



Article

Rapid In Vitro Propagation of *Quercus gilva* via Nodal Explants: A Protocol for Culture Establishment, Shoot Proliferation, and Ex Vitro Rooting

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Abstract

Quercus gilva is a dominant species in the subtropical evergreen broad-leaved forests of East Asia with substantial economic and ecological value. However, efficient clonal propagation methods for this species remain limited. This study aimed to establish a micropropagation protocol for *Q. gilva* using nodal stem segments from two-year-old seedlings as explants, focusing on culture establishment, shoot induction, shoot proliferation, and ex vitro rooting. Aseptic culture was effectively established by rinsing explants under running water for 15 min, followed by immersion in 0.1% HgCl₂ for 8 min, which balanced contamination control and explant viability. Explant browning was reduced by pre-soaking in 1.0 g·L⁻¹ ascorbic acid (VC) and by supplementing the Murashige and Skoog (MS) medium with 3.0 g·L⁻¹ activated charcoal. The highest shoot induction percentage (80.0%) was obtained on MS medium containing 1.0 mg·L⁻¹ 2,4-D and 0.5 mg·L⁻¹ TDZ. Shoot proliferation was achieved by subculturing induced shoots on MS medium supplemented with 1.0 mg·L⁻¹ NAA and 1.0 mg·L⁻¹ 2iP. For ex vitro rooting, regenerated shoots were dipped in a solution containing 600.0 mg·L⁻¹ IBA plus 700.0 mg·L⁻¹ NAA and then transplanted into a substrate of peat and perlite (1:1, v/v), resulting in a rooting percentage of 70.0% and well-developed root systems. This study establishes a preliminary in vitro propagation framework for *Q. gilva*, providing a methodological reference for future studies aimed at improving clonal propagation efficiency.

Keywords: anti-browning; ex vitro rooting; micropropagation; nodal stem explants; oaks; *Quercus gilva*; vegetative propagation



Academic Editor: Dong Zhang

Received: 13 January 2026

Revised: 6 February 2026

Accepted: 13 February 2026

Published: 18 February 2026

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1. Introduction

Quercus gilva is a highly valuable evergreen oak in the genus *Quercus*, section *Cyclobalanopsis* (Fagaceae), native to East Asia and occurring naturally in southern China, Japan, and Jeju Island of South Korea [1–3]. It is a dominant component of subtropical evergreen broad-leaved forests and provides precious timber, starchy nuts, and notable ecological services, thereby conferring substantial economic and ecological value for afforestation for industrial wood production and ecological restoration [4]. However, population persistence is threatened by habitat loss, fragmented distribution, historical over-harvesting,

seed constraints, and slow natural regeneration [3,5,6]. Consequently, *Q. gilva* has been included in regional assessments as a taxon of conservation concern [6], highlighting the need for efficient propagation approaches to support its germplasm conservation, breeding, and deployment.

In oak nursery production, seed propagation is still the predominant approach, yet the uniformity and quality of seedlings are often inconsistent because acorn traits and reproductive ecology vary widely among years and populations (e.g., acorn size and mass, irregular mast years) [7]. In addition, extensive interspecific hybridization and introgression are common in oaks, particularly among closely related species [8,9], which can lead to genetically mixed progeny and complicate the production of true-to-type planting materials. Clonal propagation is therefore desirable for the multiplication and deployment of elite genotypes. However, conventional vegetative propagation of *Q. gilva* via cuttings remains inefficient: rooting success is typically below 35.0% [10], and rooting capacity declines markedly with increasing donor age. Therefore, an efficient and reliable clonal propagation method is urgently needed.

Micropropagation, an in vitro approach to vegetative multiplication, offers a promising solution because it enables the rapid production of large numbers of uniform clones within a short period of time and limited space under controlled conditions [11]. Over the past decades, considerable progress has been made to develop micropropagation protocols in oak species, such as *Q. robur* [12–14], *Q. alba* [15,16], *Q. ilex* [17], *Q. aliena* [18], *Q. suber* [19,20], *Q. glauca* [21], and *Q. dumosa* [22,23]. Despite these great efforts, micropropagation in oaks remains challenging due to strong phenolic exudation, recalcitrance to organogenesis, low shoot multiplication, and poor rooting ability [24–26].

Early work primarily focused on the browning caused by phenolic oxidation during culture initiation of oak explants and showed that antioxidants (e.g., ascorbic acid, PVP) and adsorbents (e.g., activated charcoal) can suppress phenolic oxidation and improve explant survival [27,28]. Subsequent advances demonstrated that regeneration competence can be retained even in mature donor material, enabling shoot induction from adult trees when explant type and culture conditions are optimized [14,17,29,30]. Shoot multiplication remains a bottleneck in many oak systems, but improvements have been achieved through optimization of plant growth regulator (PGR) combinations and concentrations, pulse treatments, and subculture timing [15,31,32]. Root induction is often the most recalcitrant stage and has been enhanced by optimizing auxin concentration and exposure time, applying dark treatments, and adopting ex vitro rooting strategies [21,33–35]. In many cases, high-concentration auxin dips (e.g., indolebutyric acid, IBA) followed by ex vitro rooting outperform in vitro rooting by reducing excessive callus formation and improving acclimatization [26,33]. To date, a complete micropropagation pathway for *Q. gilva* has not been reported.

The objective of this study was to develop an efficient micropropagation protocol for *Q. gilva* using stem segments bearing axillary buds from two-year-old seedlings. Specifically, we optimized (i) aseptic establishment and anti-browning treatments for culture initiation, (ii) PGR regimes for shoot induction and multiplication, and (iii) auxin treatments for ex vitro rooting. The resulting protocol provides a technical basis for large-scale clonal propagation and conservation of *Q. gilva* and may inform the optimization of micropropagation systems in other oak species.

2. Materials and Methods

2.1. Plant Materials and Culture Conditions

2.1.1. Plant Materials

Stem bearing axillary buds from two-year-old seedlings of *Q. gilva* were used as explants. Tender current-year shoots (15–20 cm in length) were collected from April to September 2023. Sampling was conducted on sunny days between 12:00 and 13:00. The collected shoots were placed in ice bags and transported to the laboratory for cultivation on the same day.

2.1.2. Culture Condition

All media were supplemented with 30 g·L⁻¹ sucrose (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and 5.5 g·L⁻¹ agar (Sangon Biotech Co., Ltd., Shanghai, China) and adjusted to pH 5.6–5.8 prior to autoclaving at 121 °C for 15 min. All culture jars and trays were kept in a controlled growth room at 25 ± 2 °C under a photosynthetic photon flux density (PPFD) of 60 µmol·m⁻²·s⁻¹, with a 16 h photoperiod provided by white LED lamps (Philips Lighting, Eindhoven, The Netherlands).

2.2. Culture Initiation

2.2.1. Disinfection of Explants

Shoots were trimmed to 5 cm segments and gently brushed to remove surface debris. They were then rinsed under running tap water for different durations. Subsequently, shoots were soaked in 0.1% mercuric chloride (HgCl₂) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) containing two drops of Tween 20 (Solarbio Science & Technology Co., Ltd., Beijing, China) for different durations. After disinfection, explants were rinsed five times with sterile distilled water under aseptic conditions. The disinfected shoot segments (1–2 cm in length, each containing one node) were placed on Murashige and Skoog (MS) medium (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) without PGRs. Contamination and browning percentages were recorded after 14 days.

All procedures involving HgCl₂ were performed in accordance with institutional laboratory safety regulations. Preparation and handling were conducted in a chemical fume hood (FH-1800(A), Shanghai Boxun Medical Biological Instrument Corp., Shanghai, China) while wearing appropriate personal protective equipment. Hg-containing waste solutions and contaminated materials were collected separately and disposed of as hazardous chemical waste through the university's authorized waste management system, in compliance with national environmental and laboratory safety guidelines.

2.2.2. Anti-Browning of Explants

Collected shoots were trimmed into 5 cm segments and soaked in ascorbic acid (VC) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at different concentrations for 10 min. They were then rinsed under running tap water for 15 min, followed by immersion 0.1% HgCl₂ for 8 min. The disinfected shoots were further trimmed to 1–2 cm segments and placed on MS supplemented with different concentrations of activated charcoal (Solarbio Science & Technology Co., Ltd., Beijing, China). Browning percentage was measured after 14 days.

2.2.3. Shoot Induction

Successfully established explants were transferred to MS or Woody Plant Medium (WPM) (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) supplemented with 0.5 g·L⁻¹ casein hydrolysate (Solarbio Science & Technology Co., Ltd., Beijing, China) and 1.0 g·L⁻¹ myo-inositol (Sigma-Aldrich, St. Louis, MO, USA). Different concentra-

tions of 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich, St. Louis, MO, USA) and thidiazuron (TDZ) (Sigma-Aldrich, St. Louis, MO, USA) were added for shoot induction. Shoot induction percentage and shoot growth characteristics were recorded after 30 days of cultivation.

2.3. Shoot Multiplication

Induced shoots were excised above the callus region and moved to MS medium containing $0.5 \text{ g}\cdot\text{L}^{-1}$ casein hydrolysate and $1.0 \text{ g}\cdot\text{L}^{-1}$ myo-inositol. The medium was supplemented with different concentrations of N^6 -($\text{de}\alpha^2$ -isopentenyl) adenine (2iP) (0.5, 1.0, and $1.5 \text{ mg}\cdot\text{L}^{-1}$) and/or naphthaleneacetic acid (NAA) (0.5, 1.0, and $2.0 \text{ mg}\cdot\text{L}^{-1}$) (Sigma-Aldrich, St. Louis, MO, USA). Shoot multiplication was evaluated after 30 days of culture.

2.4. Ex Vitro Rooting and Acclimatization

Vigorous shoots (~4 cm in length with three leaves) were taken out from culture vessels and dipped for 3 s in solutions containing different concentrations of indole-3-butyric acid (IBA) (400, 600, and $800 \text{ mg}\cdot\text{L}^{-1}$) (Sigma-Aldrich, St. Louis, MO, USA) and/or NAA (500, 700, and $900 \text{ mg}\cdot\text{L}^{-1}$). Shoots were then transplanted into 32-cell plug trays (Zhejiang Taizhou Agricultural Plastic Co., Ltd., Taizhou, China) containing a potting mix of 50% peat (Klasmann-Deilmann GmbH, Geeste, Germany) and 50% perlite (Xinyang Mingzhu Perlite Co., Ltd., Henan, China) for ex vitro rooting. Trays were maintained in the controlled growth room under the conditions described in Section 2.1.2 and covered with a transparent plastic dome to maintain high humidity. The dome was gradually ventilated during acclimatization and eventually removed. Rooting percentage and average root number were recorded 30 days after transplanting.

2.5. Experimental Design and Statistical Analysis

All experiments were performed using a completely randomized design with three replicates per treatment. In disinfection, anti-browning, and shoot induction experiments, each replicate consisted of 20 explants. In shoot multiplication and ex vitro rooting experiments, each replicate included 10 shoots.

A Nikon D90 camera (Nikon Corporation, Tokyo, Japan) was used to photograph and document the growth of explants, induced shoots, proliferated shoots, and rooted plantlets.

Data were analyzed using one-way analysis of variance (ANOVA) with IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). Percentage data were subjected to arcsine square-root transformation prior to analysis to meet the assumptions of normality and homogeneity of variance; untransformed means are presented in tables for clarity. When significant differences were detected, means were compared using Duncan's Multiple Range Test (DMRT) at $p < 0.05$. Each treatment consisted of three independent replicates, and the replicate was considered the experimental unit for statistical analysis.

3. Results

3.1. Aseptic Establishment and Shoot Induction

Different combinations of running tap water, rinsing, and 0.1% HgCl_2 exposure produced significant differences in both contamination and browning percentages of *Q. gilva* stem explants (Table 1). Increasing rinsing and exposure time tended to reduce contamination but was accompanied by a progressive increase in browning. This trend suggests that prolonged physical cleaning and chemical sterilization may impose physiological stress on explant tissues, which has been widely associated with enhanced phenolic oxidation in woody species. Among all treatments, running water rinsing for 15 min, followed

by immersion in 0.1% HgCl₂ for 8 min, resulted in the lowest contamination (8.3%) and browning percentages (11.7%).

Table 1. Effects of running water rinsing and 0.1% HgCl₂ treatment on contamination and browning of explants.

Running Water Rinsing (min)	0.1% HgCl ₂ Soaking (min)	Contamination Percentage (%)	Browning Percentage (%)
10	8	33.3 ± 4.4 a	13.3 ± 3.3 e
10	15	31.7 ± 1.7 a	18.3 ± 3.3 de
10	20	25.0 ± 2.9 a	25.0 ± 2.9 cd
15	8	8.3 ± 1.7 b	11.7 ± 1.7 e
15	15	13.3 ± 1.7 b	18.3 ± 3.3 de
15	20	15.0 ± 5.0 b	25.0 ± 2.9 cd
20	8	13.3 ± 3.3 b	30.0 ± 2.9 bc
20	15	13.3 ± 1.7 b	35.0 ± 2.9 ab
20	20	13.3 ± 1.7 b	40.0 ± 2.9 a
Significance			
Running water rinsing		ns	**
0.1% HgCl ₂ soaking		**	**
Running water rinsing × 0.1% HgCl ₂ soaking		ns	ns

Values represent means ± SE of three independent replicates (each replicate consisted of 20 explants, and the replicate was treated as the experimental unit). Different letters within a column indicate significant differences at $p < 0.05$. ** indicates significant differences at $p < 0.01$; ns indicates no significant differences at $p < 0.05$.

The application of VC and activated charcoal effectively reduced browning compared with the control (CK), with all treatments showing significantly lower browning percentages (Table 2). Overall, explants soaked in 0.5 g·L^{−1} VC exhibited relatively higher browning percentages (31.7–36.7%) compared with those treated with higher VC concentrations. Browning was lowest (8.3%) with 1.0 g·L^{−1} VC-soaked culture on MS medium supplemented with 3.0 g·L^{−1} activated charcoal. Browning did not further decrease with increasing VC concentration, suggesting that exceeding a threshold does not suppress phenolic oxidation or enhance antibrowning efficiency. Therefore, soaking in 1.0 g·L^{−1} VC combined with 3.0 g·L^{−1} activated charcoal was identified as the most effective anti-browning treatment under the conditions tested.

Table 2. Effects of VC and activated charcoal on browning of explants.

VC (g·L ^{−1})	Activated Charcoal (g·L ^{−1})	Browning Percentage (%)
0	0	58.3 ± 5.0 a
0.5	2.0	31.7 ± 5.0 bcd
0.5	3.0	33.3 ± 4.4 bc
0.5	4.0	36.7 ± 5.0 b
1.0	2.0	26.7 ± 1.7 cde
1.0	3.0	8.3 ± 3.3 g
1.0	4.0	13.3 ± 1.7 fg
1.5	2.0	23.3 ± 1.7 de
1.5	3.0	20.0 ± 2.9 ef
1.5	4.0	25.0 ± 2.9 cde
Significance		
VC		**
Activated charcoal		*
VC × activated charcoal		*

Values represent means ± SE of three independent replicates (each replicate consisted of 20 explants, and the replicate was treated as the experimental unit). Different letters within a column indicate significant differences at $p < 0.05$. ** and * indicate significant differences at $p < 0.01$ and $p < 0.05$, respectively.

Shoot induction was significantly affected by the type of basal medium and the concentration of 2,4-D, whereas TDZ showed no significant main effect (Table 3). MS consistently produced higher induction percentages and more vigorous shoots than WPM when supplemented with the same PGR combination. Within each basal medium, 1.0 mg·L⁻¹ 2,4-D was associated with higher shoot induction than lower concentrations. Although TDZ concentration did not show a statistically significant effect, the highest induction percentage (80.0%) was observed on MS + 1.0 mg·L⁻¹ 2,4-D + 0.5 mg·L⁻¹ TDZ, producing vigorous green shoots (Figure 1A–D).

Table 3. Effects of 2,4-D and TDZ on shoot induction percentage and morphological characteristics of induced shoots on MS or WPM media.

Medium	2,4-D (mg·L ⁻¹)	TDZ (mg·L ⁻¹)	Induction Percentage (%)	Morphology of Induced Shoot
MS	0.0	0.0	21.7 ± 3.3 e	Light-yellowish shoots, short in height, with relatively small leaves
MS	0.5	0.5	58.3 ± 4.4 cd	Green shoots with relatively short internodes
MS	0.5	1.0	61.7 ± 2.9 bc	Pale-yellowish shoots with small leaves
MS	1.0	0.5	80.0 ± 2.9 a	Green shoots showing relatively normal shoot development
MS	1.0	1.0	70.0 ± 2.9 ab	Green shoots that appeared healthy
WPM	0.0	0.0	15.0 ± 2.9 e	Light-green shoots, short in height, with elongated leaves
WPM	0.5	0.5	48.3 ± 4.4 d	Light yellow shoots that appeared healthy
WPM	0.5	1.0	50.0 ± 5.0 d	Green shoots with relatively small leaves
WPM	1.0	0.5	61.7 ± 4.4 bc	Bright-green shoots that appeared healthy
WPM	1.0	1.0	56.7 ± 4.4 bcd	Light-green shoots that appeared healthy
Significance				
Medium			**	
2,4-D			**	
TDZ			ns	
Medium × 2,4-D			ns	
Medium × TDZ			ns	
2,4-D × TDZ			ns	
Medium × 2,4-D × TDZ			ns	

Values represent means ± SE of three independent replicates (each replicate consisted of 20 explants, and the replicate was treated as the experimental unit). Different letters within a column indicate significant differences at $p < 0.05$. ** indicates significant differences at $p < 0.01$; ns indicates no significant differences at $p < 0.05$.

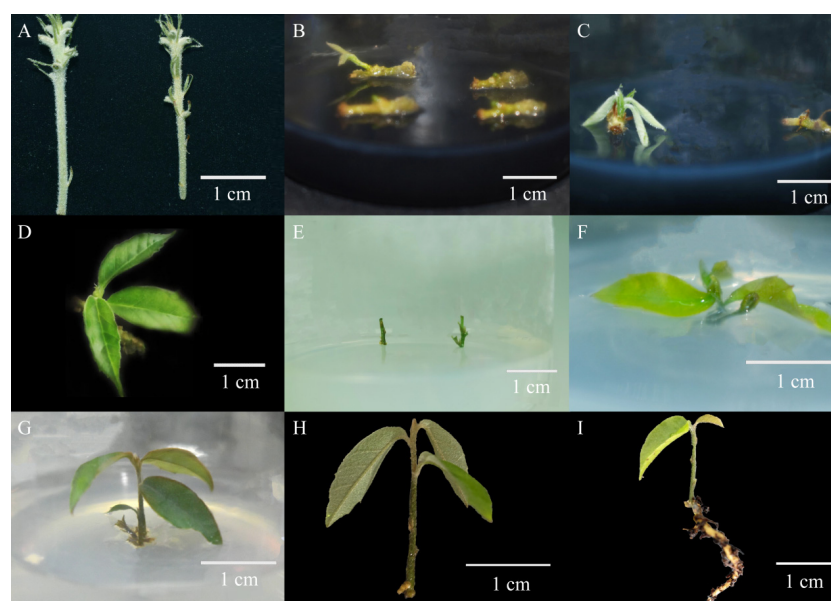


Figure 1. The micropropagation using the stem with axillary buds of *Quercus gilva* seedlings. (A) Stems with axillary buds from two-year-old seedlings were used as explants. (B) Shoots induced from explants on the initiation medium MS supplemented with 1.0 mg·L⁻¹ 2,4-D, 0.5 mg·L⁻¹

TDZ, and 3.0 g·L⁻¹ activated charcoal after 10 days of culture. (C) The growth of induced shoots after 20 days of culture. (D) The growth of induced shoots after 30 days of culture. (E) Induced shoots were transferred to MS shoot multiplication medium containing 1.0 mg·L⁻¹ NAA and 1.0 mg·L⁻¹ 2iP. (F) Shoot proliferation after 15 days of culture. (G) Shoot proliferation after 30 days of culture. (H) Vigorous shoots dipped with 600 mg·L⁻¹ IBA and 700 mg·L⁻¹ NAA for ex vitro rooting. (I) Ex vitro rooted plantlet after 15 days of rooting.

3.2. Shoot Multiplication

Overall multiplication performance of *Q. gilva* was limited, and no multiplication occurred in the control (Table 4). Only NAA showed a significant main effect on multiplication percentage, whereas 2iP and the interaction term were not significant. At a constant 2iP level, the multiplication percentage tended to increase with NAA concentration up to 1.0 mg·L⁻¹ and declined at higher concentrations. However, absolute multiplication rates remained modest across treatments, and several treatments were associated with excessive callus formation, abnormal shoot coloration, weak growth, or explant decline. These observations indicate that, although MS supplemented with 1.0 mg·L⁻¹ NAA + 1.0 mg·L⁻¹ 2iP represented the best-performing treatment among those tested (36.7%) (Figure 1E–G), the multiplication response of *Q. gilva* remains biologically limited under the present conditions.

Table 4. Effects of 2iP and NAA concentrations on shoot multiplication.

2iP (mg·L ⁻¹)	NAA (mg·L ⁻¹)	Multiplication Percentage (%)	Morphology of Proliferated Shoot
0	0	0.0 c	Died gradually after inoculation
0.5	0.5	13.3 ± 3.3 b	Pale green, thin, and weak shoots
1.0	0.5	20.0 ± 5.7 ab	Pale green with excessive callus, inhibiting proliferation
1.5	0.5	23.3 ± 8.8 ab	Pale green, elongated shoots with poor vigor
0.5	1.0	20.0 ± 5.8 ab	Light yellow with weak growth
1.0	1.0	36.7 ± 8.8 a	Green with slow growth
1.5	1.0	23.3 ± 3.3 ab	Dark red with small leaves and basal callus
0.5	2.0	16.7 ± 3.3 b	Dark green, slow-growing, dwarf shoots
1.0	2.0	3.3 ± 6.7 b	Light yellow with weak growth
1.5	2.0	0.0 c	Callus formation without shoot proliferation
Significance			
2iP		ns	
NAA		*	
2iP × NAA		ns	

Values represent means ± SE of three independent replicates (each replicate consisted of 10 explants, and the replicate was treated as the experimental unit). Different letters within a column indicate significant differences at $p < 0.05$. * indicates significant differences at $p < 0.05$; ns indicates no significant differences at $p < 0.05$.

3.3. Ex Vitro Rooting

Auxin treatment significantly improved ex vitro rooting percentage compared with the control. IBA and the interaction between IBA × NAA showed highly significant effects on rooting percentage ($p < 0.01$), whereas NAA alone showed a significant but weaker effect ($p < 0.05$) (Table 5). Average root number did not differ significantly among treatments. These results indicate that IBA exhibited a stronger statistical influence on rooting percentage in the factorial design tested. When the concentration of IBA reached 800 mg·L⁻¹, the rooting response declined compared with 600 mg·L⁻¹. The highest rooting percentage (70.0%) was achieved at 600 mg·L⁻¹ IBA + 700 mg·L⁻¹ NAA. Although this treatment also showed the highest mean root number (5.0), differences in root number among treatments were not statistically significant.

Table 5. Effects of IBA and NAA concentrations on rooting.

IBA (mg·L ⁻¹)	NAA (mg·L ⁻¹)	Rooting Percentage (%)	Average Root No.
0	0	20.0 ± 0.0 f	3.0 ± 0.6 a
400.0	500.0	23.3 ± 3.3 ef	3.7 ± 0.3 a
400.0	700.0	43.3 ± 3.3 cd	4.8 ± 1.7 a
400.0	900.0	46.7 ± 3.3 bc	3.3 ± 0.6 a
600.0	500.0	56.7 ± 8.8 b	5.0 ± 1.0 a
600.0	700.0	70.0 ± 5.8 a	5.0 ± 0.6 a
600.0	900.0	26.6 ± 3.3 ef	4.5 ± 0.8 a
800.0	500.0	40.0 ± 5.8 cd	4.2 ± 0.6 a
800.0	700.0	33.3 ± 3.3 de	4.0 ± 0.0 a
800.0	900.0	33.3 ± 3.3 de	3.2 ± 1.7 a
Significance			
IBA		**	ns
NAA		*	ns
IBA × NAA		**	ns

Values represent means ± SE of three independent replicates (each replicate consisted of 10 explants, and the replicate was treated as the experimental unit). Different letters within a column indicate significant differences at $p < 0.05$. ** and * indicate significant differences at $p < 0.01$ and $p < 0.05$, respectively; ns indicates no significant differences at $p < 0.05$.

4. Discussion

4.1. Effects of Disinfection and Anti-Browning Treatments on Aseptic Establishment

Disinfection of stem explants is the first and crucial step in establishing an in vitro propagation system, and both the type of disinfectant and the duration of treatment play decisive roles in successful culture initiation [36,37]. Mercuric chloride (0.1% HgCl₂) is commonly used as a sterilizing agent in woody plant tissue culture and has been widely applied in the disinfection of explants. Across *Quercus* species, nodal explant sterilization typically relies on immersion in 0.1% HgCl₂ for 5–10 min, which represents a compromise between effective microbial control and minimization of tissue injury and phenolic browning [13,15,38]. Consistent with these reports, our results demonstrated that an 8 min exposure to 0.1% HgCl₂ combined with running water rinsing (15 min) provided optimal sterilization for *Q. gilva* stem explants with significantly lower contamination and browning percentages compared with other treatments ($p < 0.01$).

Browning control is another critical step in the establishment of a rapid propagation protocol, as phenolic oxidation often hampers culture initiation. Browning can be reduced by selecting appropriate explants, lowering culture temperature, maintaining explants in darkness during the initial culture period, and incorporating antioxidants, adsorbents, and competitive inhibitors [39,40]. Traditionally, the application of various browning inhibitors has been used to prevent browning in plant tissue culture. Both VC (antioxidant type) and activated charcoal (adsorbent type) are widely used anti-browning agents. VC protects against oxidative stress that damages cellular structure and maintains phenolic homeostasis. Pretreating explants of *Q. robur* and *Q. petraea* with VC (100 mg·dm⁻³) showed a significantly positive effect against the rapid and harmful browning of explants [27]. Activated charcoal operates through intermolecular hydrogen bonding and van der Waals forces to absorb substances that cause browning and is often used in conjunction with other browning inhibitors. For instance, a combination of 1.0 g·L⁻¹ activated charcoal and 30 g·L⁻¹ Na₂S₂O₃ effectively inhibits browning in *Prunus avium* [41]. In this study, we tested these two types of anti-browning agents and found that the optimal anti-browning treatment for *Q. gilva* stem explants was 1.0 g·L⁻¹ VC soaking combined with 3.0 g·L⁻¹ activated charcoal.

4.2. Effects of Plant Growth Regulators on Shoot Induction, Shoot Multiplication, and Ex Vitro Rooting

Plant growth regulators (PGRs) play essential roles in shoot induction, shoot multiplication, and ex vitro rooting. The effects of PGR types and concentrations vary across species, explant ages, explant cut positions, nutritional status, and culture conditions. The effect of genotype on the optimal PGR treatment was shown to be one of the limiting factors in the commercial micropropagation of oaks [42].

Among different oak species, the optimal type and concentration of PGRs for shoot induction varied considerably. Half-strength WPM with $2.0 \text{ mg}\cdot\text{L}^{-1}$ BA produced more new buds per nodal segment, and the induced shoots were more vigorous than other treatments in the initiation culture of *Q. aliena* [18,38] observed that the concentration of BA needed to be periodically reduced following a 6-week cycle of *Q. ilex* shoots. For the initial culture of *Q. robur* shoot explants, Zeatin and BA resulted in greater elongation compared with 2iP [43]. In our preliminary study, we tried the most commonly used cytokinin BA, which yielded a low percentage of shoot induction in *Q. gilva*. Here, we investigated the effect of TDZ, a type of cytokinin that has been widely used in the micropropagation of diverse species of the family Fagaceae [25], on shoot induction in *Q. gilva*. Strong shoot induction responses were observed with the combinations of $0.5 \text{ mg}\cdot\text{L}^{-1}$ TDZ and $1.0 \text{ mg}\cdot\text{L}^{-1}$ 2,4-D.

The effect of PGR type and concentration on shoot multiplication varies considerably among oak species. In *Q. serrata*, WPM supplemented with $2.0 \text{ mg}\cdot\text{L}^{-1}$ BA produced the highest number of shoots [44]. In *Q. aliena*, $1.0 \text{ mg}\cdot\text{L}^{-1}$ BA + $0.01 \text{ mg}\cdot\text{L}^{-1}$ IBA significantly promoted shoot multiplication [17]. In *Q. ilex*, the highest shoot proliferation percentage was achieved through alternating two-week subcultures on a much lower cytokinin concentration [17]. In *Q. gilva*, MS medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ 2iP and $1.0 \text{ mg}\cdot\text{L}^{-1}$ NAA produced the highest shoot multiplication percentage among the treatments tested; however, the overall multiplication response remained relatively low. This limited proliferation capacity is consistent with the well-recognized recalcitrance of many *Quercus* species at the multiplication stage of micropropagation [18]. In the present study, several treatments also induced undesirable responses such as callus overgrowth, abnormal coloration, or weak shoot vigor, indicating that the hormonal balance and culture conditions are not yet optimal. Therefore, the identified treatment represents the best-performing condition under the tested parameters rather than a fully optimized multiplication system, and further refinement of PGR combinations or culture strategies will be required to improve propagation efficiency.

Certain woody perennial species, particularly oaks, often exhibit limited capacity for adventitious root formation. Root induction has therefore been widely investigated through optimization of auxin concentration and exposure time, dark treatment, and the adoption of ex vitro rooting approaches [45,46]. Ex vitro rooting with high-concentration auxin dips is frequently applied to enhance rooting by reducing callus formation and promoting more direct root development. Previous studies have reported that such treatments can markedly increase rooting percentage in oaks, in some cases reaching up to 80%, although responses are strongly genotype-dependent [47]. For example, a quick dip of microshoot bases in $1.0 \text{ g}\cdot\text{L}^{-1}$ IBA for 1 min resulted in high rooting percentages (54–80%) in *Q. petraea* [48]. Consistently, in the present study, superior rooting performance was achieved using ex vitro dips of $600 \text{ mg}\cdot\text{L}^{-1}$ IBA and $700 \text{ mg}\cdot\text{L}^{-1}$ NAA in *Q. gilva*. Ex vitro rooting is generally considered more efficient and less labor-intensive than in vitro rooting. Rooted microshoots produced via this method are commonly reported to acclimatize more successfully than in vitro-rooted plantlets. However, the post-acclimatization survival rate was not evaluated in the present study and warrants further investigation.

5. Conclusions

In this study, stem segments bearing axillary buds from two-year-old seedlings of *Q. gilva* were used as explants to establish an in vitro micropropagation pathway encompassing aseptic establishment, shoot induction, shoot multiplication, and ex vitro rooting. The optimal sterilization procedure was rinsing under running water for 15 min followed by immersion in 0.1% HgCl₂ for 8 min. Explant browning was effectively reduced by pre-soaking in MS medium containing 1.0 g·L⁻¹ VC and 3.0 g·L⁻¹ activated charcoal. The highest shoot induction percentage (80.0%) was achieved on MS medium supplemented with 1.0 mg·L⁻¹ 2,4-D and 0.5 mg·L⁻¹ TDZ, while shoot multiplication was obtained on MS medium containing 1.0 mg·L⁻¹ NAA and 1.0 mg·L⁻¹ 2iP. Ex vitro rooting was most successful when shoots were dipped in 600.0 mg·L⁻¹ IBA and 700.0 mg·L⁻¹ NAA, yielding a maximum rooting percentage of 70.0% and vigorous root systems. This study provides a foundational protocol for in vitro propagation of *Q. gilva*. Further studies evaluating genotype-dependent responses, clonal fidelity, performance of mature donor material, and post-acclimatization survival are necessary before large-scale operational deployment.

Author Contributions: Conceptualization, X.Q. and H.L.; formal analysis, X.-C.H. and X.Z.; funding acquisition, H.L.; investigation, X.-C.H., X.-F.Z. and L.-Q.C.; methodology, X.Z. and L.L.; project administration, H.L.; resources, L.O.; software, X.-C.H. and X.Z.; supervision, H.L.; validation, L.L.; visualization, X.-F.Z., T.-G.C. and L.-Q.C.; writing—original draft, X.-C.H.; writing—review and editing, H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Hunan Provincial Department of Education Excellent Youth Project [23B0251].

Data Availability Statement: All data are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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