

The effect of a modified DKW nutrient medium and antioxidant-adsorbent synergy on the *in vitro* regeneration of *Liriodendron tulipifera* L.

R.R. Khaydaraliyev* and Kh.Kh. Kushiev

Research Institute Agrobiotechnology and Biochemistry of Gulistan State University,
Uzbekistan

*Corresponding author email: rasuljonxaydaraliyev@gmail.com

ABSTRACT

The study aimed to improve the *in vitro* regenerative response of *Liriodendron tulipifera* L. by optimizing the mineral composition of the DKW nutrient medium and reducing phenolic oxidation, a major limitation in woody plant micropropagation. Agrochemical analysis of donor plant soils revealed deficiencies in phosphorus, zinc, and boron, which were corrected through targeted fertilization to enhance explant physiological quality. A modified DKW medium enriched with KH_2PO_4 (+30 mg L⁻¹), FeNaEDTA (+10.37 mg L⁻¹), and increased Mn and Zn concentrations significantly improved morphogenic performance compared with the standard DKW control. Among the hormonal treatments, the combination BAP 1.5 mg L⁻¹ + kinetin 0.5 mg L⁻¹ + GA₃ 0.2 mg L⁻¹ (RA variant) yielded the highest number of lateral buds, apical leaves, and lateral leaves after 21 days. Subsequent optimization with antioxidant-adsorbent combinations demonstrated that higher concentrations of ascorbic acid (150–250 mg L⁻¹) together with activated charcoal (0.5–1 g L⁻¹) markedly reduced phenolic oxidation and tissue necrosis. The V6 treatment (250 mg L⁻¹ ascorbic acid + 1 g L⁻¹ activated charcoal) achieved the highest explant viability (92%) and leaf formation. These results confirm that mineral-adjusted DKW medium combined with antioxidant-adsorbent synergy provides an effective protocol for the microclonal propagation of *L. tulipifera*, offering a reliable strategy for the regeneration of phenolic, woody species.

Keywords: activated charcoal, ascorbic acid, *Liriodendron tulipifera*, microclonal propagation, sterilization protocol

INTRODUCTION

Liriodendron tulipifera L., commonly known as the yellow poplar or tulip tree, is native to the eastern regions of North America - from southern New England west to southern Ontario and Michigan, extending south to Louisiana, and east to north-central Florida (Cao *et al.*, 2020). It is distributed throughout the Appalachian Mountain range, including Tennessee, North Carolina, Virginia, and Kentucky, as well as in the Ohio River Valley, Pennsylvania, New York, Alabama, Georgia, Mississippi, Illinois, and Arkansas (Meyer, 2020). The forests in these areas, particularly the Appalachian deciduous forests, represent the primary natural habitat of the tulip tree. This species grows best in deep, well-drained silt loam soils belonging mainly to the Inceptisol or Ultisol orders. Ultisol and Inceptisol soil types are generally characterized as acidic and low-fertility soils, with relatively low levels of nitrogen (N), phosphorus (P), and potassium (K) (Martínez *et al.*, 2007). These soils typically exhibit a slightly acidic pH ranging from 5.5 to 6.5. *Liriodendron tulipifera* naturally occurs in the Appalachian Mountains, the Ohio River Valley, and

the Near Eastern United States (Beck, 1962). Within such soil and climatic conditions, the species is distinguished from other forest trees by its rapid growth rate-reaching up to 40 meters in height within 50 years-its high-quality timber, and its ornamental characteristics (Russell, 1977). The straight trunk and conical crown shape make this species valuable for furniture, veneer, paper production, and as an ornamental tree in urban landscapes (Beck, 1962; Santamour and McArdle, 1984). Moreover, *L. tulipifera* is an important melliferous (honey-bearing) species and a natural source of ecologically and pharmacologically active compounds, including alkaloids, flavonoids, and phenolic acids (Parks *et al.*, 1995). However, its propagation through seeds is limited, with a germination rate of only about 10%. Vegetative propagation is also difficult due to lignification and phenolic oxidation. Therefore, the development of efficient *in vitro* microclonal propagation protocols is essential for conserving its genetic resources and for its use in breeding programs (Wilcox and Taft, 1969).

In vitro propagation protocols for various ornamental and valuable woody species have been developed using MS, WPM, and DKW nutrient media. However, the physiological characteristics of *L. tulipifera*-particularly its high susceptibility to phenolic exudation, tissue necrosis, and contamination-make the establishment of a stable protocol challenging. Consequently, adapting the mineral composition of the nutrient medium to the soil characteristics of its natural habitats, along with the use of antioxidants (ascorbic acid), adsorbents (activated charcoal), and optimized sterilization methods, is of critical importance for successful *in vitro* culture of this species.

This study aimed to develop an effective *in vitro* microclonal propagation protocol for *Liriodendron tulipifera* by using a modified DKW medium, determining the optimal phytohormone regime, reducing phenolic oxidation through the incorporation of ascorbic acid and activated charcoal into the medium, and improving sterilization protocols.

MATERIAL AND METHODS

To obtain donor plant material for the study, *Liriodendron tulipifera* trees cultivated under the soil and climatic conditions of the Fergana Valley and Syrdarya region were selected. Before explant collection, agrochemical analysis of the soils from these regions was carried out at the Scientific Research Institute of Agrobiotechnology and Biochemistry of Gulistan State University.

Experimental Site and Study Objective. The study was conducted in a controlled plant biotechnology laboratory dedicated to micropropagation research. The objective was to evaluate the *in vitro* regenerative performance of *Liriodendron tulipifera* explants cultivated on modified DKW nutrient media, assessing the influence of cytokinin-auxin balance, antioxidant supplementation, and activated charcoal on early regenerative responses.

Plant Material and Explant Establishment. Nodal segments of *Liriodendron tulipifera* were used as initial explants. Plant material was washed under running tap water, surface-sterilized using standard disinfection procedures (ethanol followed by sodium hypochlorite), then rinsed repeatedly with sterile distilled water. Explants were inoculated onto nutrient media under a laminar airflow hood to ensure aseptic conditions. Cultures were maintained at 24 ± 1 °C, under a 16 h photoperiod and a light intensity of $40\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$, conditions consistent with standard micropropagation protocols for woody species. The pH of all culture media was adjusted to 5.7 ± 0.1 before autoclaving at 121 °C for 20 minutes.

Culture Media Preparation. Three basal media were initially used for explant establishment: MS (Murashige and Skoog), WPM (Woody Plant Medium), and DKW (Driver and Kuniyuki Walnut medium). Based on internal optimization tests, DKW proved the most suitable and was subsequently modified according to the mineral profile of the species, following in situ soil analysis results.

Table 1. Composition of the modified DKW nutrient medium)

Component	Standard DKW	Modification applied	Final concentration
KH ₂ PO ₄	—	+30 mg·L ⁻¹	30 mg·L ⁻¹
FeNaEDTA	—	+10.37 mg·L ⁻¹	10.37 mg·L ⁻¹
MnSO ₄ ·H ₂ O	standard	+1.20 mg·L ⁻¹	increased
ZnSO ₄ ·7H ₂ O	standard	+1.00 mg·L ⁻¹	increased
Basal salts	5.6 g·L ⁻¹ DKW	unchanged	5.6 g·L ⁻¹
Sucrose	30 g·L ⁻¹	unchanged	30 g·L ⁻¹
Agar	7 g·L ⁻¹	unchanged	7 g·L ⁻¹
pH	adjusted	5.7 ± 0.1	—

All media used in both experimental stages contained sucrose (30 g·L⁻¹) and agar (7 g·L⁻¹), with identical cytokinin, auxin, and GA₃ concentrations unless otherwise specified.

The experiment included two stages: Stage 1 — Hormone and Antioxidant Treatments (RD, RA, RB, RC). This stage evaluated combinations of cytokinins, auxins, gibberellin, and an antioxidant, together with a treatment involving activated charcoal. Stage 2 — Antioxidant and Adsorbent Optimization (A1–A6).

This stage assessed combinations of ascorbic acid and activated charcoal under identical hormone conditions.

Table 2. Experimental variants for Stage 1 (RD, RA, RB, RC)

Experimental variant	Cytokinins BAP+Kinetin mg·L ⁻¹	Auxins IBA/ mg·L ⁻¹	Gibberellin GA ₃ /mg·L ⁻¹	Antioxidant ASA /mg·L ⁻¹	Activated charcoal
RD – Control	BAP 1.5 + Kinetin 0.5	0.1	—	—	—
RA	BAP 1.5 + Kinetin 0.5	0.1	—	100	—
RB	BAP 1.5 + Kinetin 0.5	0.1	0.2	100	—
RC	BAP 1.7 + Kinetin 0.6	0.2	—	100	300

Table 3. Experimental variants for Stage 2 (A1–A6)

Experimental Variant	Ascorbic acid (mg·L ⁻¹)	Activated charcoal (g·L ⁻¹)
V1	150	0.5
V2	200	0.5
V3	250	0.5
V4	150	1.0
V5	200	1.0
V6	250	1.0

Observation Stages and Measured Parameters

Explants were evaluated at 7, 14, and 21 days after inoculation. The following parameters were recorded: number of newly formed lateral buds; number of apical leaves; number of lateral leaves; coloration score (1–5), indicating explant physiological condition. For the color scoring assessment, the following scale was applied: 5 – fully saturated healthy green, indicating optimal growth; 4 – green but slightly lighter, suggesting minor stress or insufficient light; 3 – yellow-green, indicating nutrient deficiency or hormonal imbalance; 2 – completely yellow, associated with chlorosis (Fe or Mg deficiency) and physiological stress; 1 – brown or blackened, indicating necrosis or severe tissue degradation (George et al. 2008), (Bhojwani and Razdan 1996), and FAO/IAEA (2009)

Statistical Analysis

All quantitative data were processed using analysis of variance (ANOVA). Differences between treatments were considered statistically significant at $p < 0.05$. Means were presented with standard deviations, and statistically significant differences from the control were marked accordingly (* $p < 0.05$).

RESULTS AND DISCUSSIONS

Explants grown on WPM exhibited limited development, with reduced lateral bud activation and slower leaf expansion compared with those cultured on DKW medium (Figure 1). In contrast, DKW supported more vigorous early growth, characterized by enhanced shoot elongation, increased lateral bud formation, and improved overall explant vigor. These differences highlight the superior suitability of the DKW basal medium for initiating regenerative responses in *Liriodendron tulipifera* under antioxidant-free conditions (Figure 1).

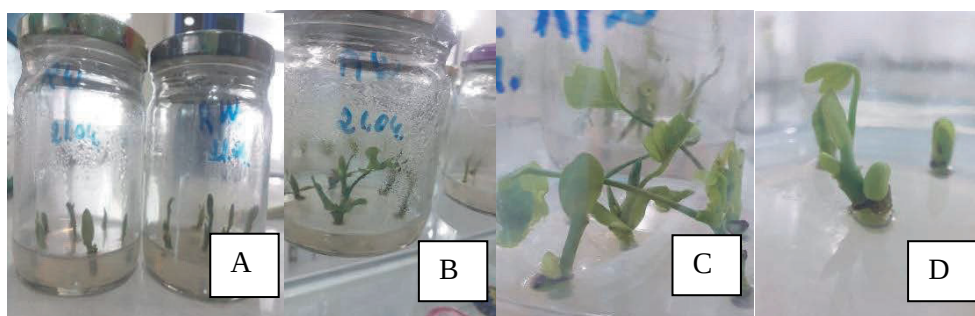


Figure 1. Comparative response of *Liriodendron tulipifera* explants cultured on WPM and DKW media in the absence of antioxidants and activated charcoal.

From left: (A) explants cultured on WPM medium on 21 April 2025, photographed on 2 May 2025; (B) explants on WPM medium photographed on 7 May 2025; (C) explants cultured on DKW medium photographed on 7 May 2025; (D) lateral shoots newly formed after 16 days of incubation on DKW medium.

Across all evaluation moments (7, 14, and 21 days), explants cultured in RA, RB, and RC variants exhibited significantly improved growth parameters compared with the RD control (Table 4). At 7 days, the antioxidant-supplemented media (RA, RB, RC) increased the number of lateral buds and early leaf initiation, indicating a reduction in oxidative stress during the initial adaptation period. By 14 days, all treated variants continued to outperform the control, particularly RA and RB, which induced higher numbers of lateral buds (1.26–1.33 vs. 1.06 in control) and lateral leaves (2.40–2.53 vs. 1.86). This demonstrates that both antioxidant (ASA) and GA₃ supplementation act synergistically in stimulating shoot and leaf development.

After 21 days, RA produced the strongest regenerative response, with the highest values for lateral bud formation (1.93) and lateral leaves (4.40), significantly exceeding the control. RB also maintained superior performance, while RC showed intermediate but still significant improvements, (Table 4).

According to the results of the study, the RA variant was identified as the most optimal, as it demonstrated the highest explant viability and new shoot formation rate. These findings provide a foundation for developing methods to propagate *Liriodendron tulipifera* using tissue culture technologies, applying biotechnology to its propagation process, and ensuring an alternative approach to regeneration, as well as for subsequent biotechnological work aimed at mass propagation and conservation.

Table 4. Schedule for the observation and recording of *in vitro* explant growth

Days	Container number	Number of new lateral bud(s)	Number of apical leaf/leaves	Number of lateral leaf/leaves	Color score (1-5)
7 day	RD control	0.33±0.48	0.46±0.51	0.8±1.01	4.86±0.35
	RA	0.4±0.5*	0.53±0.51*	1.06±1.03*	4.73±0.45
	RB	0.4±0.5*	0.66±0.48*	1.2±1.01*	4.8±0.41
	RC	0.46±0.51*	0.6±0.5*	0.93±1.03	4.73±0.45
14 day	RD control	1.06±0.45	0.86±0.63	1.86±0.91	4.8±0.41
	RA	1.26±0.88*	1.2±0.56*	2.53±1.4*	4.73±0.59
	RB	1.33±0.97*	1.13±0.63*	2.4±1.54*	4.66±0.48
	RC	1.13±0.99*	1±0.65*	2.13±1.59*	4.6±0.5
21 day	RD control	1.4±0.9	1.26±0.88	2.53±1.76	4.13±1.3
	RA	1.93±0.59*	2.06±0.59*	4.4±1.35*	4.53±0.63
	RB	1.86±0.74*	1.73±0.88*	3.86±2.06*	4.33±1.04
	RC	1.53±0.99*	1.53±0.83*	3.06±1.27*	4.2±1.08

*Note: statistical significance level compared to the control, $p < 0.05$. were considered statistically significant

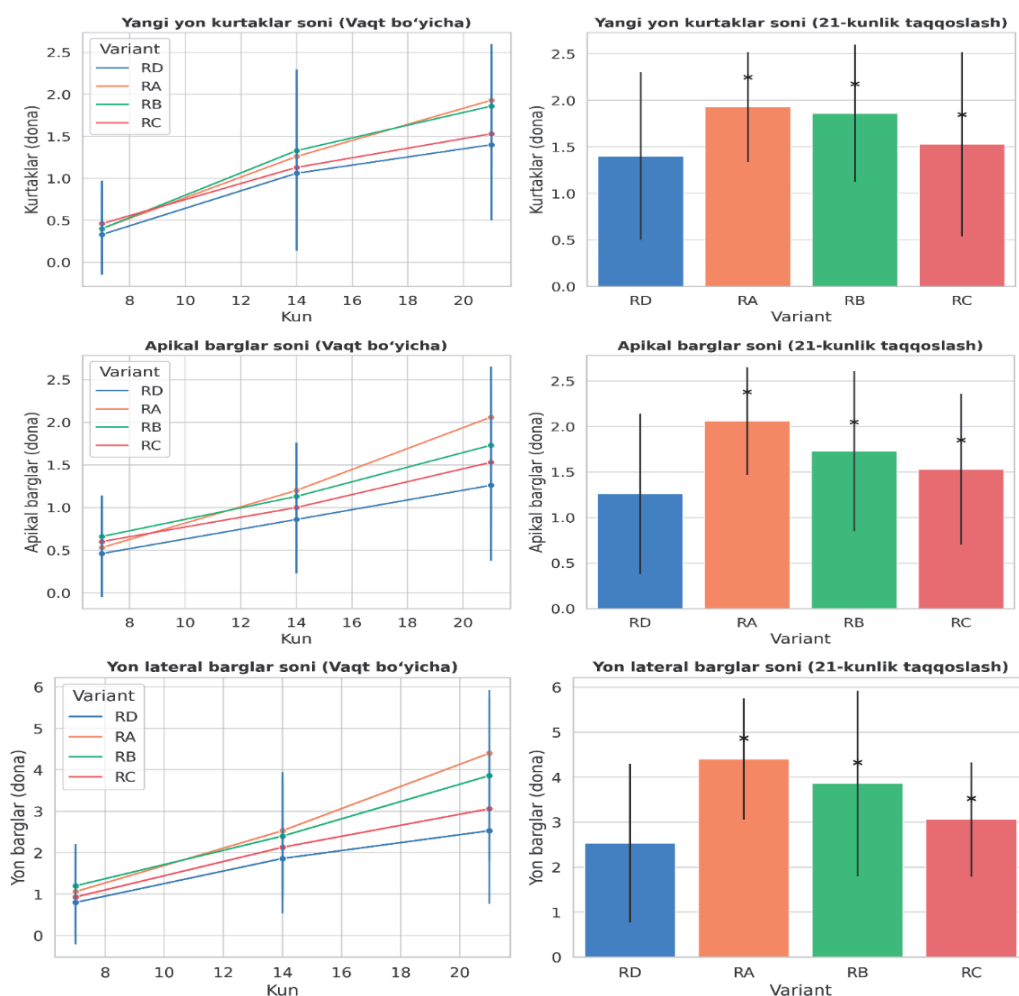


Figure 2. The line graphs illustrate the growth dynamics of newly formed lateral shoots, apical leaves, and lateral leaves in *Liriodendron tulipifera* explants during a 21-day incubation period, compared to the RD control



Figure 3. Days of growth stages of *Liriodendron tulipifera* explants (3, 7, 14 days)

Effect of increasing antioxidant and adsorbent concentrations on explant growth

In the above experiments, *Liriodendron tulipifera* explants were cultured *in vitro* using ascorbic acid (ASA) at a concentration of $100 \text{ mg}\cdot\text{L}^{-1}$ as the main antioxidant (Kang *et al.*, 2014; Zhong, 2019). The observations showed vigorous explant growth; however, since polyphenol oxidation and necrosis symptoms were not completely eliminated, activated charcoal (AC) and salicylic acid were additionally incorporated into the nutrient medium in the next experimental stage (Table 4). Polyphenol exudation at wounded sites often leads to necrosis and tissue browning (George *et al.*, 2008). Polyphenols are secondary metabolites widely distributed in woody plant species and are highly susceptible to rapid oxidation under *in vitro* conditions, leading to tissue browning and reduced regeneration efficiency (Krishnan and Siril, 2017).



Figure 4. Phenolic oxidation and medium browning observed upon the addition of $100 \text{ mg}\cdot\text{L}^{-1}$ ascorbic acid

To reduce the frequently observed phenolic oxidation, necrosis, and tissue browning in *in vitro* cultured explants (Gao *et al.*, 2019), as well as to decrease the formation of reactive oxygen species (ROS) (Şen, 2012), activate the systemic acquired resistance (SAR) mechanism, enhance stress tolerance, and stimulate bud differentiation and root induction, salicylic acid was added to the culture medium. Salicylic acid is known to enhance phenolic biosynthesis and increase the activity of antioxidant enzymes such as peroxidase (POD) and polyphenol oxidase (PPO) (Galal, 2012). Activated charcoal, on the other hand, adsorbs phenolic exudates and other toxic metabolites, but it can also partially absorb auxins and cytokinins from the medium (Weatherhead, 1978). Therefore, in the control RA variant, the concentrations of growth regulators were adjusted as follows: BAP increased from $1.5 \text{ mg}\cdot\text{L}^{-1}$ to $1.7 \text{ mg}\cdot\text{L}^{-1}$, kinetin from $0.5 \text{ mg}\cdot\text{L}^{-1}$ to $0.6 \text{ mg}\cdot\text{L}^{-1}$, and instead of $0.1 \text{ mg}\cdot\text{L}^{-1}$ IBA, $1 \text{ mg}\cdot\text{L}^{-1}$ IAA auxin was applied.

The response of *Liriodendron tulipifera* explants to different combinations of ascorbic acid (ASA) and activated charcoal (AC), (Table 4). Across all evaluation moments (7, 14, and 21 days), higher concentrations of antioxidants and adsorbents significantly influenced bud induction, leaf formation, and the intensity of phenolic oxidation.

At 7 days, low to moderate antioxidant levels ($150\text{--}200 \text{ mg}\cdot\text{L}^{-1}$ ASA) with $0.5 \text{ g}\cdot\text{L}^{-1}$ AC (variants V1 and V2) supported early bud formation and reduced phenolic exudation. Increasing ASA to $250 \text{ mg}\cdot\text{L}^{-1}$ (V3) slowed initial growth, whereas the addition of $1.0 \text{ g}\cdot\text{L}^{-1}$

AC (V4, V5, V6) inhibited elongation due to medium browning but further decreased phenolic oxidation. Medium darkening represents a serious problem in the *in vitro* culture of economically important woody plants, primarily due to high levels of lignification and accumulation of secondary metabolites. This phenomenon complicates the development of effective regeneration and genetic transformation systems across various species (Liu, et al.,2024). By 14 days, the treatments containing 200 mg·L⁻¹ ASA (V2, V5) resulted in the highest number of newly formed leaves and vigorous bud activity. The combination of 250 mg·L⁻¹ ASA + 1.0 g·L⁻¹ AC (V6) supported stable development and minimal phenolic oxidation, while 150 mg·L⁻¹ ASA + 1.0 g·L⁻¹ AC (V4) showed reduced elongation.

Table 4. *In vitro* growth of *Liriodendron tulipifera* explants in modified DKW nutrient medium supplemented with higher concentrations of ascorbic acid and different amounts of activated charcoal (observations at 7, 14, and 21 days.)

Days	Variant	Number of new lateral bud(s)	Number of apical leaf/leaves	Number of lateral leaf/leaves	Color score (1-5)
7 day	V1	0.53±0.51	0.66±0.61	1.0±0.53	4.5±0.5
	V2	0.6±0.50	0.73±0.45	1.06±0.59	4.7±0.5
	V3	0.66±0.61	0.8±0.86	1.13±0.74	4.6±0.6
	V4	0.66±0.48	0.53±0.91	1.06±0.79	4.4±0.6
	V5	0.73±0.45	0.80±1.01	1.4±0.82	4.8±0.4
	V6	0.66±0.48	0.76±0.94	1.26±0.79	4.7±0.5
14 day	V1	1.06±0.45	1.13±0.63	2.4±1.12	4.5±0.6
	V2	1.26±0.88	1.26±0.79	2.53±1.18	4.7±0.5
	V3	1.4±0.91	1.46±0.74	2.66±1.44	4.6±0.5
	V4	1.13±0.99	1.26±1.09	2.53±0.91	4.4±0.6
	V5	1.33±0.97	1.4±0.63	2.8±1.65	4.7±0.5
	V6	1.46±0.74	1.53±1.35	3.33±1.79	4.8±0.4
21 day	V1	1.73±0.59	2.13±0.83	3.73±1.27	4.3±0.8
	V2	1.8±0.67	2.00±0.92	4.00±1.51	4.6±0.5
	V3	1.93±0.70	2.53±1.35	4.53±1.18	4.5±0.6
	V4	1.53±0.99	2.46±0.55	4.26±1.48	4.2±0.8
	V5	2.13±0.51	2.66±1.04	4.66±0.97	4.7±0.5
	V6	2.26±0.70	3.06±0.27	4.93±1.27	4.5±0.6

After 21 days, the strongest responses were recorded in variants V2, V5, and V6, which reached the highest values for lateral bud formation and total leaf number. Viability reached 82% in V2, 90% in V5, and 92% in V6, indicating that high ASA combined with AC promotes long-term stability and reduces oxidative tissue damage, although excessive antioxidant levels (250 mg·L⁻¹) may slightly reduce growth rate despite high survival. These results confirm that ASA and AC play a synergistic role in reducing oxidative stress and improving explant viability under *in vitro* conditions.

CONCLUSION

The mineral modification of the DKW medium significantly enhanced the regenerative response of *Liriodendron tulipifera* explants, confirming the importance of adapting nutrient balance to species-specific ecological requirements. Among the hormonal treatments, the RA combination (BAP 1.5 mg L⁻¹, kinetin 0.5 mg L⁻¹, GA₃ 0.2 mg L⁻¹) proved to be the most effective, producing the highest number of lateral buds and leaves and achieving superior explant viability. Phenolic oxidation could not be fully controlled with 100 mg L⁻¹ ascorbic acid alone; however, the synergistic use of ascorbic acid and activated charcoal markedly reduced browning and

necrosis. The A6 treatment (250 mg L⁻¹ ASA + 1 g L⁻¹ AC) provided the best overall performance, achieving 92% viability and promoting vigorous leaf development. Overall, the optimized protocol offers a robust and efficient approach for the *in vitro* micropropagation of *L. tulipifera* and may be applied to other woody species prone to oxidative stress.

In the future, further optimization can focus on improving the rooting and ex vitro acclimatization stages, as well as applying this protocol to large-scale propagation and conservation programs for *Liriodendron tulipifera*

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