminants on explants, which complicates the optimization of both disinfectant selection and exposure duration. Therefore, rigorous screening of disinfectants and precise control of sterilization duration are essential. In the surface sterilization protocol for *Illicium henryi* Diels, 0.1% HgCl_2 was identified as the most effective sterilant for explants [7]. For young stem segments of *R. obtusum* ‘Yanzhimi’, a sequential disinfection treatment—75% ethanol for 30 s followed by 2% NaClO solution for 8 min—yielded an induction rate of 43.67% [8]. Combinatorial application of multiple disinfectants often enhances sterilization efficacy: when *Triticum aestivum* L. seeds were sterilized with NaClO and supplemented with Plant Preservative Mixture, PPMTM (PPM), pathogenic fungi belonging to the genera *Aspergillus*, *Cladosporium*, *Penicillium*, and *Bipolaris* were completely eradicated [9]. For stem segments of *R. triflorum*, a two-step chemical disinfection process—75% ethanol pretreatment for 30 s, followed by 0.1% HgCl_2 for 5 min and 2% NaClO for 10 min—resulted in a survival rate of 75% [10].

The core technical bottlenecks in the tissue culture of woody plants are concentrated on three major issues: browning, vitrification, and rooting recalcitrance. The formulation of culture media must be fully tailored to the physiological and metabolic traits of woody plants, while incorporating dynamic adjustments based on variations in explant types and culture stages [11]. Tissue culture studies on *R. dauricum* have demonstrated that different basal media exhibit significant disparities in their induction effects on explants, including leaves, stem segments, flower buds, and leaf buds: In WPM medium, calli and adventitious buds displayed robust growth and strong differentiation capacity; in Read medium, the materials showed mediocre growth performance and low differentiation efficiency; whereas in MS medium, explants almost entirely perished due to severe browning [12]. Another study revealed that calli of *Rhodomyrtus tomentosa* (Ait.) Hask could stably differentiate into adventitious buds when cultured in 1/2MS medium supplemented with $1 \text{ mg} \cdot \text{L}^{-1}$ 6-benzylaminopurine (6-BA) and $0.1 \text{ mg} \cdot \text{L}^{-1}$ 1-naphthaleneacetic acid (NAA). After transferring the regenerated plantlets to plant growth regulator-free half-strength Murashige and Skoog (1/2MS) medium, adventitious roots were observed emerging from the upper regions of the cutting stem segments within a mere 15 days [13].

Plant hormones, as pivotal regulators of explant differentiation and growth, play an irreplaceable role in the establishment of plant tissue culture systems [14]. In tissue

culture studies of *Rhododendron* species, previous researchers have extensively employed a variety of hormones to modulate growth and development. Specifically, auxins primarily include NAA, indole-3-butyric acid (IBA), IAA, and 2,4-dichlorophenoxyacetic acid (2,4-D), while cytokinins encompass thidiazuron (TDZ), zeatin (ZT), N⁶-(2-isopentenyl) adenine (2iP), Kinetin (KT), and 6-BA. Additionally, some studies have incorporated appropriate concentrations of gibberellic acid (GA₃) into the culture medium based on the growth status of plantlets to optimize cultivation outcomes [15]. Plants exhibit distinct species-specific responses to hormone combinations and concentrations, a phenomenon that has been validated across multiple plant species through empirical research. For instance, in the tissue culture of *Illicium henryi* D, the combination of 2 mg·L⁻¹ 6-BA and 0.5 mg·L⁻¹ NAA yielded optimal bud induction efficiency, whereas the combination of 1 mg·L⁻¹ 6-BA and 0.05 mg·L⁻¹ NAA was more favorable for bud elongation. The suitable hormone combination for leaf callus induction was determined to be 1.5 mg·L⁻¹ 6-BA plus 0.5 mg·L⁻¹ NAA [7]. In the indirect regeneration system of *Gerbera jamesonii* calli, 2,4-D was identified as the most potent auxin, while varying concentrations of BA or BAP exerted secondary inductive activity [16]. Hormonal regulation not only modulates regeneration efficiency but also correlates closely with critical issues such as tissue culture browning. In *Hippophae gyantsensis* Lian, cotyledons and hypocotyls could rapidly form calli in 2,4-D-only medium, yet they were susceptible to browning and failed to differentiate. Browning was effectively suppressed when the 6-BA concentration was maintained at 0.3 mg·L⁻¹ [17]. Within the genus *Rhododendron*, the regulatory effects of hormones also exhibit cultivar-specific variations: Vejsadová demonstrated that shoot tip explants of several hybrid *Rhododendron* cultivars achieved optimal bud regeneration under 2iP induction [18], whereas stem segments of *R. fortunei* successfully sprouted axillary buds when induced by ZT [19].

Leaf in vitro regeneration, characterized by a high propagation coefficient, short differentiation cycle, and operational simplicity, serves as a core technology for plant asexual propagation and the establishment of tissue culture-based rapid propagation systems. Its research focuses on three key aspects: optimization of leaf explant harvesting, screening of wounding protocols, and plant hormone regulation. The harvesting position and physiological status of leaves directly influence explant disinfection efficiency and adventitious bud induction performance—a consensus supported by studies on the genus *Rhododendron*. Using fresh functional leaves from young shoots of *R. simsii* ‘Hellmut Vogel’ as explants simplified the disinfection procedure, minimized tissue damage, and enhanced adventitious bud induction rates [20]. This pattern has been validated in other species: For *Pyrus communis* L., it was reported that combining young leaves with an appropriate hormone regime achieved a regeneration rate of up to 87.3%, confirming the high regenerative potential of young leaves [21]. In contrast, using mature apical leaves of *Lavandula vera* DC. as explants resulted in a bud differentiation rate exceeding 80% [22], providing diverse references for leaf explant harvesting strategies across woody and herbaceous plants.

Leaf wounding serves as a critical step in disrupting the epidermal barrier, promoting wound cell dedifferentiation, and initiating bud primordia. The regulatory effects of distinct wounding strategies on leaf regeneration exhibit species- and cultivar-specific variations within the genus *Rhododendron*. For *R. ‘Scintillation’*, excising leaf margins and inoculating with the abaxial surface facing downward not only yielded a high induction rate but also enabled dual regeneration pathways (both direct and indirect differentiation) [23]. The regulatory impacts of varying wounding depths and methods have been extensively validated: In the culture of *R. aureum*, removing the petiole, making light incisions on the abaxial leaf surface, and inoculating with the adaxial surface upward effectively activated cell division and achieved favorable induction outcomes [24]. This regulatory mechanism is conserved across plant species: A study on *Arabidopsis thaliana* revealed that leaf wounding

induces synergistic crosstalk between jasmonic acid (JA) and abscisic acid (ABA) signaling pathways, ensuring adventitious root regeneration under stress conditions [25]. Similarly, research on *Pyrus communis* L. demonstrated that wounding combined with a TDZ + NAA hormone regime significantly enhanced the efficiency of leaf-derived adventitious bud regeneration [21].

This study employed a winter-flowering naturally mutated individual of *R. decorum* var. album as the experimental material. Focusing on key technical bottlenecks of this species—including high difficulty in explant surface sterilization and severe browning during adventitious bud induction from leaf explants—we systematically optimized the sterilization system and regeneration culture conditions, and established a tissue culture-based rapid propagation system characterized by operational simplicity, high induction efficiency, and strong specificity. Adopting a “bud-propagated-by-bud” proliferation mode, this system successfully yielded a large number of phenotypically consistent mutant plantlets while ensuring genetic stability. The findings not only provide reliable technical support for clonal variety breeding and industrial-scale propagation of *R. decorum*. album but also offer theoretical references and practical insights for constructing tissue culture systems of other recalcitrant woody plants.

2. Materials and Methods

2.1. Plant Material

The experimental materials used in this study were current-year, disease- and pest-free young shoots of a winter-flowering naturally mutated individual of *R. decorum* with altered flowering phenology (Figure 1). These materials were collected from the dedicated *Rhododendron* cultivation greenhouse of the College of Landscape Architecture and Horticulture, Yunnan Agricultural University, between April and July 2021. No specific permits were required for the collection of these samples. Semi-lignified stem segments bearing terminal or axillary buds, as well as young leaves, were selected as explants. Immediately after collection, all explant materials were subjected to a 12 h pretreatment at 4 °C and then stored for subsequent use.



Figure 1. A winter-flowering naturally mutated individual of *Rhododendron decorum* Franch.

2.2. Media and Culture Conditions

In this experiment, WPM medium was used as the basic medium [26,27]. The formula for the conventional proliferation and differentiation medium was as follows: 30 g·L⁻¹ sucrose (Yunnan Lingyuexuan Food Co., Ltd., Kunming, China), 7.5 g·L⁻¹ agar powder (Coolaber Science & Technology, Beijing, China, Cat. No. CA1331-500g), 0.56 g·L⁻¹ Ca(NO₃)₂ (Tianjin Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China), and 100 mg·L⁻¹ VC (L-Ascorbic acid, L-Ascorbic acid (Tianjin Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China)). For the rooting medium, the sucrose concentration was adjusted to 20 g·L⁻¹,

and $1 \text{ g} \cdot \text{L}^{-1}$ activated carbon powder was added. According to the experimental design, different concentrations of cytokinin, auxin, and other functional additives were added to the medium at each stage. All media were adjusted to pH 5.4, dispensed into 240 mL specialized tissue-culture glass jars at 30–35 mL per jar, and sterilized at 121°C for 30 min before use. The culture conditions were set as follows: the temperature for stem segment explants was $23 \pm 2^\circ\text{C}$; leaf explants and detached leaves were placed in a temperature-controlled incubator and cultured under a 21°C (12 h, light)/ 15°C (12 h, dark) day–night alternating temperature regime. The light cycle for all explants was $12 \text{ h} \cdot \text{d}^{-1}$, with a light intensity of 1500–2000 lx.

2.3. Explant Surface Sterilization

Semi-lignified bud-bearing stem segments were harvested, with senescent leaves removed and cut into 2–3 cm explants, each containing 2–3 buds. The explants were placed in a glass container with 0.1% (*v/v*) aqueous detergent solution (commercial dishwashing detergent), treated in an ultrasonic cleaner for 30 min, subjected to a gentle brushing of the surfaces of stems and buds using a soft brush, and then rinsed under running tap water for 30 min. Pre-treated explants were transferred to a laminar flow hood for surface sterilization: initially disinfected with 75% ethanol for 30–60 s and rinsed twice with sterile water. Two types of disinfection strategies were compared in this study: single-agent disinfection with toxic mercuric chloride (0.1% HgCl_2), and mercury-free combined-disinfectant systems consisting of $\text{NaClO} + \text{H}_2\text{O}_2$ or $\text{KMnO}_4 + \text{H}_2\text{O}_2$. For HgCl_2 -only treatments, three exposure durations (8, 12, 16 min) were tested. For combined-disinfectant treatments, different soaking durations for each component were arranged as shown in Table 1. After sterilization, explants were thoroughly rinsed with sterile water, and browning tissues at both ends of the stem segments were excised before immediate inoculation onto the medium. Inoculation procedures for each treatment were as follows: For both the 0.1% HgCl_2 group and the combined disinfectant group, 5 stem explants were placed in each culture vessel, with 2 vessels per treatment and 3 biological replicates, yielding a total of 30 stem explants per treatment. Contamination rate and browning rate of explants were recorded at 20 days post-culture, and survival rate was determined at 60 days post-culture.

Table 1. Sterilization program for *R. decorum* stem segments.

Treatment Scheme	Disinfectant Exposure Duration (min)			
	0.1% HgCl_2	5% NaClO	10% H_2O_2	1% KMnO_4
Single-agent: 0.1% HgCl_2				
1	16	-	-	-
2	12	-	-	-
3	8	-	-	-
Combined-disinfectant groups				
4	-	15	2	-
5	-	10	4	-
6	-	5	6	-
7	-	-	2	15
8	-	-	4	10
9	-	-	6	5

Fresh young leaf explants were placed in a glass container with 0.1% (*v/v*) aqueous detergent solution (commercial dishwashing detergent) and treated in an ultrasonic cleaner for 10 min, followed by rinsing under running tap water for 20 min. Pre-treated tender leaves were transferred to a laminar flow hood, subjected to ultra-violet (UV) irradiation sterilization for 10 min, then surface-disinfected with 75% ethanol for 30 s and rinsed twice with sterile water. Gradient-duration sterilization treatments were implemented: explants

were treated with 1% KMnO₄ for 3, 5, and 8 min respectively. After each treatment duration, explants were rinsed 3–5 times with sterile water, then treated with 10% H₂O₂ for 0, 3, and 5 min and thoroughly rinsed with sterile water. Leaf surfaces were blotted dry using sterile filter paper, and different wounding treatments were applied to the leaf surfaces before immediate inoculation onto the induction medium supplemented with 1 mg·L⁻¹ TDZ and 0.05 mg·L⁻¹ NAA. Inoculation was performed as follows: 5 stem explants were placed in each culture vessel, with 2 vessels per treatment and 3 biological replicates, yielding a total of 30 stem explants per treatment. Contamination rate of explants was recorded at 10 days post-culture, while browning rate and callus induction rate were determined at 60 days post-culture.

2.4. Optimization of a Rapid Tissue Culture Propagation System

2.4.1. Primary Culture of *R. decorum* Stem Segments

Using the optimized disinfection protocol for stem-segment explants, we carried out a screening experiment to test TDZ concentrations for stem-segment sprouting. Since preliminary trials revealed negligible sprouting without TDZ, the 0 mg·L⁻¹ TDZ control was excluded. Explants with two bud primordia were cultured, with TDZ gradients of 1, 2, 3, and 4 mg·L⁻¹ and NAA held constant at 0.1 mg·L⁻¹. Inoculation was performed as follows: 5 stem explants were placed in each culture vessel, with 2 vessels per treatment and 3 biological replicates, yielding a total of 30 stem explants per treatment. At the end of the preset culture period, the bud induction rate and growth status of the explants were determined and statistically analyzed.

2.4.2. Subculture of *R. decorum* Stem Segments

When axillary buds had elongated to 1–2 cm in length, they were excised from the original stem segments and cut into uniformly sized bud explants under a laminar flow hood. The bud explants were then inoculated onto a transition medium supplemented with 0.02 mg·L⁻¹ TDZ and 0.01 mg·L⁻¹ NAA to induce new bud formation. Following the emergence of new buds, the explants were transferred to proliferation media containing factorial combinations of NAA (0.01, 0.05, 0.1 mg·L⁻¹) and TDZ (0.2, 0.6, 1 mg·L⁻¹) at gradient concentrations. After determining the optimal NAA concentration, the regenerated shoots were further inoculated onto media supplemented with ZT at different concentrations (0.5, 1, 2 mg·L⁻¹) to optimize proliferation efficiency. For all treatments, 5 stem explants were placed in each culture vessel, with 2 vessels per treatment and 3 biological replicates, yielding a total of 30 stem explants per treatment. After 60 days of culture, the growth status of the shoots and their proliferation rate were recorded and statistically analyzed.

In vitro plantlets with uniform growth and 5 mm in length were selected and inoculated onto media supplemented with 0.02 mg·L⁻¹ TDZ, 0.01 mg·L⁻¹ NAA, and gradient concentrations of GA₃ (0, 0.1, 0.5, 0.8, 1.5 mg·L⁻¹). After 30 days of culture, only plant height was quantitatively measured for the plantlets.

2.4.3. Rooting Culture of *R. decorum* Regenerated Shoots

Robust and uniform-height aseptic tissue culture plantlets at the subculture proliferation stage were selected and inoculated onto rooting media supplemented with gradient concentrations of NAA (0.1, 0.5, 1, 1.5 mg·L⁻¹). For all NAA treatments, 5 stem explants were placed in each culture vessel, with 2 vessels per treatment and 3 biological replicates, yielding a total of 30 stem explants per treatment. Similarly, plantlets were inoculated onto rooting media containing gradient concentrations of 2iP (1, 2, 3 mg·L⁻¹), maintaining identical sample size and replicate settings as the NAA treatments. Rooting induction is a slow-response process rather than rapid vegetative growth for *R. decorum*. Sufficient nutrients remained in the medium over the 90-day culture period to support rooting per-

formance. No subculture or medium transfer was performed throughout the whole rooting period. At the end of the 90-day culture period, the rooting rate, mean root length, and mean number of roots were determined and statistically analyzed for each treatment group.

2.4.4. Acclimatization of *R. decorum* Plantlets

The tissue-culture bottles were opened for 2 days for plantlet hardening. Rooted plantlets were transplanted into 21-cell plug trays (one plantlet per cell) filled with sterilized substrate composed of perlite, peat, and coconut coir at a volume ratio of 1:2:1 (autoclaved at 121 °C for 1 h), and the trays were covered with polyethylene film. Water was sprayed daily to maintain high relative humidity. Acclimatization was carried out in a greenhouse at 24 ± 1 °C under a 12–14 h photoperiod. During the bottle-opening hardening stage, the light intensity was 2000–2500 lx; after transplanting into the substrate, light intensity was adjusted to 3000–5000 lx, provided by fluorescent lamps. After 20 days of acclimatization, the transplant survival rate was determined and statistically analyzed.

2.5. Leaf Regeneration Induction

2.5.1. Regeneration Induction of Leaf Explants

After the leaf explants were induced to form calli, the calli were cut into uniformly sized fragments and subcultured onto differentiation media supplemented with gradient concentrations of TDZ (0.02, 0.1, 0.3 mg·L⁻¹). Preliminary trials showed that no adventitious buds could be induced from calli without TDZ supplementation; therefore, the 0 mg·L⁻¹ TDZ control group was not included in this formal experiment. The experimental design included 3 callus fragments per culture vessel, 2 vessels per treatment, and 3 biological replicates, with a total of 18 callus fragments per treatment. At the end of the 60-day incubation period, the bud induction rate, green leaf rate, and browning rate were determined and statistically analyzed, respectively.

2.5.2. In Vitro Regeneration Induction of Aseptic Leaf Explants

(1) Determination of Optimal Sampling Position for Aseptic Leaf Explants

Robust in vitro plantlets that had undergone 2 months of subculture were selected. Leaves from different node positions were collected sequentially from the apex to the base of the plantlets. After being subjected to wounding treatment, leaves from each nodal position were inoculated onto WPM medium supplemented with 0.02 mg·L⁻¹ TDZ and 0.01 mg·L⁻¹ NAA. For each of the sampling-position treatments, 3 leaf explants were placed per culture jar, with 2 jars per treatment, and the experiment was performed on 3 independent biological replicates, yielding a total of 18 leaf explants per treatment. Following 30 days of culture per treatment, the browning severity of leaves from different positions was observed and recorded by qualitative phenotypic assessment, so as to determine the optimal sampling position for in vitro leaf explants.

(2) The Effects of Different Wounding Treatments on In Vitro Growth of Aseptic Leaf Explants

Robust aseptic plantlets that had undergone 60 days of subculture proliferation were selected. Aseptic leaf explants were harvested from leaf regions with minimal browning susceptibility. Three types of mechanical wounding treatments were applied to leaf explants: wounding of the central leaf region using scissors or a scalpel, wounding of the marginal regions on both sides of the leaf, and bisection of the leaf through its central axis combined with wounding of the marginal regions. The explants were then inoculated onto WPM medium supplemented with 0.02 mg·L⁻¹ TDZ and 0.01 mg·L⁻¹ NAA. The experimental design included 3 callus fragments per culture vessel, 2 vessels per treatment, and 3 biological replicates, with a total of 18 callus fragments per treatment. After 30 days of incubation, the green leaf rate, browning rate, and contamination rate were observed and quantified.

(3) The Effects of Different Cytokinins on In Vitro Growth of Aseptic Leaf Explants

Robust aseptic plantlets subjected to 60 days of subculture proliferation were selected. Leaf explants were harvested from the previously identified optimal sampling position and pretreated with the optimized wounding method (consistent with prior experimental procedures). Using TDZ (0.02, 0.1, 0.3, 0.5 mg·L⁻¹) and ZT (0.5, 1, 2, 3 mg·L⁻¹) as test cytokinins, a screening assay for the optimal induction concentration was conducted with gradient concentrations. The experimental design included 3 callus fragments per culture vessel, 2 vessels per treatment, and 3 biological replicates, with a total of 18 callus fragments per treatment. After 60 days of incubation, the bud induction rate, browning rate, and green leaf rate were observed and quantified.

2.6. Data Statistics

All statistical analyses were performed using Excel 2016 and SPSS 23.0. One-way ANOVA was used for single-factor experiments, while two-way ANOVA was adopted for experiments with two independent factors. The significance level was set at $p < 0.05$. All percentage data, including contamination rate, callus induction rate, bud induction rate, and rooting rate, were subjected to arcsine square-root transformation before analysis to improve data normality. Multiple comparisons were performed using Duncan's multiple range test:

$$\text{Contamination rate (\%)} = (\text{number of contaminated samples} / \text{number of inoculated samples}) \times 100 \quad (1)$$

$$\text{Browning rate (\%)} = (\text{number of browning samples} / \text{number of inoculated samples}) \times 100 \quad (2)$$

$$\text{Survival rate (\%)} = (\text{number of surviving explants} / \text{number of inoculated samples}) \times 100 \quad (3)$$

$$\text{Bud induction rate (\%)} = (\text{number of induced buds} / \text{total number of inoculated stem-segment explant buds}) \times 100 \quad (4)$$

$$\text{Albinism and chlorosis rate (\%)} = (\text{number of albino and chlorotic plantlets} / \text{number of inoculated samples}) \times 100 \quad (5)$$

$$\text{Rooting rate (\%)} = (\text{number of rooted plants} / \text{total number of inoculated plants}) \times 100 \quad (6)$$

$$\text{Multiplication factor} = \text{total number of sprouts} / \text{number of inoculated sprouts} \quad (7)$$

$$\text{Mean root length} = \text{total root length} / \text{total number of roots} \quad (8)$$

$$\text{Mean number of roots} = \text{total number of roots} / \text{number of rooted plantlets} \quad (9)$$

$$\text{Transplanting survival rate (\%)} = (\text{number of surviving transplanted plantlets} / \text{total number of transplanted plantlets}) \times 100 \quad (10)$$

$$\text{Green leaf rate (\%)} = (\text{number of green leaves} / \text{number of treated leaves}) \times 100 \quad (11)$$

$$\text{Callus induction rate (\%)} = (\text{number of leaves with calli} / \text{number of treated leaves}) \times 100 \quad (12)$$

$$\text{Callus-derived bud induction rate (\%)} = (\text{number of buds regenerated from calli} / \text{total number of leaf explants forming calli}) \times 100 \quad (13)$$

3. Results

3.1. Primary Culture

3.1.1. Effects of Different Disinfection Treatments on the Growth of *R. decorum* Stem Segments

A disinfection screening assay was conducted on *R. decorum* stem segment explants using 0.1% HgCl₂. As shown in Table 2, 12 min treatment with 0.1% HgCl₂ achieved the highest explant survival rate (23.33%), significantly higher than 8 min and 16 min groups ($p < 0.05$), with a lower browning rate (38.33%). No significant difference in contamination rate was observed among groups. The 16 min treatment caused severe axillary bud brown-

ing, with new shoots only from terminal buds; 8 min and 12 min groups showed axillary bud germination, with light green and dark green leaves, respectively (Figure 2A).

Table 2. Effects of HgCl_2 sterilization time on *R. decorum* stem segments.

Treatment	Sterilization Time of HgCl_2 (min)	Contamination Rate (%)	Browning Rate (%)	Survival Rate (%)
1	8	55.00 ± 2.89 a	43.33 ± 7.26 ab	1.67 ± 1.67 a
2	12	38.33 ± 7.26 a	38.33 ± 6.01 a	23.33 ± 6.67 b
3	16	38.33 ± 4.41 a	63.33 ± 6.67 b	5.00 ± 2.89 a

Note: Data in the table represent the mean $\pm$ standard error of three replicates for the same treatment; different lowercase letters in the same column indicate significant differences ($p < 0.05$).

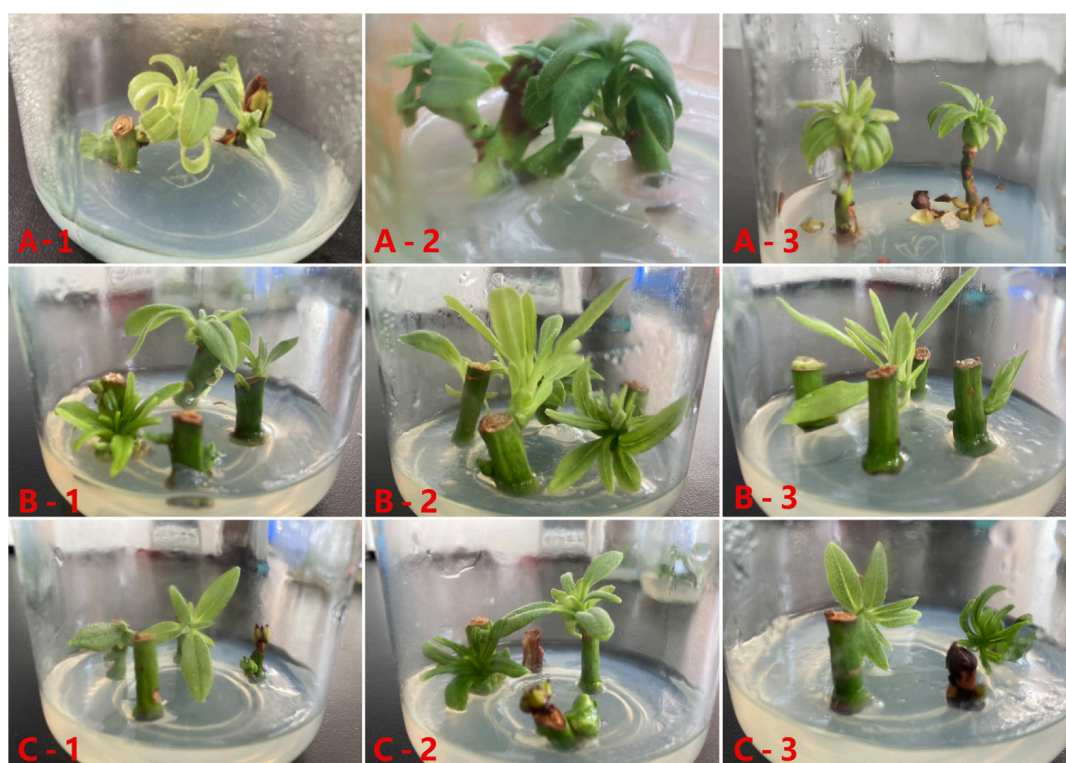


Figure 2. Effects of different sterilization methods on the sterilization of *R. decorum* stem segments. Note: (A) For the 0.1% HgCl_2 treatment, (A-1–A-3) represent sterilization times of 8 min, 12 min, and 16 min respectively. (B) For 10% H_2O_2 and 1% KMnO_4 treatments: (B-1) 10% H_2O_2 sterilization for 2 min, 1% KMnO_4 sterilization for 15 min; (B-2) 10% H_2O_2 sterilization for 4 min, 1% KMnO_4 sterilization for 10 min; (B-3) 10% H_2O_2 sterilization for 6 min, 1% KMnO_4 sterilization for 5 min, respectively. (C) For 10% H_2O_2 and 5% NaClO treatments: (C-1) 10% H_2O_2 sterilization for 2 min, 5% NaClO sterilization for 15 min; (C-2) 10% H_2O_2 sterilization for 4 min, 5% NaClO sterilization for 10 min; (C-3) 10% H_2O_2 sterilization for 6 min, 5% NaClO sterilization for 5 min.

Since 0.1% HgCl_2 failed to effectively reduce browning and contamination, gradient-time combined sterilization with 5% NaClO , 1% KMnO_4 , and 10% H_2O_2 was further tested (Table 3). Treatments 1, 2, and 5 had contamination rates $<10\%$ (Treatment 2: 0%); Treatments 3, 4, and 5 showed low browning, with higher survival rates in Treatments 4 and 5. With consistent 10% H_2O_2 duration, 1% KMnO_4 (10/15 min) groups had lower browning than 5% NaClO groups. The 1% KMnO_4 10 min + 10% H_2O_2 4 min group exhibited low contamination and high survival, with superior new bud vigor (Figure 3). This combination was identified as the optimal protocol, though the survival rate remained moderate (46.67%).

Table 3. Effects of different disinfectant combinations on the sterilization of *R. decorum* stem segments.

Treatment	Disinfectant Treatment (min)			Contamination Rate (%)	Browning Rate (%)	Survival Rate (%)
	5%NaClO	10%H ₂ O ₂	1%KMnO ₄			
1	15	2	-	6.67 ± 6.67 A	60.0 ± 10.00 C	23.33 ± 12.02 A
2	10	4	-	0.00 ± 0.00 A	36.67 ± 12.02 BC	26.67 ± 6.67 A
3	5	6	-	56.67 ± 8.82 B	10.00 ± 5.77 A	20.00 ± 5.77 A
4	-	2	15	36.67 ± 12.02 B	6.67 ± 6.67 A	40.00 ± 11.55 A
5	-	4	10	6.67 ± 6.67 A	0.00 ± 0.00 A	46.67 ± 6.67 A
6	-	6	5	46.67 ± 8.82 B	16.67 ± 8.82 AB	23.33 ± 8.82 A

Note: Data in the table represent the mean ± standard error of three replicates for the same treatment; different capital letters in the same column indicate highly significant differences ($p < 0.01$).

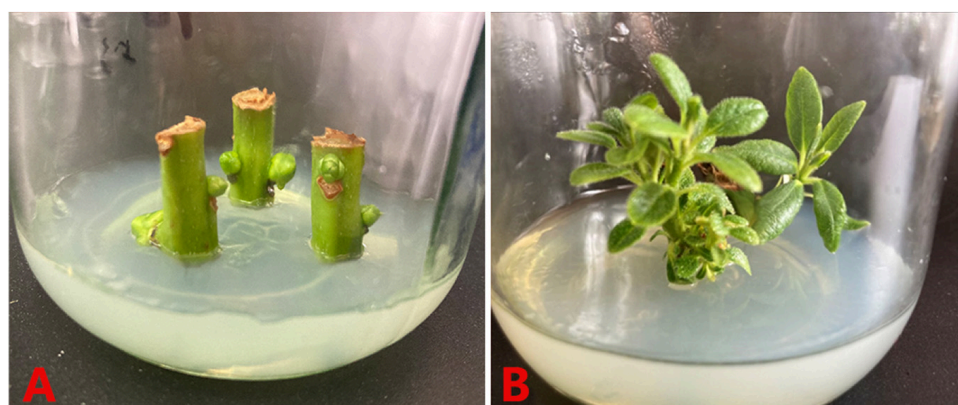


Figure 3. An amount of $3 \text{ mg} \cdot \text{L}^{-1}$ TDZ induced axillary bud growth of *R. decorum*. Note: (A) Axillary bud growth of stem segments at 14 days post-inoculation under $3 \text{ mg} \cdot \text{L}^{-1}$ TDZ treatment; (B) axillary bud growth of stem segments at 60 dpi under $3 \text{ mg} \cdot \text{L}^{-1}$ TDZ treatment.

3.1.2. Effects of Different TDZ Concentrations on Bud Induction in *R. decorum*

After treating stem segment explants with the optimal sterilization protocol screened in the previous experiment, the explants were inoculated onto media supplemented with different concentrations of TDZ to induce axillary bud germination. The axillary bud induction rate and induction time are presented in Table 4. The results showed that the bud induction rate increased with increasing TDZ concentration. When the TDZ concentration reached $3 \text{ mg} \cdot \text{L}^{-1}$, the induction rate was significantly higher than those of the other concentration groups. Concurrently, the axillary bud induction time decreased with increasing TDZ concentration. Morphological observations from Figure 2A revealed that the bud primordia of stem segments in this treatment ($3 \text{ mg} \cdot \text{L}^{-1}$ TDZ) began to swell at 10–15 days of culture, with new buds germinating after 15 days. After 2 months of culture, the growth status of the explants is shown in Figure 3B.

Table 4. Effects of different TDZ concentrations on axillary bud induction growth of *R. decorum*.

Treatment	Concentration ($\text{mg} \cdot \text{L}^{-1}$)	Bud Induction Rate (%)	Sprouting Time (Days)
A	1	$50.00 \pm 11.55 \text{ ab}$	Bud primordia swelled at 15–20 days of culture, with axillary buds sprouting after 20 days.
B	2	$40.00 \pm 5.77 \text{ ab}$	Bud primordia swelled at 12–17 days of culture, with axillary buds sprouting after 17 days.
C	3	$66.67 \pm 8.82 \text{ b}$	Bud primordia swelled at 10–15 days of culture, with axillary buds sprouting after 15 days.
D	4	$36.67 \pm 6.67 \text{ a}$	Bud primordia swelled at 10–15 days of culture, with axillary buds sprouting after 15 days.

Note: Data in the table represent the mean ± standard error of three replicates for the same treatment; different lowercase letters in the same column indicate significant differences ($p < 0.05$).

3.2. Subculture

3.2.1. Effects of Different Concentrations of TDZ and NAA on the Growth of *R. decorum* In Vitro Plantlets

Using WPM as the basal medium, a two-factor experiment was designed with different concentrations of TDZ and NAA. The results after 60 days of culture are presented in Table 5. TDZ concentration showed a significant correlation with bud growth characteristics: with the increase in TDZ concentration, the bud growth rate decreased, the proliferation coefficient increased, and the adventitious buds were arranged more closely. When the TDZ concentration was $0.6 \text{ mg}\cdot\text{L}^{-1}$, the bud proliferation coefficient was optimal, with emerald-green leaves and robust growth. When the TDZ concentration increased to $1 \text{ mg}\cdot\text{L}^{-1}$, the dense cluster buds of tissue culture plantlets made counting difficult; thus, $0.6 \text{ mg}\cdot\text{L}^{-1}$ TDZ was tentatively determined as the suitable concentration for bud proliferation of *R. decorum*. Changes in NAA concentration had no significant promoting effect on bud growth or proliferation coefficient. When the NAA concentration was $0.05 \text{ mg}\cdot\text{L}^{-1}$, the plant height uniformity of in vitro plantlets was good, and the leaf color was more emerald green than that of the $0.01 \text{ mg}\cdot\text{L}^{-1}$ and $0.1 \text{ mg}\cdot\text{L}^{-1}$ treatment groups; therefore, $0.05 \text{ mg}\cdot\text{L}^{-1}$ NAA was determined as the suitable concentration. The main-effect analysis of TDZ and NAA concentrations on bud proliferation in Table 6 indicates that TDZ exerted an extremely significant main-effect on bud proliferation, whereas increasing NAA concentration showed no obvious promoting effect. Moreover, no significant interaction was observed between TDZ and NAA during bud proliferation.

Table 5. Effects of different concentrations of TDZ and NAA on the proliferation of *R. decorum* in vitro plantlets.

Treatment	TDZ Concentration ($\text{mg}\cdot\text{L}^{-1}$)	NAA Concentration ($\text{mg}\cdot\text{L}^{-1}$)	Multiplication Times	Growing Condition
1	0.2	0.01	3.23 ± 0.23 Aa	The explants exhibited vigorous growth with vibrant green foliage.
2	0.6	0.05	5.47 ± 0.29 Bb	The explants exhibited relatively slow growth with vibrant green foliage.
3	1	0.1	Unable to count	The explants exhibited slow growth with yellowish-green foliage.
4	0.2	0.05	3.10 ± 0.30 Aa	The explants exhibited vigorous growth with vibrant green foliage.
5	0.6	0.1	4.9 ± 0.17 Bb	The explants exhibited relatively slow growth with vibrant green foliage.
6	1	0.01	Unable to count	The explants exhibited slow growth with yellowish-green foliage.
7	0.2	0.1	3.30 ± 0.21 Aa	The explants exhibited vigorous growth with vibrant green foliage.
8	0.6	0.01	5.20 ± 0.21 Bb	The explants exhibited relatively slow growth with vibrant green foliage.
9	1	0.05	Unable to count	The explants exhibited slow growth with yellowish-green foliage.

Note: Data in the table represent the mean $\pm$ standard error of three replicates for the same treatment. Different letters within the same column indicate significant differences at ($p < 0.05$). Lowercase letters denote differences among TDZ concentrations under the same NAA concentration, and uppercase letters denote differences among NAA concentrations under the same TDZ concentration. Treatments 3, 6 and 9 produced excessive and overly tiny bud points, and valid multiplication-rate data could not be obtained; accordingly, these treatments were excluded from ANOVA and multiple-comparison tests.

Table 6. Between-subject main-effect test of proliferation multiplicity of *R. decorum* in vitro plantlets with TDZ and NAA concentrations.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p Value
A × B	0.441	2	0.221	7.165	0.314
NAA concentration (A)	0.103	2	0.052	0.299	0.747
TDZ concentration (B)	17.602	1	17.602	101.878	<0.000 **
Error	2.073	12	0.173		
Total	337.740	17			

Note: Two-way analysis of variance (ANOVA) was performed for the tested indicators. A represents NAA concentration; B represents TDZ concentration. ** indicates significant difference at $p < 0.01$.

3.2.2. Effects of Different Concentrations of ZT on the Growth of *R. decorum* In Vitro Plantlets

The results of further investigations of the regulatory effects of different ZT concentrations on the growth of *R. decorum* in vitro plantlets are presented in Table 7. With the increase in ZT concentration gradient, no significant difference was observed in the bud proliferation efficiency of in vitro plantlets, and the leaves of tissue culture plantlets were generally yellowish-green. Compared with the TDZ treatment group, the promoting effect of ZT on bud proliferation was relatively weaker. Integrating the aforementioned experimental results, the optimal plant hormone combination for the subculture proliferation stage of *R. decorum* in vitro plantlets was finally determined as $0.6 \text{ mg}\cdot\text{L}^{-1}$ TDZ + $0.05 \text{ mg}\cdot\text{L}^{-1}$ NAA.

Table 7. Effects of different concentrations of ZT on the proliferation of *R. decorum* in vitro plantlets.

Treatment	ZT Concentration ($\text{mg}\cdot\text{L}^{-1}$)	NAA Concentration ($\text{mg}\cdot\text{L}^{-1}$)	Multiplication Times	Growing Condition
1	0.5	0.05	$2.40 \pm 0.29 \text{ a}$	Exhibited vigorous growth with yellowish-green foliage
2	1	0.05	$1.80 \pm 0.38 \text{ a}$	Exhibited vigorous growth with yellowish-green foliage
3	2	0.05	$2.47 \pm 0.35 \text{ a}$	Exhibited vigorous growth with yellowish-green foliage

Note: Data in the table represent the mean $\pm$ standard error of three replicates for the same treatment; different lowercase letters in the same column indicate significant differences ($p < 0.05$).

3.2.3. Effects of Different Concentrations of GA₃ on the Elongation Growth of *R. decorum* In Vitro Plantlets

During the subculture stage of *R. decorum* in vitro plantlets, growth retardation and internode elongation inhibition were observed, which limited the overall culture efficiency of the tissue-culture system. Given that GA₃ possesses the physiological function of inducing internodal cell elongation in plants, this study employed GA₃ to regulate the elongation growth of in vitro plantlets. Results from the single-factor experiment (Figures 4 and 5) showed that compared with the control group without GA₃ supplementation, exogenous application of GA₃ promoted an increase in plant height of in vitro plantlets to a certain extent, but the difference did not reach a significant level. Qualitative morphological observation from representative plantlets revealed a tendency toward longer internodes with elevated GA₃ concentration, accompanied by thinner stems. Among tested GA₃ treatments, $2 \text{ mg}\cdot\text{L}^{-1}$ GA₃ exhibited the most favorable trend for plantlet elongation, although no statistically significant differences in plant height were detected across all GA₃ groups.

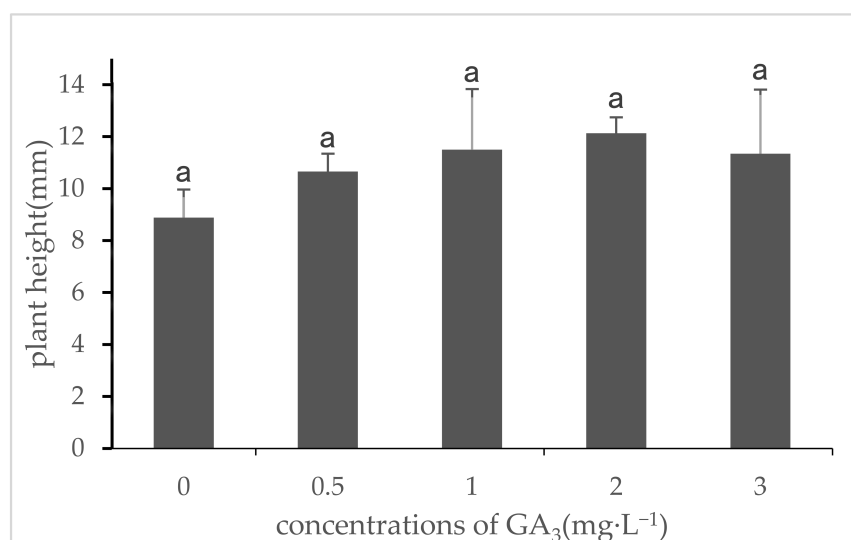


Figure 4. Effects of different concentrations of GA_3 on the height growth of *R. decorum* in vitro plantlets. Note: Different lowercase letters indicate significant differences at the 0.05 probability level ($p < 0.05$).

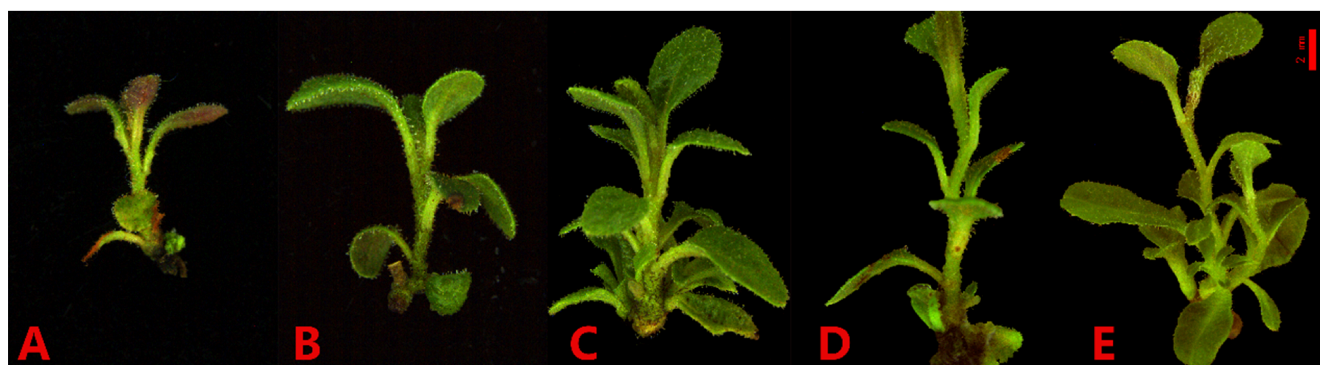


Figure 5. Morphological comparison of *R. decorum* in vitro plantlets under different concentrations of GA_3 treatment. Note: (A) $0 \text{ mg} \cdot \text{L}^{-1}$ GA_3 treatment; (B) $0.5 \text{ mg} \cdot \text{L}^{-1}$ GA_3 treatment; (C) $1 \text{ mg} \cdot \text{L}^{-1}$ GA_3 treatment; (D) $2 \text{ mg} \cdot \text{L}^{-1}$ GA_3 treatment; (E) $3 \text{ mg} \cdot \text{L}^{-1}$ GA_3 treatment. Scale bar: 2 mm.

3.3. Rooting Culture

3.3.1. Effects of Different Concentrations of NAA on Rooting in *R. decorum* In Vitro Plantlets

To investigate the regulatory effect of NAA on rooting and determine the optimal application concentration for *R. decorum* in vitro plantlets, this study conducted rooting experiments with different NAA concentrations, and the results are presented in Table 8. No rooting was observed in the *R. decorum* in vitro plantlets in the blank control group (without NAA addition). Under low NAA concentrations, the rooting performance of in vitro plantlets was poor, with all rooting indicators remaining at low levels. The rooting rate of in vitro plantlets in the $1.5 \text{ mg} \cdot \text{L}^{-1}$ NAA treatment group was the highest among all treatments, but there was no significant difference compared with the $0.8 \text{ mg} \cdot \text{L}^{-1}$ NAA treatment group. The average root number and average root length of in vitro plantlets in the $0.8 \text{ mg} \cdot \text{L}^{-1}$ NAA treatment group were significantly superior to those in other groups, reaching the optimal level. When the NAA concentration was increased to $1.5 \text{ mg} \cdot \text{L}^{-1}$, both the average root number and average root length of in vitro plantlets showed a significant downward trend. Based on the comprehensive performance of the three core rooting indicators, this experiment identified $0.8 \text{ mg} \cdot \text{L}^{-1}$ NAA as the optimal concentration for rooting culture of leaf explants of *R. decorum*.

Table 8. Effects of different concentrations of NAA on the rooting of *R. decorum*.

Treatment	NAA Concentration (mg·L ⁻¹)	Rooting Rate (%)	Mean Number of Roots	Mean Root Length (mm)
1	0	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
2	0.1	6.67 ± 3.33 a	3.67 ± 0.88 bc	5.94 ± 1.06 bc
3	0.5	26.67 ± 3.33 b	3.33 ± 0.93 b	6.22 ± 2.12 bc
4	0.8	40.00 ± 5.77 bc	5.17 ± 0.33 c	8.03 ± 2.12 c
5	1.5	46.67 ± 6.67 c	2.17 ± 0.17 b	2.89 ± 0.36 ab

Note: Data in the table represent the mean ± standard error of three replicates for the same treatment; different lowercase letters in the table indicate significant differences ($p < 0.05$).

To investigate the regulatory effect of NAA on rooting and to screen suitable NAA concentrations for *R. decorum* in vitro plantlets, this study carried out rooting experiments with gradient NAA concentrations, and the results are presented in Table 8. No rooting was observed in the blank control group without NAA supplementation. Under low NAA concentrations, plantlets exhibited poor rooting performance, and all rooting-related indicators remained at low levels. The highest rooting rate was achieved under 1.5 mg·L⁻¹ NAA, which was not significantly different from that of the 0.8 mg·L⁻¹ NAA treatment. Nevertheless, plantlets treated with 0.8 mg·L⁻¹ NAA possessed significantly higher mean root number and longer mean root length compared with most other treatments. When NAA concentration further increased to 1.5 mg·L⁻¹, both mean root number and mean root length decreased significantly. Considering the trade-off between rooting rate and root system quality (mean number of roots and mean root length), 0.8 mg·L⁻¹ NAA was selected as a compromised suitable concentration for rooting culture of *R. decorum* in vitro plantlets.

3.3.2. Effects of Different Concentrations of 2iP on Rooting in *R. decorum* In Vitro Plantlets

Under the condition of a fixed NAA concentration at 0.8 mg·L⁻¹, this study set three concentration gradients of 2iP treatments to investigate their effects on the rooting rate, mean root length, and mean root number of *R. decorum* in vitro plantlets. It should be noted that no 0 mg·L⁻¹ 2iP internal control was included in this experiment. The 0.8 mg·L⁻¹ NAA treatment without 2iP, which serves as the reference, originated from the independent single-factor rooting experiment shown in Table 8, rather than being an internal control within Table 9. The results are presented in Table 9 and Figure 6. Among all 2iP treatment groups, the 2 mg·L⁻¹ 2iP group exhibited the highest rooting rate, though no significant differences were observed across all treatment groups. The mean root number showed a trend of first increasing and then decreasing with the elevation of 2iP concentration, whereas the mean root length gradually shortened as the 2iP concentration increased. Considering the comprehensive performance of rooting-related indicators, the combination of 0.8 mg·L⁻¹ NAA and 2 mg·L⁻¹ 2iP was selected as a suitable hormone combination for in vitro rooting of *R. decorum* plantlets.

Table 9. Effects of different concentrations of 2iP on the rooting of *R. decorum* in vitro plantlets.

2iP Concentration (mg·L ⁻¹)	NAA Concentration (mg·L ⁻¹)	Rooting Rate (%)	Mean Number of Roots (Strip)	Mean Root Length (mm)
1	0.8	36.67 ± 3.33 a	4.00 ± 0.77 a	5.85 ± 0.22 b
2	0.8	50.00 ± 5.77 a	7.67 ± 0.88 b	4.83 ± 0.37 ab
3	0.8	40.00 ± 5.77 a	5.67 ± 0.88 ab	4.58 ± 0.43 a

Note: Data in the table represent the mean ± standard error of three replicates for the same treatment; different lowercase letters in the table indicate significant differences ($p < 0.05$).

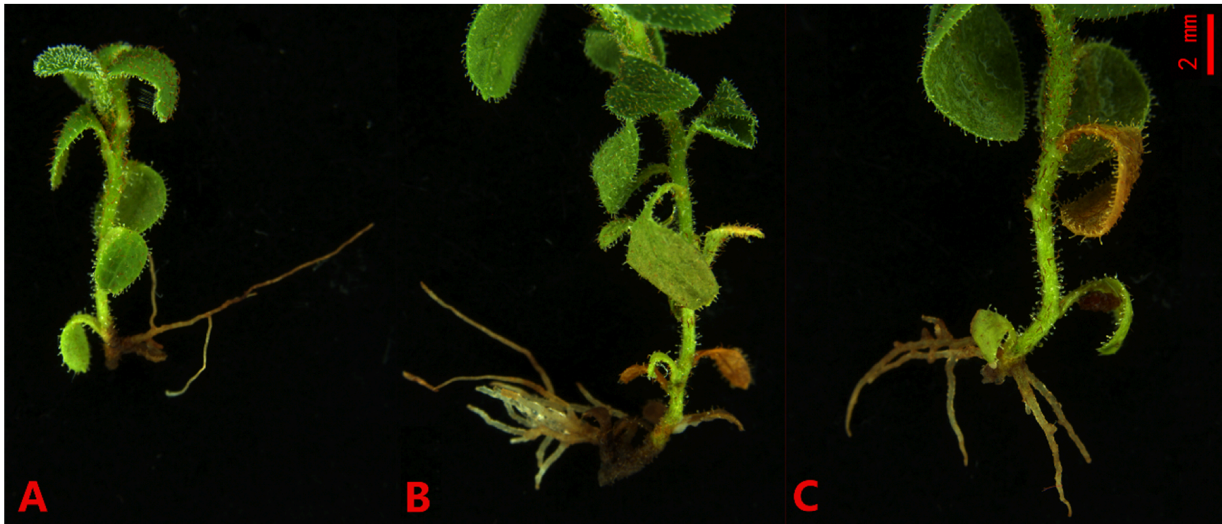


Figure 6. Effects of different concentrations of 2iP on the rooting of *R. decorum* in vitro plantlets. Note: (A) 1 mg·L^{−1} 2iP treatment; (B) 2 mg·L^{−1} 2iP treatment; (C) 3 mg·L^{−1} 2iP treatment. Scale bars: 2 mm.

3.4. Acclimatization

Tissue culture plantlets following rooting induction were transplanted to a substrate mixture with a volume ratio of perlite: peat: coconut coir = 1:2:1 for acclimatization. After 20 days of transplantation, the transplanting survival rate reached 83.33%.

3.5. Leaf Induction from Explants of *R. decorum*

3.5.1. Effects of Different Disinfection Treatments on Leaf Induction from Explants

Building on the robust disinfection efficacy of the 1% KMnO₄ and 10% H₂O₂ combination observed during the stem segment sterilization phase, this study employed this composite disinfection system for the sterilization of leaf explants of *R. decorum*. The results are presented in Table 10. No significant differences were detected in the contamination rate or browning rate of explants across the three sterilization treatments. Notably, the treatment combination of 1% KMnO₄ for 5 min followed by 10% H₂O₂ for 3 min exhibited a relatively lower contamination rate, with a callus induction rate higher than that of the other treatment groups.

Table 10. Effects of different disinfection methods on leaf induction of *R. decorum*.

1%KMnO ₄ (min)	10%H ₂ O ₂ (min)	Browning Rate (%)	Contamination Rate (%)	Callus Induction Rate (%)
5	3	27.67 ± 5.33 a	44.33 ± 11.33 a	27.67 ± 5.33 b
8	0	22.33 ± 5.33 a	72.33 ± 5.33 a	5.67 ± 5.67 a
3	5	22.00 ± 11.00 a	66.67 ± 16.67 a	11.33 ± 5.67 ab

Note: Data in the table represent the mean ± standard error of three replicates for the same treatment; different lowercase letters in the table indicate significant differences (*p* < 0.05).

Morphological observations following 60 days of culture (Figure 7) revealed the following developmental sequence: At approximately 25 dpi, the leaf incision sites exhibited thickening and swelling (Figure 7A). At 45 dpi, yellow spherical calli differentiated at the incision regions (Figure 7B). By day 60 of culture, the calli gradually transitioned from yellow to green and developed bud primordia, which subsequently underwent enlargement (Figure 7C). After 60 days of culture, new buds germinated from the bud primordia; however, the spherical calli exhibited browning, leading to a relatively low callus-derived bud induction rate. Subsequent studies should further optimize the hormone concentration

ratio in the culture medium to mitigate callus browning and reduce explant mortality caused by leaf browning.

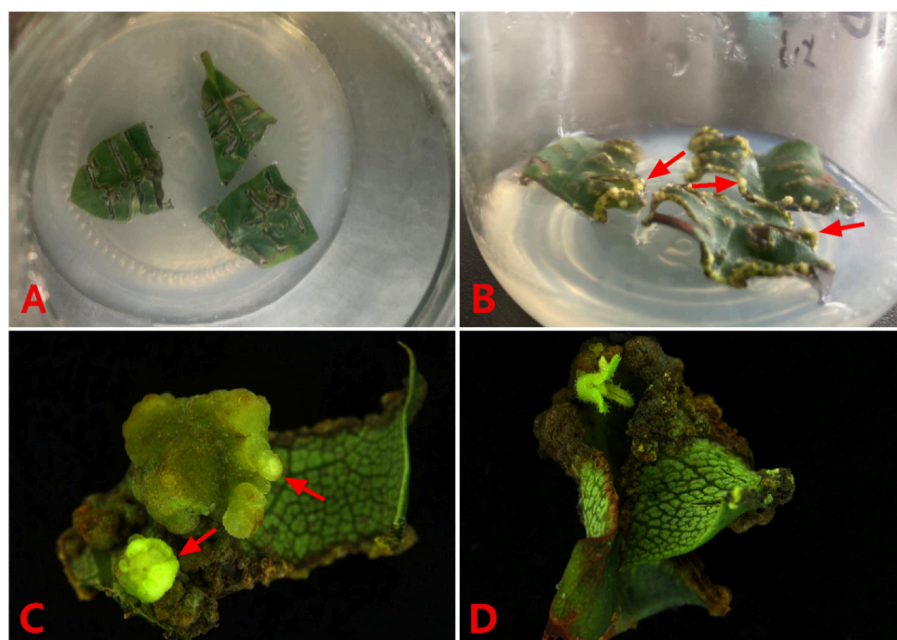


Figure 7. Callus and shoot-bud induction process from leaf explants of *R. decorum*. Note: (A) Leaf performance at 25 days after inoculation; (B) globular callus formation on leaf explants at 45 days after inoculation, arrows indicate globular callus; (C) emergence of adventitious buds on leaf explants at 60 days after inoculation, arrows indicate adventitious buds; (D) shoot sprouting from leaf explants at 60 days after inoculation.

3.5.2. Effects of Different TDZ Concentrations on Leaf Callus Induction

Leaf explants with induced calli were transferred to media supplemented with reduced TDZ concentrations for subsequent culture, and the results are presented in Table 11. Across the three tested TDZ concentration gradients, no significant differences were observed in explant browning rate or green leaf rate; notably, the $0.1 \text{ mg}\cdot\text{L}^{-1}$ TDZ treatment group exhibited a relatively lower browning rate and a higher green leaf rate. Combined with the analysis of Figure 8, distinct differences were found in the effects of varying TDZ concentrations on the callus-derived bud induction rate: The callus-derived bud induction rate of the $0.1 \text{ mg}\cdot\text{L}^{-1}$ TDZ treatment group was significantly superior to the other two groups, reaching a level of extremely significant difference.



Figure 8. Effects of different TDZ concentrations on adventitious-bud induction from leaf-derived calli of *R. decorum*. Note: (A) $0.02 \text{ mg}\cdot\text{L}^{-1}$ TDZ treatment; (B) $0.1 \text{ mg}\cdot\text{L}^{-1}$ TDZ treatment; (C) $0.3 \text{ mg}\cdot\text{L}^{-1}$ TDZ treatment.

Table 11. Effects of different TDZ concentrations on callus formation of leaf explants of *R. decorum*.

TDZ Concentration (mg·L ⁻¹)	NAA Concentration (mg·L ⁻¹)	Browning Rate (%)	Green Leaf Rate (%)	Callus-Derived Bud Induction Rate (%)
0.02	0.01	38.89 ± 5.56 a	61.11 ± 5.56 a	27.78 ± 5.56 a
0.1	0.01	22.22 ± 14.70 a	77.78 ± 14.70 a	66.67 ± 9.62 b
0.3	0.01	55.56 ± 11.11 a	44.44 ± 11.11 a	5.56 ± 5.56 a

Note: Data in the table represent the mean ± standard error of three replicates for the same treatment; different lowercase letters in the table indicate highly significant differences ($p < 0.01$).

3.6. In Vitro Induction from Leaf Explants of *R. decorum*

3.6.1. Determination of Explant Sampling Position

Selection of in vitro leaf explants is a critical determinant of bud regeneration efficiency from leaf explants of *R. decorum*; suitable explants can significantly enhance the success rate of in vitro regeneration culture. Given the susceptibility of *R. decorum* leaf explants to browning upon in vitro incubation, this study subjected leaves from different nodal positions of in vitro plantlets to wounding treatment. The browning severity was evaluated by qualitative phenotypic observation without a predefined browning rating scale, and the optimal explant sampling site was determined on this basis (Figure 9). Browning initiated uniformly at the wounded sites: leaves from the lowest stem nodes exhibited the most severe browning, eventually leading to near-complete browning and necrosis; as the sampling nodal position shifted upward, browning severity gradually diminished, with the apical leaves of the plantlets being the least affected by browning. Based on these observations, the fully expanded apical leaves from *R. decorum* in vitro plantlets were identified as the optimal explants for in vitro leaf regeneration.

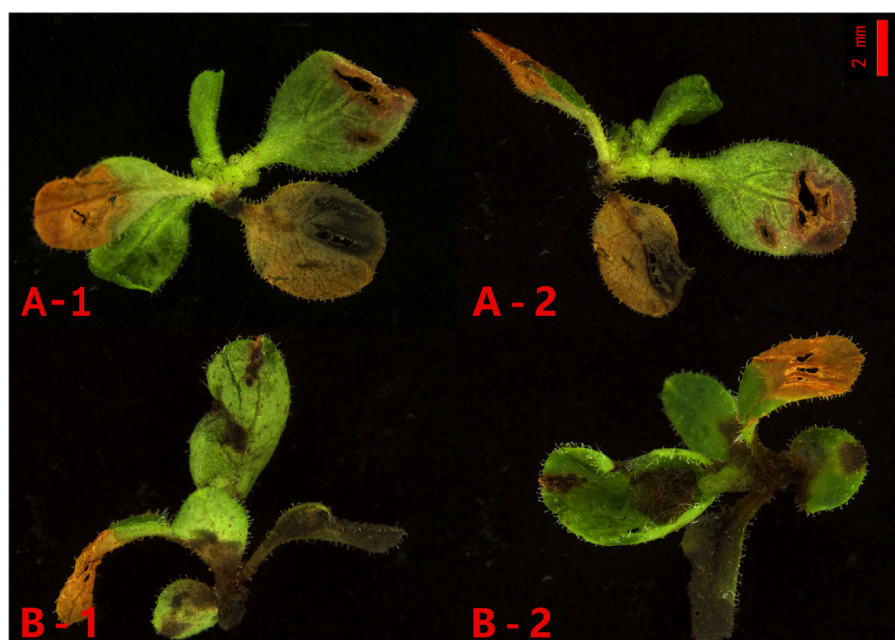


Figure 9. Effects of different positions of leaf puncture wound on browning of *R. decorum*. Note: (A-1) plant bottom view; (A-2) plant side view; (B-1) plant main view; (B-2) plant top view. Scale bars: 2 mm.

3.6.2. Effects of Different Wounding Treatments on Responses of Leaf Explants

The selection of wounding incision strategies represents a critical procedure in the in vitro regeneration culture of leaf explants of *R. decorum*. An optimal incision site can effectively reduce explant browning mortality and enhance bud induction efficiency. As

indicated in Table 12, no significant differences were observed in leaf browning rate, green leaf rate, or callus-derived bud induction rate among the three wounding treatments. The browning rate of all treatments exceeded 50%, with the combined incision treatment (mid-leaf + leaf margin) exhibiting the highest numerical browning rate (90%). In contrast, the single leaf-margin incision treatment showed the numerically highest green leaf retention and bud-induction values, although these differences were not statistically significant. Combined with the morphological observation results in Figure 10, browning occurred at the wounded sites of leaves in all treatments 15 days after incision. Among them, the mid-leaf incision treatment exhibited the largest browning spread range, while the shallow leaf-margin incision treatment showed the mildest browning symptoms. By the 30th day of culture, both the single leaf-margin incision and the “mid-leaf incision followed by leaf-margin incision” treatments formed clustered calli at the leaf margins (Figure 10(B-1,B-2)); the transverse incision treatment only produced a small amount of scattered spherical calli at the wound sites; for the combined mid-leaf + leaf-margin incision treatment, no calli differentiated at the mid-leaf wound, and new buds only germinated at the leaf-margin incision sites (Figure 10(C-2)). Based on comprehensive phenotypic performance, including browning severity and callus-formation characteristics, the single leaf-margin incision was selected as the preferable wounding treatment for leaf explants of *R. decorum*.

Table 12. Effects of different wounding treatments on the growth of in vitro-derived leaf explants of *R. decorum*.

Treatment	Browning Rate (%)	Green Leaf Rate (%)	Callus-Derived Bud Induction Rate (%)
Transverse mid-leaf incision	70 ± 15.28 a	30.00 ± 15.28 a	6.67 ± 6.67 a
Combined mid-leaf and leaf-margin incision	90 ± 4.08 a	10.00 ± 4.08 a	6.67 ± 4.08 a
Leaf-margin incision	76.67 ± 8.50 a	23.33 ± 8.50 a	20.00 ± 6.23 a

Note: Data in the table represent the mean ± standard error of three replicates for the same treatment; different lowercase letters in the table indicate significant differences ($p < 0.05$).

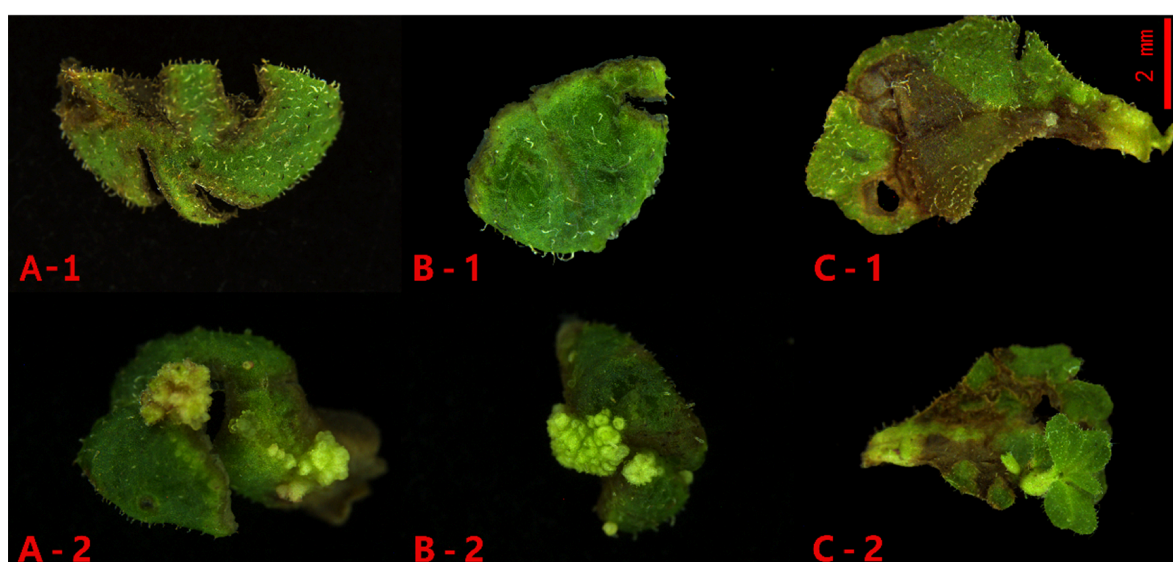


Figure 10. Effects of different wounding treatments on the growth of in vitro-derived leaf explants of *R. decorum*. Note: (A) Leaf-margin incision ((A-1) 15 days of culture; (A-2) 30 days of culture); (B) transverse mid-leaf incision ((B-1) 15 days of culture; (B-2) 30 days of culture); (C) combined mid-leaf and leaf-margin incision ((C-1) 15 days of culture; (C-2) 30 days of culture). Scale bars: 2 mm.

3.6.3. Effects of Different Cytokinins on Responses of Leaf Explants

The type and concentration of cytokinins exert a decisive regulatory effect on leaf regeneration. In this study, experiments were conducted by supplementing the culture medium with different concentrations of TDZ and ZT, and the results are presented in Table 13. The three evaluated traits, namely browning rate, green leaf rate, and callus-derived bud induction rate, responded differently to cytokinin treatments, and no single treatment performed best across all three parameters. Both cytokinins exhibited certain regulatory effects on the degree of leaf browning: the 0.02 mg·L⁻¹ TDZ treatment group showed the most severe leaf browning. As the TDZ concentration increased, the browning rate first decreased and then increased, with the 0.3 mg·L⁻¹ TDZ treatment group showing a relatively low browning rate among all TDZ treatments; the browning rate rebounded when the concentration rose to 0.5 mg·L⁻¹. The 1 mg·L⁻¹ ZT treatment group yielded the lowest browning rate across all treatments, which was significantly different from that of the 0.02 mg·L⁻¹ TDZ treatment group. For green leaf retention, the 1 mg·L⁻¹ ZT group achieved the numerically highest green leaf rate. In terms of callus-derived bud regeneration efficiency, the 0.3 mg·L⁻¹ TDZ treatment produced the significantly highest bud induction rate of 36.67%, even though its browning and green leaf performance were not the best among all groups. By comprehensive trade-off considering browning rate, green leaf rate, and bud-induction performance together, 0.3 mg·L⁻¹ TDZ was selected as the preferable cytokinin concentration for bud induction from *R. decorum* leaf explants. The growth dynamics of leaves treated with 0.3 mg·L⁻¹ TDZ are illustrated in Figure 11: At 10 dpi, the leaf tissue at the incision sites exhibited a faded color, with localized blackish-brown discoloration; at 30 dpi, browning spread from the incision sites to the entire leaf blade in some samples, while others showed only localized browning, and a subset of leaves had already differentiated into calli and new buds; at 90 dpi, the calli on the leaves differentiated into bud clusters, whereas some of the initially inoculated maternal leaves showed browning during the culture period.

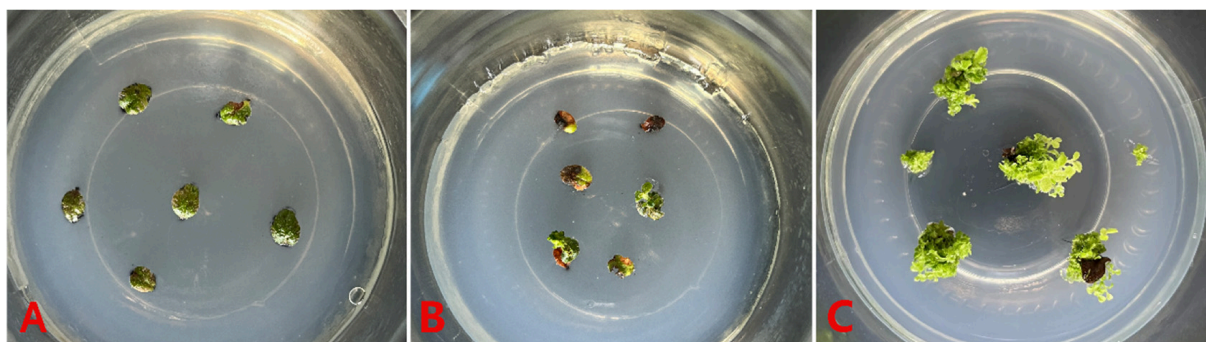


Figure 11. Growth of in vitro-derived leaf explants of *R. decorum* in the bottle at different times. Note: (A) leaf inoculation after 10 days; (B) leaf inoculation after 30 days; (C) leaf inoculation after 90 days. The plant hormone in the medium was 0.3 mg·L⁻¹ TDZ + 0.01 mg·L⁻¹ NAA.

Table 13. Effects of different concentrations of TDZ and ZT on the growth of in vitro-derived leaf explants of *R. decorum*.

TDZ Concentration (mg·L ⁻¹)	ZT Concentration (mg·L ⁻¹)	Browning Rate (%)	Green Leaf Rate (%)	Callus-Derived Bud Induction Rate (%)
0.02		93.33 ± 4.08 c	6.67 ± 4.08 a	6.67 ± 4.08 a
0.1		76.67 ± 4.08 abc	23.33 ± 4.08 abc	13.33 ± 3.33 a
0.3		60.00 ± 11.30 ab	40.00 ± 11.30 bc	36.67 ± 12.25 b
0.5		86.67 ± 8.16 bc	13.33 ± 8.17 ab	13.33 ± 8.17 a

Table 13. Cont.

TDZ Concentration (mg·L ⁻¹)	ZT Concentration (mg·L ⁻¹)	Browning Rate (%)	Green Leaf Rate (%)	Callus-Derived Bud Induction Rate (%)
	0.5	66.67 ± 5.27 abc	33.33 ± 5.27 abc	16.67 ± 5.27 ab
	1	56.67 ± 12.47 a	43.33 ± 12.47 c	23.33 ± 6.67 ab
	2	73.33 ± 8.50 abc	26.67 ± 8.50 ab	20.00 ± 6.24 ab
	3	80.00 ± 9.72 abc	20.00 ± 9.72 abc	10.00 ± 4.08 a

Note: Data in the table represent the mean ± standard error of three replicates for the same treatment; different lowercase letters in the table indicate significant differences ($p < 0.05$).

4. Discussion

Explant browning and microbial contamination represent core technical bottlenecks limiting the establishment of efficient rapid propagation systems for woody plant tissue culture. The selection of suitable disinfectant combinations is a critical strategy to reduce browning incidence, inhibit contamination spread, and promote explant bud differentiation. The type of disinfectant and treatment duration should be flexibly adjusted according to the physiological state and tissue type of explants, as different materials exhibit significant variations in their responses to disinfection conditions [28,29]. In the tissue culture system of *Musa* spp., explant browning displays distinct stage specificity: during the primary culture and subculture proliferation stages, browning primarily occurs at explant cutting wounds; in the cluster bud proliferation and rooting induction stages, however, browning is more prevalent in young leaf tissues, and severe cases can lead to shoot necrosis and the death of tissue culture plantlets [30]. When walnut stem segments were treated with 0.1% HgCl₂ for 8 min followed by 14 days of culture, explants exhibited severe browning with a survival rate of only 38.7% [31]. The underlying cause of these phenomena is the enzymatic oxidation of phenolic compounds in plants [32].

As a representative alpine woody ornamental plant, *R. decorum* faces a high risk of browning throughout the entire tissue culture cycle. Effective prevention and control of browning is a core step to ensure stable bud emergence of explants and accelerate the rapid propagation process. Previous studies have shown that both 0.1% HgCl₂ and 10% NaClO can induce the initiation of bud differentiation in explants such as leaf buds, flower buds, sepals, and stem segments of *R. decorum*. However, young stem and leaf tissues are highly sensitive to disinfection duration: prolonged treatment tends to cause severe browning, while shortened treatment fails to effectively inhibit microbial contamination [33]. This pattern is largely consistent with the experimental results of HgCl₂ and NaClO treatments with different durations in this study. This study employed a combined disinfection protocol involving KMnO₄, NaClO, and H₂O₂ to treat young stem segment and leaf explants of *R. decorum*. Results indicated that the preferable sterilization regime for stem segments was sequential treatment with 1% KMnO₄ for 10 min followed by 10% H₂O₂ for 4 min—this combination exhibited a significantly lower browning-inducing effect compared to HgCl₂ treatment. For leaf explants, the most favorable disinfection outcome was achieved with 1% KMnO₄ for 5 min and 10% H₂O₂ for 3 min. A possible underlying mechanism is speculated as follows: Strongly oxidizing KMnO₄ and NaClO may undergo redox reactions with reductive H₂O₂, which might mitigate the toxic effects of oxidative or reductive stress imposed by single disinfectants on plant tissues. Notably, when the NaClO treatment duration was extended, its efficacy in controlling browning and contamination was significantly inferior to that of KMnO₄; this discrepancy is speculated to be attributed to the lower tolerance of *R. decorum* explants to NaClO relative to KMnO₄. In summary, the combined disinfection protocol of KMnO₄ and H₂O₂ represents the preferable choice for young stem segment and leaf explants of *R. decorum*.

Beyond the selection of appropriate disinfectants, the basal medium and supplemented plant hormones are equally critical. In this study, WPM medium was employed as the basal medium throughout the entire culture process. For *Vaccinium corymbosum*, WPM medium yielded the highest number of buds, with buds exceeding 10 mm in length [34]—consistent with the basal medium used for establishing rapid propagation systems of *R. delavayi* and *R. fortunei*.

The success of in vitro regeneration hinges on exogenous plant growth regulators, which reprogram the developmental fate of explant cells toward differentiation capacity, thereby enabling subsequent organogenesis or somatic embryogenesis. TDZ is a potent regulator of in vitro morphogenesis, particularly for woody plants—prior studies have identified TDZ as the optimal trigger for morphogenesis in explant cultures of mulberry [35] and *Camellia oleifera* Abel [36]. TDZ is also widely employed as a cytokinin in *Rhododendron* stem segment and leaf cultures: for *R. mucronulatum* Turcz., leaves treated with $0.12 \text{ mg} \cdot \text{L}^{-1}$ TDZ under dark conditions achieved a maximum callus-derived bud induction rate of 75% [37]. In this study, TDZ was selected as the cytokinin for inducing bud formation in stem segments of *R. decorum* explants, yielding favorable outcomes: the bud induction rate reached 66.67% at $3 \text{ mg} \cdot \text{L}^{-1}$ TDZ, with bud primordia swelling within 10–15 days. For leaf calli, treatment with $0.1 \text{ mg} \cdot \text{L}^{-1}$ TDZ resulted in an optimal bud formation effect, confirming its concentration specificity for callus-derived bud differentiation in *R. decorum*; excessive TDZ triggered browning and inhibited regeneration, while suboptimal concentrations failed to activate bud primordia. This concentration-dependent regulatory pattern was further validated in a study on *Paeonia ostii* 'Fengdan' [38], where TDZ concentrations exceeding $0.5 \text{ mg} \cdot \text{L}^{-1}$ significantly elevated the browning rate of woody plant tissue culture explants and suppressed bud differentiation capacity.

During the subculture stage, this study concurrently evaluated the effects of different concentration combinations of TDZ, ZT and NAA on the proliferation and growth of *R. decorum* in vitro plantlets. Results indicated that compared with all ZT-NAA combinations, the TDZ-based complex systems exhibited superior proliferation efficiency; however, growth-related traits of the tissue culture plantlets—including plant height, leaf expansion, and root development—were significantly inferior to those in the ZT-regulated groups. For leaf explants, the combination of $1 \text{ mg} \cdot \text{L}^{-1}$ ZT and $0.3 \text{ mg} \cdot \text{L}^{-1}$ TDZ showed better browning control than other treatments, yet TDZ yielded a higher adventitious bud induction rate than ZT. This discrepancy essentially reflects the physiological specificity of different cytokinins: TDZ prioritizes the induction of bud primordium differentiation to enhance the proliferation coefficient, whereas ZT exerts a more pronounced promoting effect on robust bud growth and vegetative organ development. This regulatory pattern is consistent with findings in related plant studies: Research on *R. delavayi* demonstrated that the $0.08 \text{ mg} \cdot \text{L}^{-1}$ TDZ + $0.01 \text{ mg} \cdot \text{L}^{-1}$ NAA combination significantly improved regeneration capacity compared to the $0.01 \text{ mg} \cdot \text{L}^{-1}$ NAA single treatment, with endogenous ZT content peaking during the critical leaf shoot-induction period (15–22 d) [39]—further revealing the synergistic regulatory mechanism of exogenous TDZ and endogenous ZT on bud differentiation. Additionally, a tissue culture study on *Eugenia involucrate* confirmed that combined TDZ-NAA application significantly promoted bud differentiation and clustered bud formation [40]. Together, these findings validate the critical regulatory role of cytokinin–auxin crosstalk in the subculture proliferation of woody plants. Elevating the TDZ concentration to $1 \text{ mg} \cdot \text{L}^{-1}$ resulted in excessive bud differentiation density, which not only increased the difficulty of bud count quantification but also induced stunted growth of tissue culture plantlets; in severe cases, browning and necrosis were observed. Analogously, in tissue culture of ancient *Acer truncatum* trees [41], it was reported that while high TDZ concentrations maintained a certain level of differentiation capacity, they led

to weakened growth of in vitro plantlets—consistent with the findings of this study. Low concentrations of TDZ enhance the proliferation coefficient by activating the expression of cell division-related genes, whereas an optimal concentration of NAA balances bud differentiation and growth, thereby preventing developmental imbalance induced by a single hormone. This regulatory mechanism was also validated in a tissue culture study of *Acer negundo* ‘Aurea’ [42]. For plants exhibiting stunted growth, the application of GA₃ that promotes internode elongation and enhances leaf and bud development has been shown to mitigate slow propagation [43]. In this study, exogenous GA₃ tended to stimulate internode elongation of *Rhododendron* plantlets based on morphological observations from Figures 4 and 5. Compared with the GA₃-free control, a trend of increased stem thickness and larger leaf size was also observed, although these phenotypic changes were not supported by quantitative statistical data.

Precise screening of hormone types and concentrations in rooting media is a core step for enhancing rooting efficiency and shortening the rapid propagation cycle of woody plant tissue culture plantlets—a conclusion widely validated in tissue culture studies of *Rhododendron* and other ornamental woody plants. For the new ornamental crabapple cultivar *Malus* ‘Baiyun’, either a single treatment with 0.5 mg·L^{−1} NAA or a combined treatment with 0.6 mg·L^{−1} NAA and 0.7 mg·L^{−1} IBA achieved a 100% rooting rate; notably, the combined treatment also induced robust lateral roots, significantly optimizing root system quality, which fully demonstrates the promoting effect of appropriate hormone combinations and concentrations on woody plant rooting [44]. In this study, a single application of 0.8 mg·L^{−1} NAA resulted in a rooting rate of 46.67% for *R. decorum* tissue culture plantlets, which is comparable to the 45% rooting rate reported under the combined treatment of 0.5 mg·L^{−1} NAA + 3 mg·L^{−1} IBA [33]. It is worth noting that the combined treatment of 2 mg·L^{−1} 2iP and 2 mg·L^{−1} IBA increased the rooting rate of *R. decorum* tissue culture plantlets to 64%, with root length and root number significantly superior to the 2 mg·L^{−1} 2iP + 0.8 mg·L^{−1} NAA combination in this study [45]. In contrast, the introduction of 2iP in the present experiment did not exert a positive regulatory effect on rooting. This discrepancy is speculated to be closely related to the synergistic effects of hormone ratios and concentrations: the relatively low NAA concentration in this study failed to form effective hormone signal complementarity when paired with 2iP, thereby precluding the efficient activation of downstream rooting-related gene expression. Rooting induction is a slow-response process rather than rapid vegetative growth. Sufficient nutrients remained in the medium over the 90-day culture period to support rooting performance, consistent with existing published protocols for related *Rhododendron* and *Vaccinium* species, in which rooting cultures lasted 8–12 weeks without medium renewal [46,47].

Leaf explants are ideal for plant rapid propagation due to their ease of acquisition and high proliferation coefficient. However, leaf explants of *R. decorum* are prone to chlorosis, browning, and death after in vitro isolation, severely limiting regeneration efficiency. Polyphenolic oxidation triggered by tissue injury is the primary driver of browning in woody Ericaceae leaf explants, and genotypic differences strongly determine the tolerance to oxidative stress [48]. To address this challenge, the present study explored strategies to minimize browning and optimize adventitious bud induction by evaluating three key factors: leaf selection, wounding method, and hormone induction.

Results indicated that apical young leaves exhibited the lowest browning incidence. This finding is consistent with a report on *R. delavayi* var. *delavayi*, where the two fully expanded leaves immediately below the apex were identified as optimal [49]. Juvenile apical leaves maintain higher cellular activity and lower phenolic-compound accumulation compared with older basal leaves, which partly explains their improved performance [50]. But it cannot be generalized as a genus-wide fixed pattern, because the optimal leaf position

varies across genotypes and culture conditions. This confirms a genus-wide pattern of leaf sampling position influencing browning and regeneration in *Rhododendron*.

Wounding method and location had significant effects, likely reflecting differential differentiation potential across leaf regions. Mechanical wounding activates cell reprogramming but simultaneously accelerates phenolic exudation and browning responses [51]. The present study found that the leaf margin supported higher adventitious bud induction rates with controllable browning; slight wounding of the margin while preserving leaf integrity effectively mitigated browning, highlighting species-specific leaf regeneration traits in woody plants. Leaf marginal tissues contain more morphogenetically competent cells in many rhododendron genotypes, whereas heavy central-lamina injury aggravated oxidative browning and reduced regeneration potential, which agrees with previous observations on related evergreen rhododendrons [52].

Hormone screening demonstrated that TDZ-NAA combinations were far more effective for leaf-explant regeneration than ZT-NAA combinations in *R. decorum*. TDZ is widely recognized as a highly potent cytokinin for triggering organogenesis in recalcitrant woody *Rhododendron* species, frequently outperforming zeatin-based treatments, though excessive TDZ concentrations aggravate callus overgrowth and browning risk [38]. Notably, leaf explants of *R. decorum* could generate adventitious buds either directly or via callus differentiation—distinct from reports where explant leaves only produce buds indirectly. This discrepancy is hypothesized to relate to TDZ concentration, the compactness of leaf parenchyma tissue, and wounding method, though the underlying mechanisms require further investigation.

5. Conclusions

This study mainly optimized three key links of the *R. decorum* tissue-culture system: explant disinfection, adventitious-bud induction from leaf explants, and rooting culture. The optimized protocol improved explant survival rate and reduced browning incidence, and achieved stable adventitious-bud regeneration. Owing to differences in starting materials and culture parameters in previous reports, direct comparison of total culture duration with earlier published protocols is not feasible. This study provides critical technical insights for establishing an in vitro culture system using stem segments, intact leaves, and leaf explants of *R. decorum*. By optimizing contamination prevention and control strategies, hormone selection and concentration regulation, and incision methodologies, the bud induction rate and bud differentiation quality were effectively improved, while browning and contamination issues during tissue culture were significantly mitigated. In primary culture, the combined disinfection regime of 1% KMnO₄ and 10% H₂O₂ achieved better survival performance than HgCl₂ treatment. Suitable TDZ-NAA hormone combinations were screened for stem-segment proliferation, leaf-explant adventitious-bud regeneration, and in vitro-leaf induction. Auxin–cytokinin combinations of NAA and 2iP improved rooting performance, although root length remained limited. Key limitations include unclear mechanisms underlying slow subculture growth and poor in vitro rooting, absence of ex vitro rooting and multi-hormone synergy research, as well as limited mechanistic understanding of hormone-mediated leaf regeneration. Future work should analyze dynamic changes during bud formation via histology, endogenous hormone profiling, and browning-related enzyme assays, screen effective anti-browning agents, and explore the molecular mechanisms related to winter-flowering characteristics.

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Informed Consent Statement: The species of *Rhododendron decorum* does not fall within the category of endangered wild plant species, and the collection of materials is in accordance with both Chinese and international guidelines. The authors obtained the consent of the landowner to collect materials.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

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