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Concentration-Dependent Role of 2,4-Dichlorophenoxyacetic Acid in Callus Induction, Somatic Embryogenesis, and Antioxidant Responses in Rubber Tree (*Ficus elastica)*

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ABSTRACT

This study examined the influence of 2,4-dichlorophenoxyacetic acid (2,4-D) on callus development, embryogenic potential, and antioxidant metabolism in Ficus elastica. Leaf explants were cultured on Murashige and Skoog medium containing 0.0, 0.5, 1.0, 1.5, or 2.0 mg L^{-1} *(unit revised from mg·L⁻¹)* 2,4-D. Auxin concentration significantly affected morphogenetic and biochemical parameters ($p < 0.01$) *(sentence revised as suggested)*. The optimal response occurred at 1.0 mg L^{-1}, where callus induction reached 85.2 ± 1.1% with a biomass of 3.18 ± 0.14 g $explant^{-1}$ *(unit revised from g·explant⁻¹)* and the shortest initiation time of 22.6 ± 0.5 days ($p = 0.007$). Callus induction (%) = (Number of explants forming callus / Total number of explants cultured) × 100 *(formula added)*. Somatic embryogenesis was most successful at this level, producing 74.4 ± 1.2% embryo induction, a quality index of 5.1 ± 0.4, and 54.6 ± 1.6% plantlet conversion ($p = 0.006$). Somatic embryogenesis (%) = (Number of explants producing somatic embryos / Total number of explants cultured) × 100 *(formula added)*. Regenerated plantlets from the same treatment showed superior rooting (91.4 ± 1.4%) and survival following acclimatization (85.3 ± 1.4%; $p = 0.006$). Antioxidant enzyme activity was also highest at 1.0 mg L^{-1}, with superoxide dismutase, catalase, ascorbate peroxidase, and peroxidase activities significantly enhanced ($p = 0.006$), while oxidative stress markers such as H_2O_2, malondialdehyde, and electrolyte leakage were at their lowest. Phenolic compounds (1.77 ± 0.05 mg GAE g^{-1} FW; $p = 0.007$) and free proline (2.27 ± 0.06 µmol g^{-1} FW; $p = 0.007$) also peaked at this concentration. In contrast, 2.0 mg L^{-1} suppressed morphogenesis and increased oxidative damage ($p = 0.007$). Collectively, these findings confirm that 2,4-D regulates morphogenesis and stress physiology in a concentration-dependent manner, with 1.0 **mg L^{-1}** identified as the most effective for optimizing regeneration in *F. elastica*.

***Keywords*:** Rooting and acclimatization, Callus Induction, Phenolic compounds, Hydrogen peroxide (H_2O_2), *Ficus elastic*.

INTRODUCTION

Plant tissue culture provides a controlled environment for propagation, crop enhancement, maintenance, cellular and developmental processes, documented as modern plant biotechnology [1,2]. Production of valuable secondary metabolites, propagation of elite genotypes, and conservation of endangered germplasm are a wide range of applications of tissue culture [3]. Many tissue culture systems exhibit a phenomenon that allows somatic cells to enter a totipotent state, which lies in the capability of plant cells to undertake reprogramming and dedifferentiation [4]. Somatic embryogenesis and Callus formation serve as the foundation for genetic engineering systems and plant regeneration that characterize one of the most vital outcomes of this reprogramming [5]. Callus induction is a preliminary step in which plant explants grow in proliferative cell masses and are unorganized under nutritional and hormonal conditions [6]. Auxins are most frequently employed in plant tissue culture for initiating dedifferentiation, such as Callus induction, and recognized as growth regulators [7]. Furthermore, synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) induces cellular proliferation and callogenesis in a diversity of species due to its strong capability [8]. 2,4-D establishes the conditions essential for supplementary morphogenesis by changing gene expression and modulating endogenous hormonal balance that promotes re-entry of somatic cells into the cell cycle [9]. Low to moderate levels of 2,4-D concentration play a pivotal role in shaping developmental outcomes, often

promoting embryogenesis and callus formation, while oxidative stress, inhibited morphogenesis, and abnormal tissue growth consequences at supra-optimal concentrations [10]. While higher concentrations lead to reduced regeneration productivity and tissue necrosis, the optimal window of 2,4-D exposure is essential for effective induction of embryogenic calli [11]. The hormonal necessities for embryogenesis vary significantly among plant taxa [12]. Developmental reprogramming to form bipolar structures is a unique regeneration pathway in Somatic embryogenesis, where somatic cells undergo zygotic embryogenesis [13]. These somatic embryos can develop into whole plants, assembling a precise system for genetic transformation and large-scale propagation [14]. Growth regulators play an important role in stress-related signaling, the physiological state of the explant, and the development of somatic embryos [15,16]. It also plays a dual role, serving as both inducers and inhibitors of development and embryogenesis under endogenous and exogenous stress conditions [17]. Hydrogen peroxide, Morphogenesis, and dedifferentiation are closely associated with oxidative stress within cultured tissues and are generated as byproducts of metabolic activity [18,19]. While modest levels of ROS trigger developmental reprogramming, act as signaling molecules, and extreme accumulation can lead to cellular damage [20]. Furthermore, the non-enzymatic metabolites, such as proline and phenolic complexes, play vital roles in embryogenesis and in stress adaptation [21]. Phenolics act as antioxidants by defending cellular structures and scavenging free radicals, while Proline serves as a stabilizing protein under stress [22, 23]. Accumulation of these metabolites improved tolerance to in vitro stress conditions with good embryogenic potential [24, 25].

Ficus elastica, belongs to the Moraceae family, frequently recognized as the rubber plant, and is valued for its capacity to improve indoor air quality and as an ornamental foliage plant [26]. Its wide-ranging physiological resilience and adaptability make it a striking candidate for large-scale propagation and for biotechnological improvement [27]. However, associated with other members of the Moraceae, such as Morus alba and Ficus carica, comparatively limited studies have been conducted on regeneration and in vitro culture of F. elastica [28]. Ficus elastica remains comparatively underexplored and lacks a well-established standardized in vitro regeneration protocol, highlighting the necessity of the present study**.** This knowledge gap limits the expansion of transformation systems and reliable propagation, which are important for their wider horticultural application and future improvement. Therefore, this study was conducted to clearly investigate the role of 2,4-D in regulating callus induction and somatic embryogenesis in F. elastica and to establish a standardized and efficient micropropagation protocol.

Materials And Methods

1-Experimental design, plant material

Young, healthy explants (young leaf segments, approximately 0.5–1.0 cm in length) of *Ficus elastica* were excised from the apical and sub-apical regions of disease-free, 1–2-year-old field-grown mother plants in southern Iraq. The selected explants were physiologically active, free from visible damage, infection, and stress symptoms. Five 2,4-D treatments (0.0, 0.5, 1.0, 1.5, and 2.0 $mg \cdot L^{-1}$) were used, and each treatment comprised three replicates; each replicate contained 30 explants (total explants per treatment = 90).

2-Explant preparation and sterilization

The Explants 0.5–1.0 cm segments were trimmed and treated under aseptic conditions in a laminar-flow cabinet. Systematically cleaned for 20 min under a continuous stream of tap water, and sterilized for 30 seconds by immersion in 70% ethanol, and mercuric chloride solution 0.1% (w/v) under aseptic conditions for 5 min with gentle agitation. Explants were washed three times with sterile distilled water to eliminate traces of sterilizing agents, blotted dry on sterile filter paper, and directly transported to culture medium [29].

3-Culture medium and conditions

Murashige and Skoog (MS) basal medium and vitamins were used, enhanced with 3% (w/v) sucrose, agar-0.7% (w/v), and coagulated. Before autoclaving, at 121 °C for 15 min, the medium pH was adjusted to 5.8. Stock solution (1,000 $mg \cdot L^{-1}$) of 2,4-D (analytical grade) was prepared in a small volume of 1 N NaOH, filter-sterilized (0.22 µm), and added aseptically to cooled medium to reach desired levels viz; (0.0 (control), 0.5, 1.0, 1.5, and 2.0 $mg \cdot L^{-1}$). For callus induction, these desired levels were incorporated into the medium**.** At 25 ± 2 °C, cultures were incubated in complete darkness. Subculturing was achieved every 28 days to keep callus proliferation [30 , 31].

4-Callus Induction and Somatic Embryogenesis Assessment

The callus formation was measured by recording the mean number of days from inoculation until the first noticeable appearance of callus in the receptive explants. To determine callus biomass, samples were harvested on the 28^{th} day, carefully blotted on sterile filter paper to eliminate excess surface moisture, and immediately weighed using an analytical balance with an accuracy of 0.01 g. The morphological structures of the callus were observed under aseptic conditions and classified into separate categories, with compact, friable, embryogenic, and non-embryogenic

forms. Explants were sited separately on MS callus induction medium (one explant per culture tube). The callus induction proportion was calculated as described by previously [32 , 33]:

$$\textbf{Callus induction (\%)} = \frac{\textbf{Number of explants forming callus}}{\textbf{Total number of explants cultutred}} \times \mathbf{100}$$

5-Somatic embryogenesis, embryo scoring, and plantlet conversion

Calli were assessed after 8–12 weeks for somatic embryo development. Embryo induction frequency was calculated as the percentage of calli forming embryos from the total detected. Embryo quality was scored on a 1–5 scale, where 1 designated necrotic and 5 showed well-formed bipolar embryos. Healthy embryos were transferred to regeneration medium (½ MS or MS without auxin, with or without 0.1–0.5 mg·L^{-1} BAP), where BAP treatments were applied to specific groups of embryos, while a control group was maintained on medium without BAP for comparison and conversion efficiency was determined as the percentage of embryos producing plantlets. Shoots ≥2 cm were removed and moved to rooting medium (MS + 2.0 mg·L^{-1} IBA). [34]

$$\textbf{Somatic embryogenesis (\%)} = \frac{\textbf{Number of calli forming embryos}}{\textbf{Total number of calli observed}} \times \mathbf{100}$$

$$\textbf{Conversion (\%)} = \frac{\textbf{No. embryos producing plantlets}}{\textbf{Total embryos transferred}} \times \mathbf{100}$$

6-Rooting and acclimatization

After 4–6 weeks on MS medium, rooting was assessed, and the medium was enhanced with IBA. Rooting proportion was calculated as the plantlets that developed roots relative to the total number transported. The average number of roots per plantlet was noted by counting roots longer than 1 cm, and the mean root length was determined from five representative roots per plantlet. Acclimatization success was assessed under gradually reduced humidity as the percentage of plantlets that survived four weeks after transfer to a peat: sand (1:1) substrate as described by [35].

7-Biochemical sampling and general extraction procedure

Callus samples were collected for biochemical assays at the same time of day on day 28. ~0.5–1.0 g fresh weight (FW) of callus was harvested from each treatment. Flash-frozen in liquid nitrogen and stored at −80 °C until analysis. All extractions were performed on ice. For enzyme assays, in liquid nitrogen, tissue (0.5 g FW) was crushed and homogenized in 5 mL ice-cold extraction buffer (50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1% (w/v) polyvinylpolypyrrolidone (PVPP), and 0.1% Triton X-100). Homogenates were centrifuged at 12,000 × g for 15 min at 4 °C, and supernatants were collected for assays. Protein extracts were quantified by using bovine serum albumin (BSA) as a standard; enzyme activities are expressed per mg protein as described [36, 37, 38].

8- Antioxidant Enzyme Activity and Oxidative Stress Markers

Enzymatic antioxidant activities from fresh callus tissue extracts were examined: The SOD (activity of superoxide dismutase) was determined following the protocol drawn by Marklund and Marklund [39], with Absorbance at 560 nm and with slight alterations by Hasanuzzaman *et al.,* [40]. Catalase (CAT) activity was recorded by the adapted method of Aebi [37]. The decline in absorbance of H_2O_2 at 240 nm was used to quantify enzyme activity [5]. Peroxidase activity was measured using guaiacol and monitoring the rise in absorbance at 470 nm due to guaiacol oxidation [41]. The absorbance (at 290 nm), ascorbate peroxidase (APX), was determined due to ascorbate oxidation [42]. Coefficient for ascorbate reduction (ε = 2.8 $mM^{-1}\cdot cm^{-1}$ at 290 nm) as used and expressed as µmol ascorbate oxidized min^{-1} mg^{-1} protein with analogous calculation to CAT. Hydrogen peroxide (H_2O_2) measured by the potassium iodide reaction, with absorbance at 390 nm; H_2O_2 content shown as µmol H_2O_2 g^{-1} FW by means of a calibration curve organized with known H_2O_2 concentrations [43]. Lipid peroxidation (MDA) was assessed by the TBA reaction, MDA content absorbance at 532 and 600 nm to correct for non-specific turbidity [44]. Initial conductivity (C1) is measured after incubating callus tissue in deionized water at room temperature for a fixed period, then samples are boiled to release total electrolytes and final conductivity (C2) is recorded; electrolyte leakage is then calculated using these values).

CAT activity = ΔA240/ (∈ × l) × (V total)/ (V enzyme) × 1/ (Protien (mg))

POD activity = ΔA470/ (∈ × l) × (V total)/ (V enzyme) × 1/ (Protien (mg))

MDA (nmol g^{-1} FW) = ((A532-A600))/(∈TBA) × (V total)/W

Electrolyte leakage (%) = C1/C2 ×100

9- Determination of Total Phenolic Content

Following the Folin–Ciocalteu method, the total phenolic content was estimated [45]. Almost 0.5 g of fresh callus tissue was centrifuged at 10,000 rpm for 15 min and homogenized in 10 mL of 80% methanol. 0.5 mL supernatant

was incubated for 5 min and was mixed with 2.5 mL Folin–Ciocalteu reagent (1:10 dilution). Then, sodium carbonate solution 2.0 mL of 7.5% was added, and the mixture was set aside at room temperature for 30 min. A UV–V is spectrophotometer was used, and absorbance was recorded at 765 nm. The gallic acid standard curve was used for measuring the concentration of phenolics, and the outcomes were expressed as mg gallic acid equivalent (GAE) per g fresh weight (FW) using the equation:

Total phenolics (mg GAE\cdotpg⁻¹ FW) = $\frac{C \times V}{M}$

Where:

CCC = concentration from calibration curve (mg·mL⁻¹), VVV = extract volume (mL), MMM = mass of fresh callus tissue (g)

10- Determination of Free Proline Content

By the technique of Bates, Waldren, and Teare [46], Free proline content was measured. 0.5 g of fresh callus tissue, at 10,000 rpm for 15 min, was centrifuged and homogenized in 10 mL of 3% sulfosalicylic acid. Two milliliters of the extract were mixed in a test tube (with acid ninhydrin reagent 2 mL and 2 mL of glacial acetic acid), followed by heating at 100 °C for 1 h. The absorbance was recorded at 520 nm. And 4 mL toluene was added to extract the chromophore. The reaction was stopped in an ice bath. Proline absorption was measured from a standard curve equipped with L-proline and expressed as µmol·g⁻¹ FW using the following formula:

Proline (µmol\cdotpg⁻¹ FW) = $\frac{A \times Vt}{115.5 \times W \times Vs}$

Where: A= proline concentration from standard curve (µg·mL⁻¹), Vt= total extract volume (mL), W= fresh weight of sample (g), VsV= volume of sample used (mL), 115.5 = molecular weight of proline.

11- Statistical Analysis

One-way analysis of variance (ANOVA) was performed, and Significant differences were recorded by using Duncan's Multiple Range Test (DMRT) at a significance level of $p \leq 0.05$. Data were expressed as mean ± standard error.

Results

The response of Ficus elastica explants to 2,4-D at various levels is shown in Table 1. A basal medium without 2,4-D (control) showed a very inadequate callus induction rate (6.2 ± 0.7%), which was significantly lower than all auxin-supplemented treatments ($p < 0.01$). At 0.5 mg·L⁻¹ 2,4-D, callus induction improved substantially to 66.6 ± 2.2% with an average callus fresh weight of 1.77 ± 0.16 g per explant. However, callus initiation was relatively delayed, appearing after 33.5 ± 1.3 days ($p = 0.006$ compared with the control). The maximum callogenic response was found at 1.0 mg·L⁻¹ 2,4-D, which documented 85.2 ± 1.1% induction with a mean fresh weight of 3.18 ± 0.14 g per explant and the shortest time to visible callus formation (22.6 ± 0.5 days). Statistical analysis confirmed that these values were significantly higher than those of both lower and higher concentrations ($p = 0.007$**)**. A further increase to 1.5 mg·L⁻¹ resulted in a modest decline in induction frequency (72.3 ± 1.4%) and biomass (2.72 ± 0.15 g), though these values remained statistically significant compared with the control ($p = 0.005$**)**. At 2.0 mg·L⁻¹ 2,4-D, callus induction dropped to 54.3 ± 2.7%, with a reduced fresh weight of 1.22 ± 0.12 g per explant and delayed initiation (35.4 ± 1.4 days). These reductions were statistically significant when compared with the optimal concentration ($p = 0.006$).

Table 1. Callus induction frequency, mean days to visible callus, and callus fresh weight after 28 days on MS medium with varying 2,4-D concentrations.

2,4-D (mg·L⁻¹)	Callus induction (%)	Days to visible callus (d)	Callus fresh weight (g · explant⁻¹)	Significance (p-value)
0.0 (Control)	6.2 ± 0.7a			
0.5	66.6 ± 2.2b	33.5 ± 1.3 c	1.77± 0.16 b	0.006
1	85.2± 1.1c	22.6± 0.5a	3.18± 0.14 c	0.007
1.5	72.3± 1.4c	25.5± 0.6b	2.72± 0.15c	0.005
2	54.3± 2.7b	35.4 ± 1.4d	1.22± 0.12 a	0.006

Note: NS = not significant; different letters (a, b, c, d) indicate statistically significant differences according to Duncan's multiple range test ($p < 0.05$).

The effect of 2,4-D concentration on somatic embryo induction, embryo morphology, and conversion efficiency is summarized in Table 2. No embryogenic response was recorded in the control treatment, confirming that auxin supplementation was essential for initiating somatic embryogenesis in Ficus elastica. At 0.5 mg·L⁻¹ 2,4-D, embryo

induction reached 34.2 ± 1.6%, with a mean embryo quality index of 2.5 ± 0.4 and a plantlet conversion rate of 22.1 ± 1.4%. These values were significantly greater than the control ($p = 0.005$). The most effective result was observed at 1.0 mg·L⁻¹, which produced the highest embryo induction frequency (74.4 ± 1.2%), superior embryo quality (5.1 ± 0.4), and the greatest plantlet conversion rate (54.6 ± 1.6%). Statistical analysis confirmed that this concentration was significantly different from all other treatments ($p = 0.006$). A moderate decline was observed at 1.5 mg·L⁻¹, where induction decreased to 57.4 ± 2.2% with a lower embryo quality index (3.2 ± 0.2) and conversion efficiency (43.4 ± 1.7%), though the results remained significantly higher than the control ($p = 0.005$). By contrast, the highest concentration (2.0 mg·L⁻¹) strongly inhibited embryogenesis, reducing induction to 21.6 ± 1.4%, embryo quality to 1.9 ± 0.4, and conversion efficiency to 14.6 ± 1.4%. These values were significantly lower compared with the optimal treatment ($p = 0.007$).

Table 2. Frequency of somatic embryo induction (8–12 weeks), embryo quality index (1–5 scale; 5 = best morphology), and embryo-to-plantlet conversion (%) after transfer to regeneration medium.

2,4-D (mg·L⁻¹)	Embryo induction (%)	Embryo quality index	Conversion to plantlet (%)	p-value
0.0 (Control)	0.0 ± 0.0 a		0.0 ± 0.0 a	
0.5	34.2± 1.6b	2.5± 0.4 b	22.1± 1.4b	0.005
1	74.4± 1.2c	5.1± 0.4c	54.6 ± 1.6c	0.006
1.5	57.4 ± 2.2 c	3.2± 0.2 c	43.4 ± 1.7b	0.005
2	21.6± 1.4b	1.9 ± 0.4 b	14.6± 1.4 b	0.007

Note: The embryo quality index (1–5 scale) is based on visual scoring and represents a subjective assessment. NS = not significant ($p > 0.05$). Different letters indicate significant differences according to Duncan's test.

The rooting response of regenerated *F.* elastica plantlets on IBA-supplemented medium is presented in Table 3. Plantlets derived from the control callus (0.0 mg·L⁻¹ 2,4-D) exhibited the lowest rooting frequency (44.2 ± 1.2%), with an average of only 2.4 ± 0.3 roots per plantlet, a mean root length of 3.1 ± 0.4 cm, and poor survival after acclimatization (42.6 ± 2.4%). These results were not significantly different from each other (NS), reflecting the weak morphogenic potential of control-derived callus. Supplementation with 0.5 mg·L⁻¹ 2,4-D during the callus induction stage substantially improved subsequent rooting performance. Rooting frequency increased to 74.3 ± 1.4%, accompanied by a higher mean root number (4.9 ± 0.3), root length (4.9 ± 0.4 cm), and acclimatization survival (71.4 ± 2.2%), which were all statistically significant compared with the control ($p = 0.007$). The maximum rooting efficiency was obtained from plantlets derived from 1.0 mg·L⁻¹ 2,4-D, showing 91.4 ± 1.4% rooting with 3.8 ± 0.4 roots per plantlet and the longest mean root length (7.9 ± 0.3 cm). Survival after acclimatization also peaked at 85.3 ± 1.4%. These improvements were statistically significant ($p = 0.006$). Plantlets originating from 1.5 mg·L⁻¹ 2,4-D shown 79.4 ± 1.3% rooting with the highest mean root number (6.3 ± 0.3) and moderate root length (5.9 ± 0.4 cm). Acclimatization survival remained high at 79.1 ± 1.6% ($p = 0.007$). By contrast, 2.0 mg·L⁻¹ 2,4-D negatively affected rooting and survival, reducing rooting frequency to 62.4 ± 1.6%, with only 4.9 ± 0.4 roots per plantlet, a mean root length of 3.9 ± 0.4 cm, and a survival rate of 64.3 ± 2.4% ($p = 0.005$).

Table 3. Rooting percentage on IBA (2.0 mg·L⁻¹) rooting medium, mean root number and root length, and survival after acclimatization.

Origin (2,4-D)	Rooting (%)	Mean roots/plantlet	Root length (cm)	Acclimatization survival (%)	p-value
0.0 (Control)	44.2 ± 1.2d	2.4± 0.3d	3.1± 0.4d	42.6± 2.4d	NS
0.5	74.3± 1.4a	4.9± 0.3a	4.9± 0.4a	71.4± 2.2a	0.007
1	91.4± 1.4b	3.8± 0.4b	7.9± 0.3b	85.3± 1.4b	0006
1.5	79.4± 1.3b	6.3± 0.3b	5.9± 0.4 b	79.1 ± 1.6b	0.007
2	62.4± 1.6c	4.9± 0.4c	3.9± 0.4c	64.3± 2.4c	0.005

Note: NS = not significant ($p > 0.05$). Different letters indicate significant differences according to Duncan's test.

The different concentrations of 2,4-D had a significant influence on the antioxidant enzyme system and oxidative stress indicators in *F. elastica* callus tissues (Table 4). In the control group (0.0 mg·L⁻¹), relatively low activities of superoxide dismutase (SOD, 12.1 ± 0.4 U mg⁻¹ prot), catalase (CAT, 15.4 ± 0.3 U mg⁻¹ prot), ascorbate peroxidase (APX, 7.6 ± 0.4 U mg⁻¹ prot), and peroxidase (POD, 18.2 ± 1.3 U mg⁻¹ prot) were observed. In contrast, oxidative

damage was more evident, with elevated levels of hydrogen peroxide (H_2O_2, 5.9 ± 0.4 µmol g^{-1} FW), malondialdehyde (MDA, 6.5 ± 0.5 nmol g^{-1} FW), and electrolyte leakage (14.2 ± 0.1%), which together reflected a baseline stress status (p = 0.005). At 0.5 mg·L^{-1} 2,4-D, there was a noticeable enhancement in enzymatic defense. SOD, CAT, APX, and POD activities rose to 16.3 ± 0.9, 26.2 ± 1.3, 14.4 ± 0.6, and 33.2 ± 1.7 U mg^{-1} prot, respectively (p = 0.007). This enzymatic upregulation coincided with a reduction in stress indicators, as H_2O_2 content dropped to 4.6 ± 0.4, MDA to 3.9 ± 0.6, and electrolyte leakage to 11.2 ± 0.5%. The most pronounced response was recorded at 1.0 mg·L^{-1}, where maximum enzyme activities were achieved: SOD (27.6 ± 1.2), CAT (43.1 ± 1.5), APX (24.2 ± 1.4), and POD (52.4 ± 1.5 U mg^{-1} prot) (p = 0.006). Simultaneously, stress markers reached their lowest levels, with H_2O_2 at 2.6 ± 0.4, MDA at 2.8 ± 0.5, and electrolyte leakage at 6.4 ± 0.4%, indicating efficient mitigation of oxidative damage. When 1.5 mg·L^{-1} 2,4-D was applied, enzyme activities remained elevated (SOD: 21.4 ± 0.6; CAT: 34.3 ± 1.3; APX: 17.1 ± 0.4; POD: 43.1 ± 1.5; p = 0.008), although values were lower compared with the 1.0 mg·L^{-1} treatment. Stress indicators showed a slight increase, with H_2O_2 at 3.3 ± 0.4, MDA at 3.7 ± 0.4, and electrolyte leakage at 8.6 ± 0.5%, suggesting partial oxidative imbalance. By contrast, a decline in antioxidant activity was evident at 2.0 mg·L^{-1}. Enzyme levels dropped significantly (SOD: 14.9 ± 0.6; CAT: 21.2 ± 1.4; APX: 12.4 ± 0.5; POD: 26.5 ± 1.4 U mg^{-1} prot; p = 0.007), while oxidative stress markers increased, with H_2O_2 (5.1 ± 0.1), MDA (5.4 ± 0.2), and electrolyte leakage (13.6 ± 0.3%) approaching control levels.

Table 4. Antioxidant enzyme activities and oxidative stress markers in callus tissue.

2,4-D (mg·L^{-1})	SOD (U mg^{-1} prot)	CAT (U mg^{-1} prot)	APX (U mg^{-1} prot)	POD (U mg^{-1} prot)	H_2O_2 (µmol g^{-1} FW)	MDA (nmol g^{-1} FW)	Electrolyte leakage (%)	p-value
0	12.1± 0.4a	15.4± 0.3a	7.6± 0.4a	18.2± 1.3a	5.9± 0.4c	6.5± 0.5d	14.2 ± 0.1c	0.005
0.5	16.3± 0.9b	26.2± 1.3b	14.4± 0.6b	33.2 ± 1.7b	4.6± 0.4 b	3.9± 0.6c	11.2± 0.5b	0.007
1	27.6± 1.2c	43.1 ± 1.5c	24.2± 1.4c	52.4± 1.5 c	2.6± 0.4a	2.8± 0.5a	6.4± 0.4a	0.006
1.5	21.4± 0.6c	34.3± 1.3c	17.1± 0.4c	43.1± 1.5 c	3.3± 0.4a	3.7± 0.4b	8.6± 0.5a	0.008
2	14.9± 0.6b	21.2± 1.4b	12.4 ± 0.5b	26.5± 1.4b	5.1 ± 0.1c	5.4± 0.2d	13.6± 0.3 c	0.007

Note: NS = not significant (p > 0.05). Different letters indicate significant differences according to Duncan's test.

The results of phenolic content and free proline accumulation in callus tissues after 28 days revealed a strong dependence on the concentration of 2,4-D (Table 5). In the control callus (0.0 mg·L^{-1} 2,4-D), phenolic compounds were detected (mg GAE g^{-1} FW 0.66 ± 0.04) and proline (µmol g^{-1} FW 0.96 ± 0.03), both representing the lowest levels, with no significant variation (NS). The addition of 0.5 mg·L^{-1} 2,4-D led to a marked increase, with phenolics reaching 1.13 ± 0.03 mg GAE g^{-1} FW (p = 0.006) and proline rising to 1.41 ± 0.06 µmol g^{-1} FW (p = 0.006). The most pronounced effect was detected at 1.0 mg·L^{-1} 2,4-D, where phenolic accumulation peaked at 1.77 ± 0.05 mg GAE g^{-1} FW (p = 0.007), and 2.27 ± 0.06 µmol g^{-1} FW (p = 0.007). At 1.5 mg·L^{-1}, both parameters declined moderately, with phenolics at 1.46 ± 0.06 mg GAE g^{-1} FW (p = 0.008) and proline at 1.81 ± 0.05 µmol g^{-1} FW (p = 0.008), though still above control. In contrast, exposure to 2.0 mg·L^{-1} resulted in a reduction, as phenolic content dropped to 0.96 ± 0.03 mg GAE g^{-1} FW (p = 0.007) and proline to 1.17 ± 0.04 µmol g^{-1} FW (p = 0.007).

Table 5. Total phenolic content and free proline in callus (day 28).

2,4-D (mg·L^{-1})	Total phenolics (mg GAE · g^{-1} FW)	Free proline (µmol · g^{-1} FW)	p-value
0.0 (Control)	0.66± 0.04a	0.96± 0.03a	NS
0.5	1.13± 0.03b	1.41± 0.06b	0.006
1	1.77± 0.05c	2.27± 0.06c	0.007
1.5	1.46± 0.06c	1.81± 0.05c	0.008
2	0.96± 0.03b	1.17± 0.04b	0.007

Note: NS = not significant (p > 0.05). Different letters indicate significant differences according to Duncan's test.

Discussion

The current study found that 2,4-dichlorophenoxyacetic acid (2,4-D) employs a vigorous concentration-dependent effect on somatic embryogenesis, antioxidant defense mechanisms, callus induction, and rooting in *Ficus elastica*. The outcomes of this study clearly found that modest auxin levels, chiefly 1.0 mg·L^{-1}, with biochemical responses, and optimal morphogenetic, while supra-optimal concentrations (2.0 mg·L^{-1}) produced a failure in physiological stability, embryogenesis, and callogenesis. The maximum biomass (3.18 g per explant) and callus induction rate

(85.2%) were attained at 1.0 mg·L⁻¹ 2,4-D, while higher levels concentrated the response. These results are consistent with *Ficus elastica* [29] and *Coffea arabica* [47], where low to moderate 2,4-D concentrations stimulated dedifferentiation, but extra auxin instigated tissue toasting and oxidative stress. Similarly, somatic embryogenesis conversion rate of 54.6%, superior embryo morphology with a peak at 1.0 mg·L⁻¹. Similar outcomes were noted that the auxin is important for embryogenic induction in *Medicago truncatula* and Arabidopsis, but with the reduction of auxin enabled embryo maturation and also described that auxin-triggered dedifferentiation and subsequent rebalancing of endogenous signals for successful bipolar embryo formation [48,49]. The enhanced plantlets after acclimatization rooting performance reached 1.0 mg·L⁻¹ 2,4-D treatments, showing a carry-over effect of auxin priming at the callus stage, with rooting reaching 91.4%, and a survival rate of 85.3. These results are consistent with previous findings in other woody species such as *Morus alba* and *Ficus carica* [50, 51]. Remarkably, although 1.5 mg·L⁻¹ 2,4-D produced more roots per plantlet, root length and survival were superior at 1.0 mg·L⁻¹. da Silva *et al.* [52] described that the higher concentrations of auxin improve root primordia initiation with the elongation and physiological quality of the roots. The higher activities of antioxidant enzymes (SOD, CAT, APX, and POD) were found at 1.0 mg·L⁻¹ 2,4-D, with the lowest levels of hydrogen peroxide, lipid peroxidation, and electrolyte leakage in this study. These results are consistent with previous studies in *Glycine max* and *Oryza sativa*, which described the improved enzymatic antioxidant defense with the optimal auxin exposure, while excessive auxin accumulation enhances cellular injury and reactive oxygen species (ROS) [53. 54]. Fehér, [33] described that increase in oxidative stress markers, disrupt cellular homeostasis leading to tissue necrosis with the higher supra-optimal auxin doses. Secondary metabolite analysis confirmed that 2,4-D powerfully controlled the accumulation of proline and phenolics and in *Ficus elastica* callus tissues with maximum metabolites were perceived at 1.0 mg·L⁻¹, with a gradual decline detected at higher auxin levels. Bielach *et al.*, [55] studied the phenolics and proline and found regulatory role in auxin-mediated morphogenesis and stress adaptation, reported it as an antioxidant for protecting embryogenic cells from oxidative damage. In addition, Kavi Kishor *et al.*, [56] also explained that phenolics and proline contribute to signaling processes and cell wall remodeling both of which are serious for embryogenic capability. Martin *et al.*, [57] described that Proline accumulation enhanced tolerance to osmotic and oxidative stress under hormonal stress during *in vitro* culture and act as an osmoprotectant. Nieves *et al.*, [58] and Awada *et al.*, [59] designed the study on *Medicago sativa* and *Coffea canephora* and increased proline levels with improved somatic embryogenesis, metabolite functions and recognized as a stress marker and developmental regulator. Jin *et al.*, [60] conducted study on Oryza sativa and reported enhanced embryogenic potential with the proline accumulation under auxin whereas extreme levels of 2,4-D suppressed metabolite amalgamation and condensed regeneration proficiency. In *Nicotiana tabacum*, proline and phenolics were both raised in auxin-induced calli with improved embryogenic responses [32]. He *et al.* [61, 62] also found Similar auxin-dependent patterns in *Vitis vinifera* with the increased phenolic accumulation in embryogenic calli.

Conclusion

This experimental study concluded that the success of in vitro regeneration is dependent on precise modulation of auxin concentration in *Ficus elastica*. This study also concluded that low concentrations are insufficient to trigger dedifferentiation and high levels impose oxidative stress, but the intermediate dose (1.0 mg·L⁻¹) provides the ideal balance for morphogenesis and biochemical stability. These insights contribute to the broader understanding of auxin-regulated developmental plasticity in plants and may serve as a reference for some recalcitrant woody species.

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الدور المعتمد على التركيز لحمض 2،4- ثنائي كلورو فينوكسي أسيتيك في تحريض الكالس ، والتطور الجنيني الجسدي ، والاستجابات المضادة للأكسدة في المطاط (*Ficus elastica*)

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الخلاصة

فحصت هذه الدراسة تأثير حمض 2،4-ثنائي كلورو فينوكسي أسيتيك (***2,4-D***) على تطور الكالس ، والكفاءة الجنينية ، والتمثيل الغذائي المضاد للأكسدة في ***Ficus elastica***. تمت زراعة نباتات الأوراق على وسط ***Murashige*** و ***Skoog*** يحتوي على (0.0 أو 0.5 أو 1.0 أو 1.5 أو 2.0 ملغم · L^{-1} ***2,4-D***) لوحظ تأثير ملحوظ لتركيز الأوكسين على المعلمات المورفولوجية والكيميائية الحيوية (***p*** < 0.01). حدثت الاستجابة المثلى عند 1.0 ملغم · L^{-1} ، حيث وصل تحريض الكالس إلى 85.2 ± 1.1٪ مع كتلة حيوية تبلغ 3.18 ± 0.14 غم لكل مستنبت $^{-1}$ وأقصر وقت بدء 22.6 ± 0.5 يوم (***p*** = 0.007). كان التطور الجنيني الجسدي أكثر نجاحا عند هذا المستوى ، حيث أنتج 74.4 ± 1.2٪ تحريض الجنين ، ومؤشر جودة(5.1 ± 0.4 ، و 54.6 ± 1.6٪)تحويل النبتة (***p*** = 0.006). أظهرت النباتات المتجددة من نفس العلاج تجذيرا متفوقا (91.4 ± 1.4٪) والبقاء على قيد الحياة بعد التأقلم (85.3 ± 1.4٪ ؛ ***p*** = 0.006). كان نشاط الإنزيم المضاد للأكسدة أعلى أيضا عند 1.0 ملغم · L^{-1} ، مع أنشطة ***superoxide dismutase, catalase, ascorbate peroxidase, and peroxidase*** التي تم تحسينها بشكل كبير (***p*** = 0.006) ، في حين أن علامات الإجهاد التأكسدي مثل H_2O_2 ، والمالونديالديهايد ، وتسرب الإلكتروليت كانت في أدنى مستوياتها. كما بلغت المركبات الفينولية (1.77 ± 0.05 ملغم ***FW*** $^{-1}$***g GAE*** ؛ ***p*** = 0.007) والبرولين الحر (2.27 ± 0.06 ميكرولتر غم ***FW*** $^{-1}$ ؛ ***p*** = 0.007) ذروتها أيضا عند هذا التركيز. في المقابل ، 2.0 ملغم · L^{-1} منع التشكل وزيادة الضرر التأكسدي (ص = 0.007). بشكل جماعي ، تؤكد هذه النتائج أن ***2,4-D*** ينظم التشكل وفسيولوجيا الإجهاد بطريقة تعتمد على التركيز ، مع 1.0 ملغم · L^{-1} تم تحديده على أنه الأكثر فعالية لتحسين التجديد في المطاط ***Ficus elastica***

الكلمات المفتاحية: التجذير والتأقلم، تحريض الكالس، المركبات الفينولية، بيروكسيد الهيدروجين (H_2O_2)، المطاط *Ficus elastica*