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Huayun He

National Forestry and Grassland Engineering Technology Research Center for *Rosa Roxburghii*, Guizhou University

Wei Yang

National Forestry and Grassland Engineering Technology Research Center for *Rosa Roxburghii*, Guizhou University

Wentao Ma

National Forestry and Grassland Engineering Technology Research Center for *Rosa Roxburghii*, Guizhou University

Hong Nan

National Forestry and Grassland Engineering Technology Research Center for *Rosa Roxburghii*, Guizhou University

Min Lu

National Forestry and Grassland Engineering Technology Research Center for *Rosa Roxburghii*, Guizhou University

Huaming An

hman@gzu.edu.cn

National Forestry and Grassland Engineering Technology Research Center for *Rosa Roxburghii*, Guizhou University

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Establishment of a Regeneration System from Hypocotyls of *Rosa roxburghii* Tratt.

Huayun He¹, Wei Yang¹, Wentao Ma^{1,2}, Hong Nan^{1,2}, Min Lu^{1,2}, Huaming An^{1,2*}

¹National Forestry and Grassland Engineering Technology Research Center for *Rosa Roxburghii*, Agricultural College, Guizhou University, Guiyang 550025, People's Republic of China

²Guizhou Technical Innovation Center of Cili Industry, Guiyang 550025, P.R. China

*Author for correspondence: hman@gzu.edu.cn

Abstract

Rosa roxburghii Tratt. is an important horticultural crop with significant commercial value due to its nutritional and medicinal properties. However, as a recalcitrant species, it imposes severe limitations on plant regeneration, thereby restricting the application of genetic transformation techniques. Therefore, there is an urgent need to develop an efficient whole-plant regeneration system for *R. roxburghii*. In this study, complete plantlets derived from hypocotyl explants were successfully regenerated for the first time. The regeneration process consisted of callus induction, adventitious shoot induction, adventitious root induction, and plantlet transplantation. Initially, hypocotyl explants of the cultivar 'Guinong 5' were inoculated onto NN69 medium supplemented with 0.05 mg·L⁻¹ 6-BA and 2.0 mg·L⁻¹ TDZ, yielding a callus induction rate of 83.70% and an adventitious shoot induction rate of 10.62%. The induced calli were then transferred to NN69 medium containing 0.1 mg·L⁻¹ 6-BA, 4.0 mg·L⁻¹ TDZ, and 2.0 mg·L⁻¹ ZT for adventitious shoot differentiation, achieving a shoot induction rate of 17.33%. Subsequently, the adventitious shoots were cultured on half-strength MS medium supplemented with 0.3 mg·L⁻¹ IBA, 300 mg·L⁻¹ activated charcoal, and 30 g·L⁻¹ glucose for root induction. The rooting rate reached 56.67%, with an average of 3.2 roots per shoot. Following acclimatization and transplantation into substrate, a total of 21 healthy plantlets were obtained. This study provides both a theoretical and technical foundation for the future development of genetic transformation systems in this rose species.

Keywords *Rosa roxburghii* Tratt., Hypocotyl, Tissue culture, Callus, Regeneration

Introduction

Rosa roxburghii Tratt., belonging to the family Rosaceae, is a fruit crop of considerable economic importance in Southwest China, particularly in Guizhou Province, where the predominant cultivar 'Guinong 5' is widely cultivated (Wu, 2023). The fruit of *R. roxburghii* is distinguished by its exceptionally high content

of L-ascorbic acid (AsA, vitamin C), which functions not only as an essential micronutrient for human health but also as a potent bioactive compound with free radical-scavenging, antioxidant, and anti-aging properties (Liu, 2023). Owing to its remarkable nutritional, economic, and ecological value, *R. roxburghii* has emerged as a pillar of regional specialty agriculture, contributing substantially to rural economic development.

The development of tissue culture protocols for *R. roxburghii* commenced in the 1980s, with two independent groups reporting preliminary results concurrently (Xie, 1986; Gao et al., 1986); these early efforts were subsequently refined (Gao et al., 1991). In a more advanced attempt, Wen (2003) successfully induced adventitious shoots from hypocotyl explants cultured on a modified MS medium supplemented with 6-BA, NAA, GA₃, sucrose, and agar. Critically, however, all adventitious shoots originated directly from epidermal cells of the explant without intervening callus formation, and no shoot differentiation was observed from callus tissue, indicating that genuine in vitro plant regeneration via indirect organogenesis remained unrealized.

Subsequent investigations explored a broader spectrum of explant types and genotypes. Studies employing anthers as explants characterized the physiological and biochemical dynamics of callus cultures and examined factors contributing to tissue browning, alongside efforts to culture unfertilized ovules; however, plant regeneration was not achieved through any of these approaches (Chen and Zhang, 2008a, 2008b; Zhang and Chen, 2009; Zhang, 2009). In a complementary line of research, callus induction systems developed from leaf and fruit explants provided a platform for cell-culture-based production of bioactive secondary metabolites (Fang et al., 2019; Fang, 2020). Building on this foundation, Li and Chen (2022) conducted a comparative evaluation of callus induction frequency and browning inhibition efficacy across leaf explants from four *R. roxburghii* genotypes ('Guinong 1', 'Guinong 2', 'Guinong 5', and 'Guinong 7').

With respect to in vitro clonal propagation, micropropagation systems for seedless *R. roxburghii* have been systematically established through axillary shoot proliferation and tender stem segment culture (Wei et al., 2007; Tang et al., 2015; Zhao et al., 2015; Li et al., 2016; Jiang et al., 2017). Furthermore, Wu et al. (2023a, 2023b) developed a stem segment-based rapid propagation protocol for thornless germplasm using vegetative shoots of Wuci 1 and Wuci 2, providing critical technical support for the propagation of thornless cultivars. Despite these advances in micropropagation, a comprehensive review of the literature reveals that successful plant regeneration in *R. roxburghii* via indirect organogenesis—i.e., the pathway involving explant dedifferentiation into callus followed by redifferentiation of adventitious shoots from callus—has not been reported to date.

Concurrently, research into *R. roxburghii* has increasingly focused on the molecular mechanisms

underlying fruit quality formation and regulation, driven by evolving market demands and industrial priorities. Notable progress includes the elucidation of the AsA biosynthetic pathway and the functional characterization of key regulatory genes such as *GGP* (An et al., 2015; Yang, 2025; Huang, 2025). However, *R. roxburghii* is a perennial woody shrub with a protracted juvenile phase, and the absence of an established regeneration system via indirect organogenesis has severely constrained the application of gene editing and genetic transformation technologies for functional genomics studies. These technical barriers have, in turn, impeded in-depth molecular dissection of the regulatory networks governing fruit quality traits. To address this critical bottleneck, the present study aims to establish, for the first time, a complete plant regeneration system for *R. roxburghii* via indirect organogenesis from hypocotyl explants, thereby laying a reliable technical foundation for future genetic improvement and molecular breeding of this economically important species.

Materials and methods

Plant material

The hypocotyl explants were sourced from seedlings germinated from cold-stored seeds harvested in the current year; cotyledons and roots were excised beforehand.

Media preparation

In this experiment, four basal media were employed: WPM, DKW, QL, and NN69. According to the requirements of different culture stages and following the manufacturer's instructions, the appropriate amount of each medium powder was accurately weighed and dissolved in distilled water together with sucrose at a concentration of 30 g·L⁻¹. Subsequently, the 6-BA (0.1 mg·L⁻¹) and 2,4-D (1.5 mg·L⁻¹) were added using a micropipette. After thorough mixing, the solution was brought to final volume, and its pH was measured with a pH meter. The pH was then adjusted to 5.8–6.0 using HCl or NaOH. Finally, agar was added at 7 g·L⁻¹, and the mixture was heated to boiling until the agar was completely dissolved. The medium was then dispensed into glass bottles and sterilized by autoclaving at 121 °C for 20–25 min. After sterilization, the media were transferred to a superclean bench to cool prior to use.

Disinfection of explants

Seedlings of *R. roxburghii* germinated from cold-stored seeds were retrieved, and residual fine sand was gently rinsed off with clean water. The seedlings were placed in tissue culture vessels, sprayed with ethanol for surface disinfection, and then transferred to superclean bench. Surface disinfection was performed by immersion in 75% (v/v) ethanol for 1 min, followed by three rinses with sterile distilled water. The seedlings were subsequently immersed in 2% (v/v) sodium hypochlorite solution for 4 min, followed by four to five

rinses with sterile distilled water. The disinfected seedlings were temporarily held in sterile containers until further use..

Effects of different basal media on the induction of hypocotyl callus

In a superclean bench, the cotyledons and root portions were excised using a scalpel, and the remaining hypocotyls were cut into segments approximately 1 cm in length. The hypocotyl segments were inoculated onto WPM, DKW, QL, and NN69 basal media and subjected to dark culture. For each treatment, 120 explants were inoculated, and the experiment was repeated three times. After 28 days, the callus induction rate was recorded, and the optimal basal medium for callus induction was selected based on the time of callus emergence and callus growth status.

Effects of different plant growth regulator combinations on the induction of hypocotyl callus

Hypocotyls were used as explants and inoculated onto the previously selected optimal basal medium supplemented with different types and concentrations of plant growth regulators (PGRs). The cultures were maintained in the dark. For each treatment, 120 explants were inoculated, and the experiment was replicated three times. After 45 days, the callus induction rate and adventitious shoot differentiation rate were recorded. Based on the number of explants producing callus and adventitious shoots, the optimal medium for hypocotyl callus formation and adventitious shoot differentiation in *R. roxburghii* was determined.

Effects of different PGR concentrations ratios on the induction of hypocotyl callus and differentiation of adventitious shoot

Hypocotyls were used as explants and inoculated onto the previously selected optimal basal medium supplemented with varying concentrations of PGRs. In one set of treatments, the original PGR ratio was maintained while the concentrations of all PGRs were reduced by half. In another set of treatments, the PGR concentrations were increased: 6-BA and IAA were each raised to 0.2 mg·L⁻¹ and TDZ to 6.0 mg·L⁻¹; in a further treatment, 6-BA and IAA were increased to 0.5 mg·L⁻¹ and TDZ to 15.0 mg·L⁻¹. The cultures were maintained in the dark. For each treatment, 100 explants were inoculated, and the experiment was replicated three times. After 45 days, the callus induction rate and adventitious shoot differentiation rate were recorded. Based on the amount of callus produced and the number of adventitious shoots regenerated, the optimal medium composition that significantly enhanced hypocotyl callus induction and adventitious shoot differentiation was determined.

Effects of different genotypes and explant types on the differentiation of adventitious shoots

Hypocotyls of the cultivars 'Guinong 5' and Wuci 1 were inoculated onto the optimal induction medium

selected from the preceding experiments and maintained under dark culture conditions.

Leaf and hypocotyl explants of *R. roxburghii* cultivar 'Guinong 5' were inoculated onto the optimal induction medium selected from the preceding experiments and maintained under dark culture conditions.

For each explant type and genotype, 100 explants were inoculated per treatment, and the experiment was replicated three times. After 45 days, the callus induction rate and adventitious shoot differentiation rate were recorded. Based on the number of explants producing callus and adventitious shoots, the differential responses of distinct explant sources to callus formation and shoot regeneration in *R. roxburghii* were comparatively evaluated.

Effects of different PGRs on adventitious shoot induction from hypocotyl callus

One-month-old hypocotyl-derived callus was used as explant material. The basal medium consisted of NN69 medium supplemented with $0.1 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $4.0 \text{ mg} \cdot \text{L}^{-1}$ TDZ. To determine the optimal PGRs for adventitious shoot induction from hypocotyl-derived callus, three treatments were applied by individually adding $2.0 \text{ mg} \cdot \text{L}^{-1}$ KT, $2.0 \text{ mg} \cdot \text{L}^{-1}$ ZT, or $1.5 \text{ mg} \cdot \text{L}^{-1}$ GA₃ to the basal medium. All cultures were maintained in darkness. For each treatment, 50 explants were inoculated, and the experiment was repeated three times. After 28 days, callus morphology was observed, and the frequency of adventitious shoot differentiation was recorded.

Effects of ZT concentration on adventitious shoot induction from hypocotyl callus

Based on the preliminary hormone screening, one-month-old hypocotyl-derived callus was used as experimental material. To further determine the optimal concentration of ZT, a gradient of 0, 0.5, 1.0, 1.5, 2.0, and $2.5 \text{ mg} \cdot \text{L}^{-1}$ was established. All cultures were maintained in darkness. For each treatment, 50 explants were inoculated, and the experiment was repeated three times. After 28 days, callus morphology was observed, and the frequency of adventitious shoot differentiation was recorded to evaluate the effect of different ZT concentrations on shoot regeneration.

Screening of the optimal medium for adventitious shoot rooting

Our preliminary experiments showed that tissue-cultured shoots derived from stem segment rapid propagation achieved a 100% rooting rate on MS medium supplemented with $0.5 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $0.05 \text{ mg} \cdot \text{L}^{-1}$ IAA. Based on this result, the same hormone combination was adopted as the foundation for the present investigation. This experiment aimed to evaluate the effects of different basal medium types and agar concentrations on adventitious shoot rooting. Vigorous adventitious shoots approximately 2 cm in height were selected and inoculated onto two basal media: MS and half-strength MS (1/2 MS). For each medium type,

three agar concentrations were tested: 5, 6, and 7 g·L⁻¹. Following the method of Wen (2003), glucose and 300 mg·L⁻¹ activated charcoal were added to the rooting media; additionally, 0.3 mg·L⁻¹ IBA was incorporated according to the protocol of Wu (2023) (Table 1). For each treatment, ten strong shoots were inoculated, and the experiment was repeated three times. Rooting progress was monitored periodically during the culture period. After 28 days, the rooting rate and the average number of roots per shoot were recorded.

Table 1 Medium for rooting adventitious shoots from hypocotyl of 'Guinong 5' *R. roxburghii*

Number	Medium
1	MS + 6-BA (0.5 mg·L ⁻¹) + IAA (0.05 mg·L ⁻¹) + sucrose (30 g·L ⁻¹) + agar (5、6、7 g·L ⁻¹)
2	1/2MS + 6-BA (0.5 mg·L ⁻¹) + IAA (0.05 mg·L ⁻¹) + sucrose (30 g·L ⁻¹) + agar (5、6、7 g·L ⁻¹)
3	1/2MS + 6-BA (0.5 mg·L ⁻¹) + IAA (0.05 mg·L ⁻¹) + activated charcoal (300 mg·L ⁻¹) + glucose (30 g·L ⁻¹) + agar (6 g·L ⁻¹)
4	1/2MS + IBA (0.3 mg·L ⁻¹) + activated charcoal (300 mg·L ⁻¹) + glucose (30 g·L ⁻¹) + agar (6 g·L ⁻¹)

Statistical Analysis

Data were analyzed by one-way ANOVA using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant. The calculation formula was as follows:

$$\text{Callus induction rate (\%)} = \frac{\text{Number of explants producing callus}}{\text{Total number of explants inoculated}} \times 100\%$$

$$\text{Adventitious shoot induction rate (\%)} = \frac{\text{Number of adventitious shoots induced}}{\text{Total number of explants inoculated}} \times 100\%$$

$$\text{Rooting rate (\%)} = \frac{\text{Number of shoots forming roots}}{\text{Total number of shoots inoculated}} \times 100\%$$

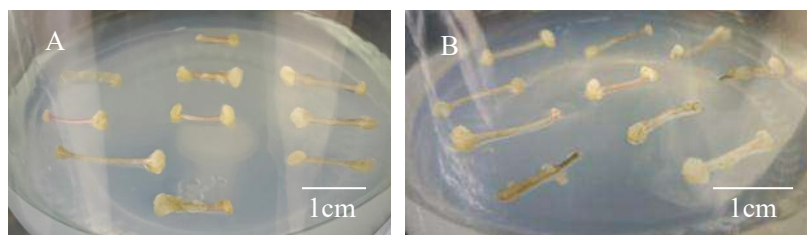
$$\text{Average number of roots per shoot} = \frac{\text{Total number of roots formed}}{\text{Number of rooted shoots}}$$

Results

Effects of different basal media on the induction of hypocotyl callus

Significant differences were observed among basal media in their effects on callus induction from hypocotyl explants of *R. Roxburghii*. No callus formation was observed on either WPM or DKW medium. In contrast, QL medium induced pale yellow callus (Fig. 1B), with an induction percentage of 77.70%. Notably, NN69 medium produced uniformly growing callus (Fig. 1A) and achieved a significantly higher induction percentage of 88.37% compared with QL medium. Based on these findings, NN69 was determined to be the

173 optimal basal medium for hypocotyl callus induction in *R. roxburghii* under the present experimental
 174 conditions.



175 **Fig.1 Callus induced from hypocotyl of *R. roxburghii*.** A: Hypocotyl callus of *R. roxburghii* Tratt. cultured on NN69
 176 medium. B: Hypocotyl callus of *R. roxburghii* cultured on QL medium.

177 **Effects of different PGR combinations on the induction of hypocotyl callus**

178 Different combinations of PGRs had varying effects on callus induction from hypocotyls of *R. roxburghii*
 179 (Table 2). Among them, adventitious shoot differentiation from callus was clearly observed on two media with
 180 specific PGR combinations (Fig. 2K, L). When KT was added to medium 1 (medium 6), the callus induction
 181 rate was higher than that of medium 1.

182 Comparison of media 1, 2, and 3 revealed that when 6-BA was used as the synergistic hormone, the
 183 induction rate with NAA was higher than that with IAA; the induction rate with TDZ was higher than that
 184 with NAA, and adventitious shoots were produced (Fig. 2A), with an induction rate of 6.84%. Early
 185 adventitious shoots (Fig. 2D) and light green, light yellow, and white shoot primordia (Fig. 2B, C) were
 186 observed on the induced callus.

187 Comparison of media 3, 4, and 5 showed that when TDZ was used as the synergistic hormone, the
 188 induction rate with IAA was higher than that with NAA, and adventitious shoots appeared (Fig. 2F), with an
 189 induction rate of 5.71%. Early adventitious shoots (Fig. 2G, I) and light green and white shoot primordia (Fig.
 190 2H, I) were visible on the callus. In contrast, the difference in callus induction rate between the medium with
 191 6-BA and that with IAA was not significant, and their adventitious shoot induction rates were also similar.

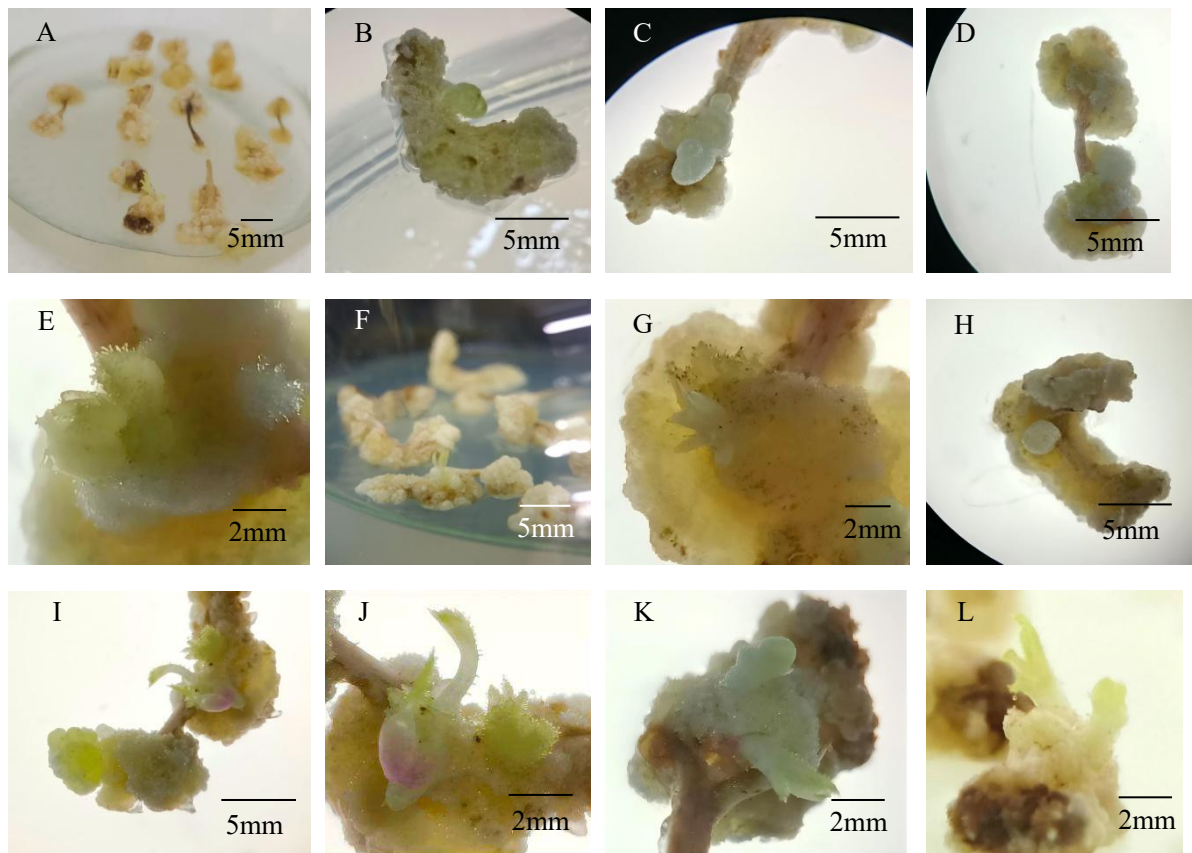
192 Therefore, the PGR combinations suitable for callus induction from *R. roxburghii* hypocotyls were
 193 determined in this experiment to be $0.1 \text{ mg} \cdot \text{L}^{-1}$ 6-BA + $4.0 \text{ mg} \cdot \text{L}^{-1}$ TDZ and $0.1 \text{ mg} \cdot \text{L}^{-1}$ IAA + $4.0 \text{ mg} \cdot \text{L}^{-1}$
 194 TDZ.

195

196 **Table 2 Effects of different PGR combinations on callus induction from hypocotyl of *R. roxburghii***

Number	PGR combination	Callus induction rate (%)	Adventitious shoot induction rate (%)
1	1.0 mg·L ⁻¹ 6-BA + 0.5 mg·L ⁻¹ IAA	75.64 ± 2.92 ^c	0
2	1.0 mg·L ⁻¹ 6-BA + 0.5 mg·L ⁻¹ NAA	89.54 ± 3.09 ^b	0
3	0.1 mg·L ⁻¹ 6-BA + 4.0 mg·L ⁻¹ TDZ	98.69 ± 0.83 ^a	6.84
4	0.1 mg·L ⁻¹ NAA + 4.0 mg·L ⁻¹ TDZ	86.38 ± 3.15 ^b	0
5	0.1 mg·L ⁻¹ IAA + 4.0 mg·L ⁻¹ TDZ	95.85 ± 5.09 ^a	5.71
6	1.0 mg·L ⁻¹ 6-BA + 0.5 mg·L ⁻¹ IAA + 0.5 mg·L ⁻¹ KT	87.38 ± 1.53 ^b	0

197



198 **Fig. 2** Callus and adventitious shoots induced from hypocotyl explants of *R. roxburghii*. **A:** Adventitious shoots of *R.*
199 *roxburghii* growing on 3 medium. **B:** Shoot primordia differentiated from hypocotyl callus on 3 medium. **C:** Shoots
200 primordia differentiated from hypocotyl callus on 3 medium. **D:** Early-stage adventitious shoots differentiated from
201 hypocotyl callus on 3 medium. **E:** Magnified view of the region shown in (D). **F:** Adventitious shoot of *R. roxburghii*
202 growing on 5 medium. **G:** Early-stage adventitious shoots differentiated from hypocotyl callus on 5 medium. **H:** Shoot
203 primordia differentiated from hypocotyl callus on 5 medium. **I:** Shoots primordia and adventitious shoot differentiated
204 from hypocotyl callus on 5 medium. **J:** Magnified view of the region shown in (I). **K, L:** Adventitious shoots differentiated
205 from hypocotyl callus.

206 **Effects of different PGR concentrations on the induction of hypocotyl callus and**

differentiation of adventitious shoot

Based on the above experimental results, concentration adjustment experiments were further conducted on the basis of the original PGR combinations; that is, the ratios of the PGRs were kept unchanged, while their concentration levels were either reduced or increased. Specifically, in one group, the original PGR concentrations were halved; in another group, the addition levels were increased, with 6-BA and IAA raised to $0.2 \text{ mg}\cdot\text{L}^{-1}$ and TDZ adjusted to $6.0 \text{ mg}\cdot\text{L}^{-1}$. Meanwhile, a higher concentration treatment was also set, in which both 6-BA and IAA were increased to $0.5 \text{ mg}\cdot\text{L}^{-1}$ and TDZ to $15.0 \text{ mg}\cdot\text{L}^{-1}$. The relevant results are presented in Table 3.

Different concentrations of PGRs had varying effects on callus induction from *R. roxburghii* hypocotyls, as reflected in both the callus induction rate and the adventitious shoot induction rate. Comparison among media 2, 4, and 6 showed that in media containing the IAA and TDZ combination, no significant differences in callus induction rate were observed regardless of whether the PGR concentrations were increased or decreased. However, in terms of adventitious shoot differentiation, no adventitious shoots were produced on medium 2, whereas the adventitious shoot induction rate on medium 4 was significantly higher than that on medium 6.

Comparison among media 1, 3, and 5 revealed that in media with the 6-BA and TDZ combination, altering the PGR concentrations also did not significantly affect the callus induction rate, but the effects on adventitious shoot formation capacity differed among treatments. Among them, no adventitious shoots were produced on medium 1, and the adventitious shoot induction rate of medium 5 was significantly lower than that of medium 3. Furthermore, when comparing media supplemented with the same concentrations of 6-BA and IAA, the adventitious shoot induction rate on the 6-BA-containing medium was always significantly higher than that on the IAA-containing medium, reaching a maximum of 10.62%.

Based on the above data analysis, the medium for inducing hypocotyl callus and adventitious shoot differentiation was determined to be NN69 + $0.05 \text{ mg}\cdot\text{L}^{-1}$ 6-BA + $2.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ.

Table 3 Effects of different PGR concentrations on callus induction from hypocotyl of *R. roxburghii*

Number	PGR concentration	Callus induction rate (%)	Adventitious shoot induction rate (%)
1	$0.2 \text{ mg}\cdot\text{L}^{-1}$ 6-BA + $6.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ	$84.35 \pm 0.37^{\text{ab}}$	0 ^e
2	$0.2 \text{ mg}\cdot\text{L}^{-1}$ IAA + $6.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ	$84.63 \pm 1.31^{\text{ab}}$	0 ^e
3	$0.05 \text{ mg}\cdot\text{L}^{-1}$ 6-BA + $2.0 \text{ mg}\cdot\text{L}^{-1}$ TDZ	$83.70 \pm 1.58^{\text{ab}}$	$10.62 \pm 1.18^{\text{a}}$

4	0.05 mg·L ⁻¹ IAA + 2.0 mg·L ⁻¹ TDZ	85.71 ± 2.71 ^a	5.83 ± 0.26 ^b
5	0.5 mg·L ⁻¹ 6-BA + 15.0 mg·L ⁻¹ TDZ	82.50 ± 0.71 ^b	4.55 ± 0.27 ^c
6	0.5 mg·L ⁻¹ IAA + 15.0 mg·L ⁻¹ TDZ	83.81 ± 0.88 ^{ab}	1.14 ± 0.16 ^d

Effects of different genotypes and explant types on the differentiation of adventitious shoots

Genotype and explant type differentially affected adventitious shoot differentiation (Table 4). Both the callus induction frequency and the adventitious shoot induction frequency of ‘Guinong 5’ were higher than those of Wuci 1. The adventitious shoot induction frequency of Wuci 1 was only 1.83%, whereas that of ‘Guinong 5’ reached 10.62%, indicating that hypocotyls of ‘Guinong 5’ are more amenable to adventitious shoot differentiation. Furthermore, when hypocotyls and leaves of ‘Guinong 5’ were used as explants on the same medium, leaf explants produced neither callus nor adventitious shoots, demonstrating that hypocotyls are more suitable for both callus induction and adventitious shoot differentiation.

Table 4 Effects of genotypes and explant types on adventitious shoot differentiation

Genotype	Explant type	Callus induction rate (%)	Adventitious shoots induction rate (%)
Wuci 1	hypocotyl	54.72	1.83
‘Guinong 5’	hypocotyl	83.70	10.62
‘Guinong 5’	leaf	0	0

Effects of different PGRs on adventitious shoot induction from hypocotyl callus

After 45 days of culture, the induced hypocotyl callus of *R. roxburghii* can be used for adventitious shoot induction. If the hypocotyl callus that has not differentiated adventitious shoots continues to grow on the induction medium for another 2 months, it will no longer differentiate adventitious shoots or produce adventitious roots, and will gradually turn brown and die. Therefore, it is necessary to supplement the induction medium with different PGRs to further screen for the optimal medium suitable for adventitious shoot differentiation from hypocotyl callus.

The effects of three PGRs on adventitious shoot differentiation differed significantly (Table 5). The adventitious shoot induction rate under GA₃ treatment was markedly lower than that under KT treatment. The experiment showed that hypocotyl callus cultured on induction medium for 1 month produced only a small number of adventitious shoots on differentiation medium supplemented with GA₃, whereas callus cultured on

induction medium for 2 or 3 months failed to produce any adventitious shoots on GA₃-supplemented differentiation medium, demonstrating that GA₃ is not suitable for adventitious shoot induction from hypocotyl callus. The adventitious shoot induction rate under ZT treatment was significantly higher than that under KT treatment, reaching a maximum of 17.33%. Therefore, under the conditions of this experiment, ZT was identified as the appropriate PGR for adventitious shoot differentiation from hypocotyl callus..

Table 5. Effects of different PGRs on adventitious shoots induction from hypocotyl callus of *R. roxburghii*

Hormone	Adventitious shoot differentiation rate (%)	Callus state
KT	4.00 ± 2.65 ^b	Yellowish-white callus with extensive browning
ZT	17.33 ± 13.01 ^a	Yellowish-white callus with large clumps; occasional browning at the apical ends
GA ₃	0.67 ± 1.16 ^c	Yellowish-white callus, abundant browned clumps.

Effects of ZT concentration on adventitious shoot induction from hypocotyl callus

No adventitious shoot differentiation was observed in the absence of ZT or at excessively high ZT concentrations (Table 6). The highest adventitious shoot induction frequencies were obtained at ZT concentrations of 0.5 mg·L⁻¹ and 2.0 mg·L⁻¹, reaching 16.28% and 13.51%, respectively. At 0.5 mg·L⁻¹ ZT, only single shoots were induced, whereas at 2.0 mg·L⁻¹ ZT, multiple shoot clusters were formed (Fig. 3A) and the individual shoots developed thick, sturdy stems (Fig. 3B). Therefore, under the conditions of this experiment, the optimal ZT concentration for adventitious shoot induction from hypocotyl callus was determined to be 2.0 mg·L⁻¹.

Table 6. Effect of ZT concentration on adventitious shoots induction from hypocotyl callus of *R. roxburghii*

ZT concentration (mg·L ⁻¹)	Adventitious shoot differentiation rate (%)	Adventitious shoot state
0	0 ^c	-
0.5	16.28 ± 1.00 ^a	No multiple shoots produced, stems slender
1	2.63 ± 0.84 ^b	No multiple shoots produced
1.5	5.88 ± 1.63 ^b	No multiple shoots produced
2	13.51 ± 3.48 ^a	Multiple shoots produced, stems stout
2.5	0 ^c	No adventitious shoots produced

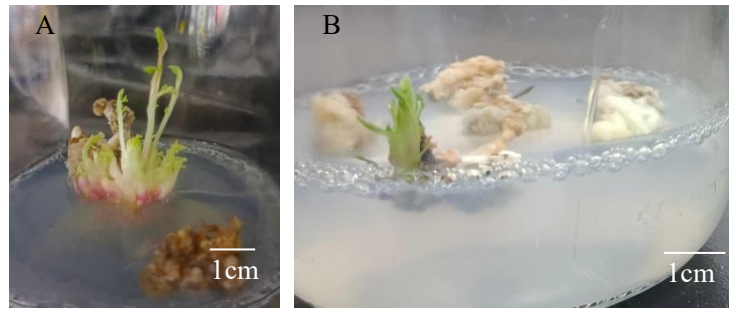


Fig. 3 Adventitious shoots induced from hypocotyl callus of *R. roxburghii*. **A:** Cluster shoots; **B:** Adventitious shoots with stout stems.

Screening of the optimal medium for adventitious shoot rooting

In preliminary experiments, the effect of agar concentration on adventitious shoot rooting was evaluated by adding 5, 6, or 7 g·L⁻¹ agar to media No. 1 and No. 2 (Table 7); no adventitious root formation was observed in any treatment. Therefore, following the method of Wen (2003), sucrose in medium No. 2 was replaced with glucose, and 300 mg·L⁻¹ activated charcoal was added (designated medium No. 3). This modification successfully induced adventitious rooting, yielding a rooting rate of 23.33% and an average of 1.17 roots per shoot. Further optimization was conducted according to the protocol of Wu (2023), in which 6-BA (0.5 mg·L⁻¹) and IAA (0.05 mg·L⁻¹) in medium No. 3 were replaced with IBA (0.3 mg·L⁻¹), yielding medium No. 4. This medium increased the rooting rate to 56.67% and raised the average root number to 3.20 (Table 7), with a maximum of six adventitious roots observed per shoot (Fig. 4B). In summary, under the conditions of this experiment, the optimal medium for adventitious root induction from shoot-bearing hypocotyl callus of *R. roxburghii* was determined to be 1/2 MS + 0.3 mg·L⁻¹ IBA + 300 mg·L⁻¹ activated charcoal + 30 g·L⁻¹ glucose.

Table 7. Effects of different media formulations on rooting of adventitious shoots of *R. roxburghii*

Number	Medium	Medium combination	Rooting rate (%)	Average rooting number
1	MS	6-BA (0.5 mg·L ⁻¹) + IAA (0.05 mg·L ⁻¹) + sucrose (30 g·L ⁻¹)	0	-
2	1/2MS	6-BA (0.5 mg·L ⁻¹) + IAA (0.05 mg·L ⁻¹) + sucrose (30 g·L ⁻¹)	0	-
3	1/2MS	6-BA (0.5 mg·L ⁻¹) + IAA (0.05 mg·L ⁻¹) + activated charcoal (300 mg·L ⁻¹) + glucose (30 g·L ⁻¹) + agar (6 g·L ⁻¹)	23.33	1.17
4	1/2MS	IBA (0.3 mg·L ⁻¹) + activated charcoal (300 mg·L ⁻¹) + glucose (30 g·L ⁻¹) + agar (6 g·L ⁻¹)	56.67	3.20

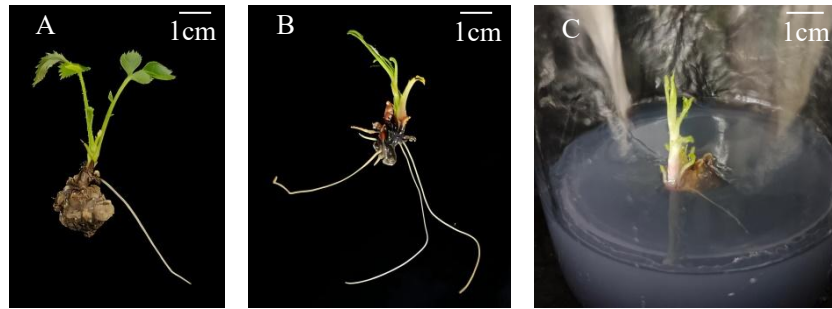


Fig. 4 Adventitious roots from adventitious shoots of *R. roxburghii*. A: A regenerated plant with 1 root; B: Regenerated plants with 6 roots; C: Regenerated plants in tissue culture bottles.

Acclimatization and transplanting of regenerated plantlets

Adventitious shoots that had rooted after one month of culture on rooting medium were transferred to rooting medium with a reduced glucose concentration (Fig. 5A, B). For acclimatization, the tissue culture bottles were opened one-third every three days at room temperature. After nine days of acclimatization, the plantlets were carefully removed and their roots rinsed with water to remove all traces of medium. The plantlets were then transplanted into a 1:1:1 (v/v/v) mixture of vermiculite, humus soil, and perlite and cultivated in a growth chamber (Fig. 5C). Two weeks after transplantation, the survival rate of the rooted plantlets was 75%. In total, 21 regenerated plantlets were obtained from this experiment.

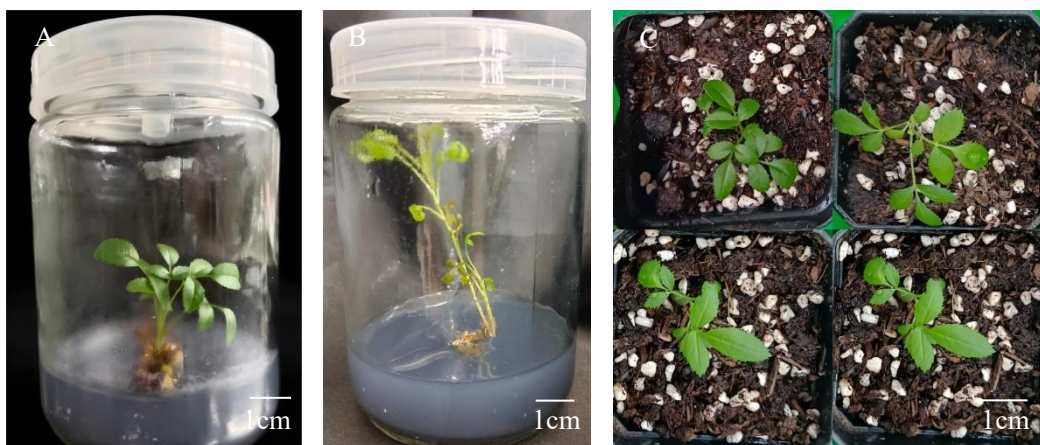


Fig. 5 Regeneration seedlings of *R. roxburghii*. A, B: Regenerated plants in tissue culture bottles; C: Tissue culture seedlings of *R. roxburghii* in an illumination incubator.

Discussion

The composition of the basal culture medium is a fundamental determinant of in vitro regeneration success, exerting profound effects on cell division, differentiation, and organogenesis. In the present study, although both NN69 and QL media supported callus induction from *R. roxburghii* hypocotyls, NN69 medium

consistently outperformed QL in terms of callus induction rate (88.37% vs. 77.70%), callus morphology, and growth uniformity. The superior performance of NN69 medium may be attributable to its distinct mineral composition—particularly its lower ammonium-to-nitrate ratio and higher calcium concentration relative to other formulations—which has been shown to favor callus proliferation in several woody species. This finding is consistent with previous reports demonstrating the suitability of NN69 medium for hypocotyl-derived callus induction in *Pyrus betulifolia* (Xiao et al., 2022) and *P. communis* (Yancheva et al., 2006), suggesting a degree of conservation in the nutritional requirements for callus initiation among Rosaceae species.

The interplay between cytokinin and auxin constitutes a central regulatory node governing callus growth and organogenic differentiation (Omori et al., 2024). According to the classical paradigm established by Skoog and Miller, a high cytokinin-to-auxin ratio promotes shoot differentiation, whereas a high auxin-to-cytokinin ratio favors root formation (Ikeuchi et al., 2019). Consistent with this principle, adventitious shoot regeneration from *R. roxburghii* hypocotyls failed under PGR combinations in which auxin activity predominated (e.g., 0.5 mg·L⁻¹ IAA + 0.1 mg·L⁻¹ 6-BA), regardless of the basal medium employed. Similarly, the combination of 1.0 mg·L⁻¹ 6-BA and 0.5 mg·L⁻¹ IAA was ineffective for shoot induction. In marked contrast, adventitious shoots were successfully induced when the PGR balance was shifted decisively toward cytokinin predominance—specifically, using combinations of 0.1 mg·L⁻¹ 6-BA + 4.0 mg·L⁻¹ TDZ, or 4.0 mg·L⁻¹ TDZ + 0.1 mg·L⁻¹ IAA. These results collectively indicate that adventitious shoot organogenesis in *R. roxburghii* hypocotyls requires a PGR environment in which cytokinin activity substantially exceeds that of auxin. Notably, the combination of TDZ with 6-BA yielded higher shoot induction rates than TDZ combined with IAA or NAA, further underscoring the primacy of cytokinin signaling during the shoot commitment phase in this species.

Among cytokinin-type growth regulators, TDZ (thidiazuron) exhibits exceptionally potent activity in woody plant tissue culture (Huetteman and Preece, 1993), and its capacity to induce adventitious shoots from explants such as hypocotyls and leaves markedly exceeds that of other cytokinins (Fasolo et al., 1989). TDZ has been shown to possess dual functionality, acting both as a cytokinin analogue and as a modulator of endogenous auxin metabolism (Taha et al., 2009; Gharari et al., 2021). The effectiveness of TDZ-containing PGR combinations in promoting adventitious shoot formation in the present study corroborates these earlier findings and reinforces the conclusion that TDZ is a critical component for overcoming the recalcitrance of *R. roxburghii* to in vitro regeneration. Moreover, the type, concentration, and combination ratio of PGRs exert decisive influence on organogenesis, and the requirements for specific hormones vary considerably among

different tissue types (Michalczuk et al., 1992; Du, 2023; Luo, 2024). Nevertheless, the precise endogenous hormonal changes elicited by exogenous TDZ application in *R. roxburghii* tissues remain to be elucidated. Future studies incorporating a systematic TDZ concentration gradient, coupled with quantification of endogenous hormone profiles, would be valuable for further refining the induction protocol.

Genotype-dependent variation in regeneration capacity is a well-documented phenomenon in plant tissue culture. For instance, hawthorn (*Crataegus* spp.) cultivars have been shown to differ markedly in adventitious shoot induction efficiency under identical culture conditions (Wang, 2025). In the present study, the adventitious shoot induction rate of ‘Guinong 5’ hypocotyls (10.62%) was substantially higher than that of Wuci 1 (1.83%), despite both cultivars being subjected to the same optimized medium. This pronounced differential response implies that the PGR formulation empirically optimized for ‘Guinong 5’ may not represent the optimal regimen for Wuci 1, and highlights the necessity of genotype-specific optimization of both basal medium composition and PGR combinations in future regeneration studies involving diverse *R. roxburghii* germplasm.

Explant type is another pivotal factor determining tissue culture outcomes. Leaves have been widely employed as explants in regeneration systems of Rosaceae fruit crops, including pear (Sun, 2014), apple (Wang et al., 2024), and hawthorn (Wang, 2025). A well-established principle in plant tissue culture is that explant juvenility is positively correlated with morphogenic competence: the more juvenile the source tissue, the greater its capacity for dedifferentiation and subsequent organogenesis (Yu et al., 2020). This relationship has been elegantly demonstrated in *Cerasus serrulata*, where leaves from in vitro-proliferated shoots readily underwent adventitious shoot regeneration, whereas leaves from mature field-grown trees were competent only for callus formation (Luo et al., 2021). Consistent with this principle, hypocotyl explants in the present study proved markedly superior to leaf explants for both callus induction and adventitious shoot differentiation; leaf explants of ‘Guinong 5’ failed to produce either callus or shoots under the conditions tested. The pronounced responsiveness of hypocotyl tissue likely reflects its embryonic origin and consequently heightened developmental plasticity relative to foliar tissues. This interpretation is further supported by findings in *Fortunella hindsii* (Deng and Zhang, 1988) and *Citrus reticulata* ‘Nanfeng’ (Duan et al., 2009), where hypocotyl segments from seed-germinated seedlings displayed strong embryogenic competence.

With respect to adventitious shoot induction from established callus cultures, the choice of cytokinin proved decisive. Cytokinins are a class of phytohormones that promote cell division and shoot regeneration, with 6-BA, KT, and ZT being among the most commonly employed in tissue culture (Hassan et al., 2018; Jia-

Jia et al., 2019). Among the three cytokinins evaluated in the present study—KT, ZT, and GA₃—ZT (zeatin) demonstrated the highest efficacy, yielding an adventitious shoot induction rate of 17.33%, which was significantly higher than that obtained with KT. In contrast, GA₃ treatment supported only minimal adventitious shoot formation from one-month-old callus and failed entirely to induce shoots from older (2–3 month) callus cultures, indicating that GA₃ is unsuitable for adventitious shoot induction from *R. roxburghii* hypocotyl callus. The superior performance of ZT is consistent with findings in blueberry (*Vaccinium* spp.), where ZT has been reported to effectively promote adventitious shoot differentiation (Yi et al., 2017; Zhang, 2022). The differential efficacy of ZT over KT may reflect differences in their affinity for cytokinin receptors, their metabolic stability in plant tissues, or their capacity to activate downstream type-B response regulators.

The effect of ZT on adventitious shoot differentiation from hypocotyl callus was distinctly concentration-dependent and non-monotonic. Optimal differentiation was observed at 0.5 mg·L⁻¹ (16.28%) and 2.0 mg·L⁻¹ (13.51%), whereas an intermediate concentration of 1.0 mg·L⁻¹ unexpectedly suppressed shoot formation, and 2.5 mg·L⁻¹ completely inhibited regeneration. Notably, shoots induced at 2.0 mg·L⁻¹ ZT were morphologically superior—developing as robust multiple-shoot clusters with thick, sturdy stems—suggesting that this concentration may be preferable from the standpoint of producing vigorous, transplantable plantlets, despite yielding a slightly lower induction frequency than 0.5 mg·L⁻¹. This non-monotonic dose–response pattern is consistent with the hypothesis that exogenous cytokinin application alters endogenous hormonal equilibria in a concentration-specific manner (Luo and Xu, 2011; Zhang et al., 2021; Zhao et al., 2022). It is plausible that ZT at low concentrations (≤ 0.5 mg·L⁻¹) augments endogenous cytokinin signaling to promote differentiation; supra-optimal levels (1.0 mg·L⁻¹) may trigger negative feedback mechanisms or metabolic inactivation; concentrations in the 1.5–2.0 mg·L⁻¹ range may re-establish a favorable hormonal balance through activation of complementary signaling pathways; and toxic concentrations (≥ 2.5 mg·L⁻¹) likely suppress morphogenic competence through cytokinin-induced cytotoxicity.

In the rooting phase, the selection of carbon source and the inclusion of activated charcoal emerged as critical factors. Consistent with the findings of Wen (2003), who reported that glucose outperformed sucrose as a carbon source for *R. roxburghii* adventitious shoot rooting (76.5% vs. 66.7% rooting rate), the present study observed that adventitious rooting was entirely absent on sucrose-containing medium but reached 23.33% when glucose and 300 mg·L⁻¹ activated charcoal were co-supplemented. Activated charcoal is presumed to adsorb residual PGRs and phenolic exudates from the culture medium, thereby creating a chemical microenvironment more permissive to rhizogenesis. However, the rooting rate achieved in the present study

(23.33%) remained substantially lower than the 72.0%–77.6% reported by Wen (2003) using 300–400 mg·L⁻¹ activated charcoal, suggesting that further optimization of the glucose-to-activated charcoal ratio and their interaction with other medium components is warranted.

Auxin supplementation, while not strictly essential for adventitious rooting, has been consistently shown to enhance both rooting percentage and root quality within an appropriate concentration range (Sun and Sun, 2000; Yin et al., 2026). The present results are in agreement with this general pattern: the rooting rate of ‘Guinong 5’ adventitious shoots increased from 23.33% (without IBA) to 56.67% upon the addition of 0.3 mg·L⁻¹ IBA, with an accompanying increase in the average root number from 1.17 to 3.20 per shoot. These findings confirm the promotive role of exogenous auxin in *R. roxburghii* rhizogenesis. Nevertheless, the optimal IBA concentration for maximizing rooting efficiency—as well as the potential synergistic effects of IBA with other auxin types or concentrations—remains to be systematically determined through subsequent experiments.

In summary, this study establishes, for the first time, a functional in vitro regeneration system for *R. roxburghii* via indirect organogenesis from hypocotyl explants, encompassing callus induction, adventitious shoot differentiation, root induction, and successful acclimatization of regenerated plantlets. While the current regeneration efficiencies—particularly the adventitious shoot induction rate (17.33%) and rooting rate (56.67%)—remain suboptimal relative to those reported for more tractable Rosaceae species such as apple (*Malus × domestica*) and pear (*Pyrus spp.*), the system described herein provides a critical proof-of-concept and a tractable experimental platform. The comparatively low regeneration efficiency underscores the recalcitrant nature of *R. roxburghii* and indicates that the conditions required for genetic transformation in this species are likely to be more demanding than those for model rosaceous crops. Further systematic optimization of PGR formulations, culture conditions, and genotype-specific protocols will be essential to elevate regeneration efficiency to levels compatible with routine genetic transformation. The successful establishment of this regeneration pathway represents an important step toward enabling functional genomics and precision breeding in this nutritionally and economically valuable crop.

Conclusion

In this study, an in vitro regeneration system from hypocotyl explants of *R. roxburghii* was successfully established, and the optimal conditions for each key stage were determined as follows: (1) the optimal basal medium for callus induction was NN69; (2) the optimal PGR combination for adventitious shoot

differentiation was 0.05 mg·L⁻¹ 6-BA and 2.0 mg·L⁻¹ TDZ; (3) the optimal cytokinin for inducing adventitious shoot differentiation from callus was 2.0 mg·L⁻¹ ZT; (4) the optimal rooting medium was 1/2 MS supplemented with 0.3 mg·L⁻¹ IBA, 300 mg·L⁻¹ activated charcoal, and 30 g·L⁻¹ glucose. The establishment of this system addresses a gap in the in vitro regeneration of *R. roxburghii* and provides a valuable foundation for molecular breeding and cultivar improvement.

Author contributions All authors contributed to the study conception and design. Material preparation, data curation and analysis were performed by He Huayun and Wei Yang. Funding acquisition, experimental design and supervision were conducted by An Huaming, Min Lu, Wentao Ma and Hong Nan. The first draft of the manuscript was written by He Huayun, and the manuscript was revised by An Huaming. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All study data that support the findings are included in the article. Further inquiries can be directed to the corresponding authors.

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Ethics declaration not applicable.

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