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Effects of 1-Naphthaleneacetic Acid, Indole-3-Acetic Acid, and ABT Rooting Powder on Rooting, Physiology, Hormones, and Active Components in *Flemingia macrophylla*

Yunfang Zhang^{1,2}, Wumei Si^{1,2}, Fuzhou Pang³, Ruirong Zhang^{1,2}, Weiting Ye²
and Kunhua Wei^{1,2,*}

¹Key Laboratory of State Administration of Traditional Chinese Medicine for Production & Development of Cantonese Medicinal Materials, Guangzhou Comprehensive Experimental Station of National Industrial Technology System for Chinese Materia Medica, Guangdong Engineering Research Center of Good Agricultural Practice & Comprehensive Development for Cantonese Medicinal Materials, School of Chinese Materia Medica, Guangdong Pharmaceutical University, Guangzhou, China

²Traditional Chinese Medicine Resource Germplasm Bank Management Center, Yunfu, China

³School of Pharmacy, Guangxi Medical University, Nanning, China

*Corresponding Author: Kunhua Wei. Email: divinekh@163.com

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ABSTRACT: *Flemingia macrophylla* (Willd.) Kuntze ex Merr. is a multipurpose legume crop with medicinal and forage value. However, the shortage of wild resources and the long cultivation cycle limit its sustainable utilization. As a preliminary screening experiment, this study evaluated the effects of 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and ABT rooting powder (a mixture of IAA and NAA), which are commonly used in production, on root growth, physiological and biochemical parameters, endogenous hormones, and the accumulation of the bioactive isoflavones genistein and genistin in tissue-cultured plantlets of *F. macrophylla*. The results showed significant differences among the regulatory effects of the three plant growth regulators (PGRs). NAA was most effective in boosting antioxidant enzyme activities while minimizing membrane lipid peroxidation; IAA significantly promoted root elongation; and the ABT treatment resulted in the highest accumulation of soluble sugars and abscisic acid (ABA). The highest genistein content ($4.59 \text{ mg}\cdot\text{g}^{-1}$) was observed under the $0.3 \text{ mg}\cdot\text{L}^{-1}$ ABT treatment, while the highest genistin content ($33.82 \text{ mg}\cdot\text{g}^{-1}$) was obtained with the $1.0 \text{ mg}\cdot\text{L}^{-1}$ ABT treatment. Spearman's correlation analysis suggested that the association patterns between active components and physiological factors were treatment-specific. Within the tested concentration range, the $1.0 \text{ mg}\cdot\text{L}^{-1}$ ABT treatment yielded the highest bioactive constituent content ($37.76 \text{ mg}\cdot\text{g}^{-1}$), indicating a relatively high level of isoflavone accumulation; in contrast, $1.0 \text{ mg}\cdot\text{L}^{-1}$ IAA and $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA exhibited relatively balanced effects among root development, stress physiology, and bioactive constituent accumulation. This exploratory study provides preliminary data to inform the selection of growth regulators for the tissue culture propagation and production of bioactive constituents in *F. macrophylla*.

KEYWORDS: Plant growth regulators; rooting; genistein; genistin; tissue culture; *Flemingia macrophylla*

1 Introduction

Flemingia macrophylla (Willd.) Kuntze ex Merr. is a perennial shrub belonging to the genus *Flemingia* (Fabaceae), widely distributed in tropical and subtropical regions. It is a traditional Chinese folk medicinal plant, and its dried roots are extensively used to treat rheumatoid arthritis and irregular menstruation [1]. Modern pharmacological studies have confirmed that genistein and genistin have been established as its

primary active principles [2], exhibiting multiple pharmacological activities including significant antioxidant, antitumor, estrogenic, and cardiovascular protective effects [3–5]. In addition, owing to its high protein content, it is also utilized as a high-quality supplemental feed resource [6]. However, with growing market demand, its wild resources are being depleted by overharvesting; cultivated plants require 3–5 years to mature, and the content of active compounds fluctuates substantially depending on the place of origin and soil conditions, which severely compromises the consistency and reliability of product quality.

Compared with conventional cultivation, tissue culture offers multiple advantages, including independence from seasonal and geographical constraints, a short propagation cycle, and high plantlet uniformity, and has been successfully applied to the rapid micropropagation and secondary metabolite production of various medicinal plants [7,8]. Thanonkeo optimized the hairy root culture medium of *Pueraria candollei* and achieved a 4.8-fold increase in genistein content compared with normal roots [9]. Our previous study demonstrated that, under suitable conditions, the genistein and genistin contents in 50–70-day-old tissue-cultured plantlets of *F. macrophylla* were higher than those in field-grown plants [10]. Based on this observation, the present study adopted 60 days as a fixed harvest time point to preliminarily evaluate the effects of different plant growth regulator (PGR) treatments on isoflavone accumulation. This phenomenon implies that by optimizing tissue culture conditions, especially by adding appropriate plant growth regulators and nutrients, it is possible not only to shorten the propagation cycle but also to actively remodel the metabolic network, thereby inducing the overaccumulation of isoflavones such as genistein and genistin. Consequently, the tissue culture system can serve the dual functions of seedling production and target metabolite acquisition.

Indole-3-acetic acid (IAA) and α -naphthaleneacetic acid (NAA) are widely used for root induction in micropropagation. Exogenous IAA compensates for endogenous auxin deficiency under *in vitro* conditions [11,12], while NAA, a synthetic auxin with higher stability, can promote root formation and accumulation of phenolic and flavonoid metabolites [13–15]. ABT rooting powder (ABT) is a combined product of IAA and NAA that exerts important regulatory effects on plant cell proliferation, differentiation, and root growth [16,17]. Nevertheless, in micropropagation studies of *F. macrophylla*, a systematic comparison of the three auxin-type regulators mentioned above is still lacking. Which auxin type and which concentration are most conducive to adventitious root formation? Do the conditions that promote root system establishment also favor the accumulation of genistein and genistin? These two key questions, which are critical for optimizing the *in vitro* propagation system of *F. macrophylla* and the efficient production of bioactive constituents, remain unresolved.

This study systematically compared the effects of NAA, IAA, and ABT, each applied at four concentration gradients, on root system establishment, physiological and biochemical parameters, endogenous hormone contents, and genistein and genistin contents in *F. macrophylla* tissue-cultured plantlets. We hypothesized that exogenous auxins can exert specific effects on root formation and secondary metabolite biosynthesis by modulating the antioxidant system and endogenous hormone balance. Specifically, this study aimed to determine the optimal auxin type and concentration for root system establishment, to investigate whether conditions that promote rooting also favor the accumulation of genistein and genistin, and to explore the potential physiological mechanisms underlying these effects, thereby providing data support for PGR selection in the micropropagation and production of bioactive constituents of *F. macrophylla*.

2 Materials and Methods

2.1 Plant Material and Culture Establishment

The plant material of *F. macrophylla* was obtained from Guangxi Botanical Garden of Medicinal Plants (Nanning, Guangxi, 108.38° E, 22.86° N). Young stem segments from *F. macrophylla* branches were selected as primary explants for tissue culture. After surface sterilization with 75% ethanol and 0.1% HgCl₂, the explants were established on Murashige and Skoog (MS) basal medium [18] with 0.1 mg·L⁻¹ NAA for bud initiation. The culture was kept at 25 ± 1°C, the illumination time was 16 h, and the illumination intensity was 1500 lux. Subculture was conducted once every 4 weeks, and the sterile plantlets from two successive subculture cycles were used as experimental seeds for subsequent rooting induction.

2.2 Experimental Treatments

Uniform stem segments (1–2 cm) each containing one axillary bud were transferred to MS medium individually fortified with NAA, IAA, or ABT rooting powder (Chembase, Beijing, China; total active ingredient 50%, containing 20% NAA and 30% IAA, with inert fillers as the balance) at four concentration levels. Based on effective concentrations reported in previous studies on legume tissue culture and the results of preliminary screening experiments [19,20], four concentration gradients (0.1, 0.3, 0.5, and 1.0 mg·L⁻¹) were established in this study for rooting culture under the same conditions as described above. A blank control group without plant growth regulators (0 mg·L⁻¹) was not included in the experimental design. Each treatment combination was replicated three times, with five culture vessels per replicate and 3–6 plantlets per vessel. After 60 days of culture under the same environmental conditions, plantlets were harvested for comprehensive analyses of rooting performance, physiological and biochemical traits, endogenous hormone profiles, and isoflavone contents.

2.3 Root Measurement

Root growth of the tissue-cultured plantlets was evaluated using rooting percentage (number of rooted plantlets/total plantlets × 100%), average root number (total number of roots/number of plantlets), and average root length (sum of mean root length per plantlet/number of plantlets) as growth indicators.

2.4 Determination of Genistein and Genistin Contents

The extraction of genistein and genistin was performed entirely following the method of Yu et al. [10]. The cultured plantlets were dried and ground into powder. A 1.5 g powder sample was extracted with ethanol by ultrasonication and then transferred into methanol. A 1.5-μL aliquot of the methanol-soluble fraction was injected into an Agilent 1260 high-performance liquid chromatography (HPLC) system equipped with a Stable Bond Analytical C18 column (4.6 × 250 mm, 5 μm). The mobile phase consisted of methanol (solvent A) and 0.1% aqueous phosphoric acid (solvent B), running at a flow rate of 1.0 mL·min⁻¹ with the following gradient profile: 25%–65% A over 0–35 min, 65%–25% A over 35–37 min, and re-equilibration at 25% A for 37–40 min. Genistein and genistin were identified and quantified by comparison with authentic standards (Chengdu Pufeide Biotechnology Co., China) at 260 nm. All extractions and injections were performed in triplicate.

2.5 Enzyme Activities and Metabolite Contents

2.5.1 Sample Preparation

Harvested materials were immediately sealed in bags, flash-frozen in liquid nitrogen for 5 min, and then stored at -80°C until further use. For enzyme extraction, the frozen tissue was ground and homogenized in phosphate buffer (pH 6.8) containing polyvinylpyrrolidone (PVP). After centrifugation at 14,000 rpm for 10 min at 4°C , the enzyme-containing supernatant was collected for subsequent assays.

2.5.2 Antioxidant Enzymes

Peroxidase (POD) activity was determined spectrophotometrically by monitoring the absorbance change at 470 nm in phosphate buffer (pH 6.8) containing guaiacol and H_2O_2 [21,22]. One unit (U) of POD activity is defined as the amount of enzyme required to increase the absorbance at 470 nm by 0.01 per minute under the assay conditions. POD activity was calculated using the following formula:

$$\text{POD (U} \cdot \text{g}^{-1}\text{FW} \cdot \text{min}^{-1}) = \frac{\Delta A_{470} \times V_T}{W \times V_S \times t \times 0.01}$$

ΔA_{470} is the change in absorbance at 470 nm during the reaction time, V_T is the total volume of the enzyme extract (mL), V_S is the volume of sample used for the assay (mL), W is the fresh weight of the sample (g), t is the reaction time (min), and 0.01 represents the absorbance change corresponding to one enzyme activity unit.

Superoxide dismutase (SOD) activity was assessed based on its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), and the absorbance was measured at 560 nm [23]. One unit (U) of SOD activity is defined as the amount of enzyme required to inhibit the photochemical reduction of NBT by 50% under the assay conditions. SOD activity was calculated using the following formula:

$$\text{SOD (U} \cdot \text{g}^{-1}\text{FW} \cdot \text{h}^{-1}) = \frac{(A_0 - A_1) \times V_T \times 60}{A_0 \times 0.5 \times W \times V_S \times t}$$

A_0 is the absorbance of the control tube under light, A_1 is the absorbance of the sample tube, V_T is the total volume of the enzyme extract (mL), W is the fresh weight of the sample (g), V_S is the volume of enzyme solution used in the assay (mL), t is the illumination time (min), and 0.5 represents the 50% inhibition rate.

2.5.3 Nitrogen Metabolism Enzymes

Glutamic-oxaloacetic transaminase (GOT) activity was determined based on its ability to produce pyruvate in GOT substrate solution [24,25]. Glutamic-pyruvic transaminase (GPT) activity was assayed similarly to GOT activity, except that DL-alanine was used in place of DL-aspartic acid in the GPT substrate solution. Both were quantified by measuring the absorbance at 520 nm. One unit (U) of GOT or GPT activity is defined as the amount of enzyme required to catalyze the formation of 1 μg of pyruvate within 30 min under the assay conditions. Enzyme activity was calculated using the following formula:

$$\text{GOT/GPT (U} \cdot \text{g}^{-1}\text{FW} \cdot (30\text{min})^{-1}) = \frac{C \times V_T}{W \times V_S}$$

C is the pyruvate content (μg) obtained from the pyruvate standard curve, V_T is the total volume of the enzyme extract (mL), V_S is the volume of enzyme solution used in the assay (mL), and W is the fresh weight of the sample (g).

2.5.4 Membrane Lipid Peroxidation

We measured malondialdehyde (MDA) using the thiobarbituric acid (TBA) method [26,27]. The resulting MDA–TBA chromophore was read at 532 nm and 600 nm, and its concentration was derived from the molar extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$. MDA concentration was calculated using the following formulas:

$$C_{MDA} = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

$$\text{MDA } (\mu\text{mol} \cdot \text{g}^{-1}\text{FW}) = \frac{C \times V}{1000 \times W}$$

A_{532} , A_{600} , and A_{450} are the absorbance values at the corresponding wavelengths, C is the MDA concentration in the reaction solution ($\mu\text{mol} \cdot \text{L}^{-1}$), V is the total volume of the extract (mL), and W is the fresh weight of the sample (g).

2.5.5 Soluble Protein and Soluble Sugar

Soluble protein was assayed by the Coomassie Brilliant Blue G-250 dye-binding technique, employing a commercial reagent kit (Tiangen Biotech, Beijing; catalog PA102) [28].

$$\text{soluble protein } (\text{mg} \cdot \text{g}^{-1}\text{FW}) = \frac{C \times V}{W \times V_S}$$

C is the protein content (mg) determined from the standard curve, V is the total volume of the extract (mL), V_S is the volume of enzyme solution used in the assay (mL), and W is the fresh weight of the sample (g).

Soluble sugars were quantified by the anthrone-sulfuric acid method, measuring absorbance at 620 nm against a glucose standard curve [29].

$$\text{Soluble sugars } (\text{mg} \cdot \text{g}^{-1}\text{FW}) = \frac{C \times V_T}{W \times V_S \times 1000}$$

C is the glucose content (μg) obtained from the standard curve, V is the total volume of the extract (mL), V_S is the volume of enzyme solution used in the assay (mL), W is the fresh weight of the sample (g), and 1000 is the conversion factor from μg to mg.

2.6 Endogenous Hormone Profiling by ELISA

Endogenous abscisic acid (ABA), gibberellic acid (GA_3), zeatin (ZT), brassinosteroid (BR), and methyl jasmonate (MeJA) were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (mlbio®, Shanghai Enzyme-linked Biotechnology Co., China; catalog numbers ml077235, ml077232, ml077233, ml036308, and ml077337). All procedures were performed strictly according to the manufacturer's instructions [30,31].

2.7 Statistical Analysis

All quantitative data, except rooting percentage, were subjected to ANOVA with auxin type and concentration as fixed factors, followed by *post-hoc* multiple comparisons using Bonferroni correction ($\alpha = 0.05$). Rooting percentage data were analyzed using a generalized linear model with a binomial distribution and complementary log-log link function. In addition to the overall comparisons, we also

performed subgroup comparisons within each of the NAA, IAA, and ABT treatment groups. For pairwise comparisons within each auxin series (NAA, IAA, or ABT), Bonferroni correction was applied separately for each series to control the family-wise error rate at $\alpha = 0.05$. To explore potential covariation patterns between physiological parameters and isoflavone contents, Spearman's rank correlation coefficients were computed separately for each auxin treatment group. Raw p -values from correlation analyses were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) procedure, with a family-wise threshold of $q < 0.05$. All statistical computations were performed in SPSS 27.0 (IBM Corp., Armonk, NY, USA) and Microsoft Excel 2003.

For all tabulated data, values are expressed as the mean $\pm$ standard deviation (SD) derived from three biological replicates ($n = 3$). Different superscript letters denote statistically significant distinctions at the adjusted threshold of $p < 0.05$.

3 Results

3.1 Effects of Different PGR Treatments on Rooting

Rooting responses varied markedly across PGR treatments (Table 1, Fig. 1). Because no blank control group without any plant growth regulators was included in this experiment, all statistical analyses and statements are based on relative comparisons among the treatment groups. The letter markings reflect only the relative differences among treatments, rather than effects relative to the untreated state.

Under NAA treatment, the rooting percentages at 0.1, 0.3, and 0.5 $\text{mg}\cdot\text{L}^{-1}$ were all 100%, while that at 1.0 $\text{mg}\cdot\text{L}^{-1}$ was 95.24%; pairwise comparisons within the group showed no significant differences in rooting percentage among the NAA concentrations ($p > 0.05$). Under IAA treatment, the rooting percentages at 0.1, 0.5, and 1.0 $\text{mg}\cdot\text{L}^{-1}$ were all 100%, while that at 0.3 $\text{mg}\cdot\text{L}^{-1}$ was 91.43%; within-group comparisons showed that the rooting percentage at 0.3 $\text{mg}\cdot\text{L}^{-1}$ was significantly lower than those at 0.1, 0.5, and 1.0 $\text{mg}\cdot\text{L}^{-1}$ ($p = 0.010$). In the ABT treatment group, the rooting percentage was only 78.93% at 0.3 $\text{mg}\cdot\text{L}^{-1}$, the lowest value among all 12 treatment combinations, and reached the group maximum of 96.43% at 0.5 $\text{mg}\cdot\text{L}^{-1}$; within-group comparisons showed that the rooting percentage at 0.3 $\text{mg}\cdot\text{L}^{-1}$ was significantly lower than those at 0.5 and 1.0 $\text{mg}\cdot\text{L}^{-1}$ ($p = 0.002$).

With respect to root architecture, under NAA treatment the average root number was highest at 0.3 $\text{mg}\cdot\text{L}^{-1}$ (5.78 roots), and the average root length was also longest at 0.3 $\text{mg}\cdot\text{L}^{-1}$ (5.02 cm). Under IAA treatment, the average root number was highest at 0.3 $\text{mg}\cdot\text{L}^{-1}$ (5.72 roots), and the average root length was longest at 0.5 $\text{mg}\cdot\text{L}^{-1}$ (6.28 cm). Under ABT treatment, the average root number was highest at 1.0 $\text{mg}\cdot\text{L}^{-1}$ (6.57 roots), but the average root length under this treatment was only 4.43 cm. Pairwise comparisons within each hormone group showed no significant differences in root length or root number among the different concentrations within the NAA, IAA, or ABT series ($p > 0.05$).

The generalized linear model confirmed that auxin type, concentration, and their interaction exerted highly significant effects on rooting percentage ($p < 0.001$). Both NAA and IAA induced significantly higher rooting percentages than ABT ($p < 0.001$), with no statistical difference between the two (Table 1, Fig. 1A).

Two-way ANOVA revealed that PGR type had a significant effect on root length (IAA > NAA and ABT, $p < 0.05$), whereas the effects of concentration and the interaction term on root length did not reach statistical significance. The average root length under IAA treatment was significantly greater than those under NAA and ABT treatments (Table 1, Fig. 1B). PGR type, concentration, and their interaction had no significant effect on root number ($p > 0.05$, Table 1, Fig. 1C).

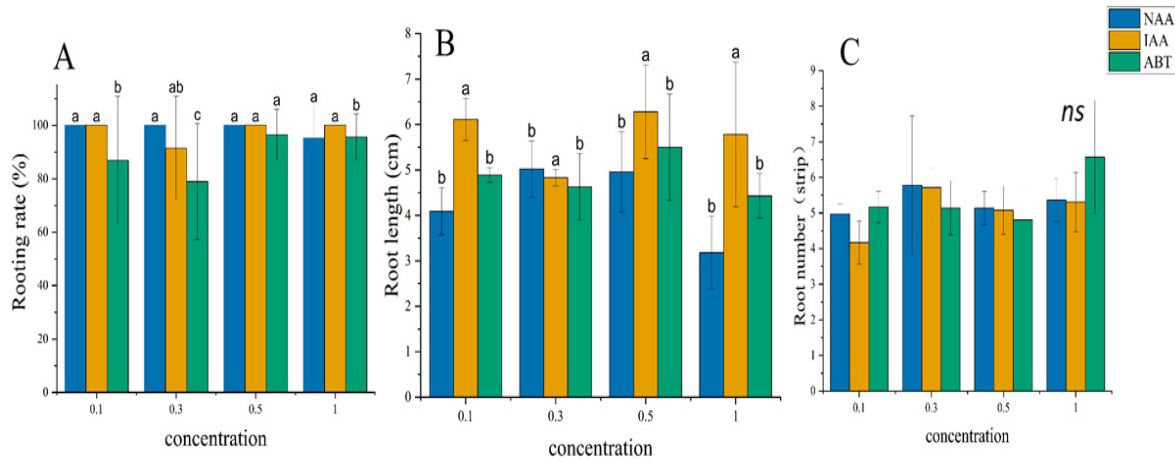


Figure 1: Effects of NAA, IAA, and ABT on rooting parameters of *F. macrophylla*. (A): rooting rate; (B): root length; (C): root number. Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$); the same letter indicates no significant difference ($p > 0.05$). Rooting rate (A) was analyzed using a generalized linear model; letters are based on pairwise comparisons among all 12 treatment combinations. For root length (B), only the auxin type main effect was significant (IAA > NAA and ABT, $p < 0.05$); therefore, IAA is labeled “a” and NAA and ABT are labeled “b”. For root number (C), no significant (ns) differences were detected. All statistical comparisons are limited to treatment groups; no hormone-free control was included.

Table 1: Effects of different concentrations of NAA, IAA, and ABT on rooting of *F. macrophylla*.

Treatment	Concentration	Rooting Rate (%)	Root Length (cm)	Root Number
NAA	0.1	100.00 ± 0.00a	4.09 ± 0.52b	4.97 ± 0.29
	0.3	100.00 ± 0.00a	5.02 ± 0.62b	5.78 ± 1.95
	0.5	100.00 ± 0.00a	4.96 ± 0.88b	5.14 ± 0.47
	1	95.24 ± 12.10a	3.18 ± 0.80b	5.36 ± 0.60
IAA	0.1	100.00 ± 0.00a	6.11 ± 0.46a	4.17 ± 0.60
	0.3	91.43 ± 19.47ab	4.83 ± 0.18a	5.72 ± 0.54
	0.5	100.00 ± 0.00a	6.28 ± 1.03a	5.08 ± 0.68
	1	100.00 ± 0.00a	5.78 ± 1.59a	5.31 ± 0.83
ABT	0.1	86.91 ± 23.96b	4.89 ± 0.16b	5.17 ± 0.44
	0.3	78.93 ± 21.68c	4.63 ± 0.73b	5.14 ± 0.75
	0.5	96.43 ± 9.65a	5.50 ± 1.17b	4.81 ± 0.91
	1	95.71 ± 8.52b	4.43 ± 0.49b	6.57 ± 1.59

Note: Values are means ± SD ($n = 3$). Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$); the same letter indicates no significant difference ($p > 0.05$). For rooting rate, letters are based on pairwise comparisons among all 12 treatment combinations using a generalized linear model. For root length, only the auxin type main effect was significant (IAA > NAA and ABT, $p < 0.05$); therefore, IAA is labeled “a” and NAA and ABT are labeled “b”. All comparisons are limited to treatment groups; no hormone-free control was included.

3.2 Effects of Different PGRs on Physiological and Biochemical Parameters

PGR type, concentration, and their interaction had highly significant effects on POD activity ($p < 0.001$). Among all 12 treatment combinations, the POD activity in tissue-cultured plantlets under the 0.5 mg·L⁻¹ NAA treatment reached the highest value of 21,710.53 U·g⁻¹ FW·min⁻¹, which was significantly higher than that in all other treatments. Within the NAA series, POD activity at 0.5 mg·L⁻¹ was significantly higher than those at 0.1, 0.3, and 1.0 mg·L⁻¹ ($p = 0.001$, 0.007, and 0.001, respectively); within the IAA series, POD activity at 1.0 mg·L⁻¹ was significantly higher than that at 0.5 mg·L⁻¹ ($p = 0.026$). No significant differences

in POD activity were observed among the different concentrations within the ABT series ($p > 0.05$). The membrane lipid peroxidation indicator MDA content showed no significant differences among treatments, but reached its lowest value of $0.104 \mu\text{mol}\cdot\text{g}^{-1}$ FW under the $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA treatment, indicating that $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA represents a critical turning point for maintaining redox homeostasis and that plantlets under this treatment exhibited the strongest stress resistance (Table 2, Fig. 2). SOD activity was significantly affected by PGR type.

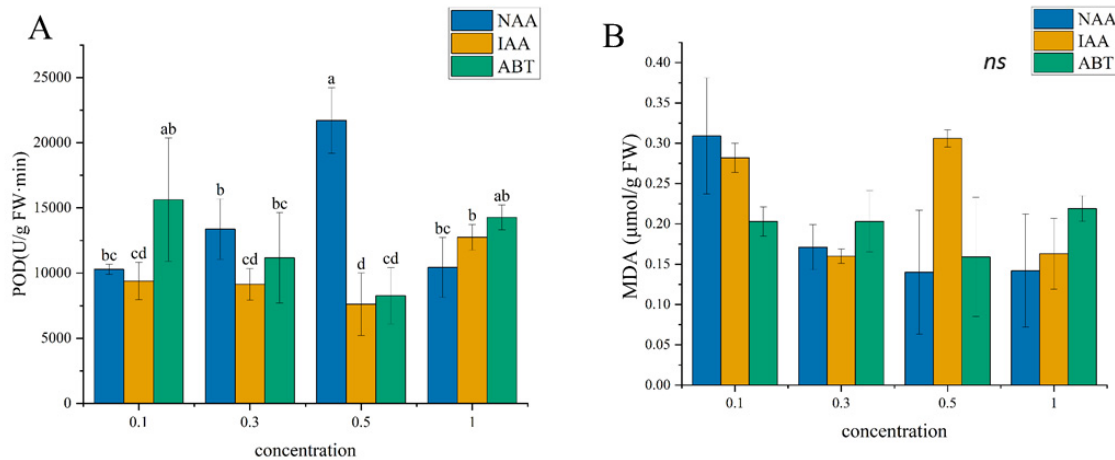


Figure 2: Effects of different concentrations of NAA, IAA, and ABT on POD activity and MDA content. (A): Peroxidase (POD) activity; (B): Malondialdehyde (MDA) content. Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$); the same letter indicates no significant difference ($p > 0.05$). For POD (A), the auxin type $\times$ concentration interaction was significant ($p < 0.05$), hence letters are based on comparisons among all 12 treatment combinations. MDA (B) showed no significant (ns) main effects or interaction ($p > 0.05$), hence no letters. All statistical comparisons are limited to treatment groups; no hormone-free control was included.

Table 2: Effects of different concentrations of NAA, IAA, and ABT on physiological and biochemical parameters of *F. macrophylla*.

Treatment	Concentration ($\text{mg}\cdot\text{L}^{-1}$)	POD ($\text{U}\cdot\text{g}^{-1}\text{FW}\cdot\text{min}^{-1}$)	GPT ($\mu\text{g}\cdot\text{g}^{-1}\text{FW}\cdot 30\text{ min}^{-1}$)	GOT ($\mu\text{g}\cdot\text{g}^{-1}\text{FW}\cdot 30\text{ min}^{-1}$)	SOD ($\text{U}\cdot\text{g}^{-1}\text{FW}\cdot\text{h}^{-1}$)	MDA ($\mu\text{mol}\cdot\text{g}^{-1}\text{FW}$)
NAA	0.1	$10,284.39 \pm 387.49\text{bc}$	$2524.28 \pm 152.51\text{a}$	$4657.73 \pm 473.74\text{a}$	$1533.71 \pm 6.16\text{a}$	$0.309 \pm 0.072\text{a}$
	0.3	$13,371.56 \pm 2314.04\text{b}$	$3044.92 \pm 332.65\text{a}$	$4047.27 \pm 647.95\text{ab}$	$1481.46 \pm 138.37\text{a}$	$0.171 \pm 0.028\text{a}$
	0.5	$21,710.53 \pm 2515.50\text{a}$	$2990.13 \pm 188.93\text{a}$	$3448.59 \pm 633.60\text{b}$	$1553.51 \pm 32.91\text{a}$	$0.104 \pm 0.077\text{a}$
	1	$10,425.29 \pm 2301.78\text{bc}$	$2105.58 \pm 201.12\text{a}$	$3109.55 \pm 706.09\text{b}$	$1533.26 \pm 67.57\text{a}$	$0.142 \pm 0.070\text{a}$
IAA	0.1	$9386.88 \pm 1429.14\text{cd}$	$2135.23 \pm 92.66\text{ab}$	$3431.48 \pm 412.57\text{b}$	$1361.02 \pm 18.86\text{a}$	$0.282 \pm 0.018\text{a}$
	0.3	$9136.08 \pm 1225.57\text{cd}$	$2523.45 \pm 450.11\text{ab}$	$3222.05 \pm 486.86\text{b}$	$1476.58 \pm 28.41\text{a}$	$0.160 \pm 0.009\text{a}$
	0.5	$7606.59 \pm 2392.43\text{d}$	$3025.45 \pm 1071.95\text{ab}$	$4265.86 \pm 505.91\text{ab}$	$1490.84 \pm 9.89\text{a}$	$0.306 \pm 0.011\text{a}$
	1	$12,745.22 \pm 989.70\text{b}$	$1546.62 \pm 116.60\text{ab}$	$4243.30 \pm 567.72\text{ab}$	$1409.73 \pm 45.01\text{a}$	$0.163 \pm 0.044\text{a}$
ABT	0.1	$15,635.36 \pm 4721.70\text{ab}$	$2010.99 \pm 1111.20\text{b}$	$1089.48 \pm 25.11\text{c}$	$1168.56 \pm 266.67\text{b}$	$0.203 \pm 0.018\text{a}$
	0.3	$11,166.40 \pm 3460.89\text{bc}$	$1634.30 \pm 526.18\text{b}$	$1384.40 \pm 621.05\text{c}$	$1341.58 \pm 88.16\text{b}$	$0.203 \pm 0.038\text{a}$
	0.5	$8260.25 \pm 2157.53\text{cd}$	$1822.61 \pm 569.86\text{b}$	$2414.60 \pm 451.96\text{c}$	$1414.88 \pm 132.27\text{b}$	$0.159 \pm 0.074\text{a}$
	1	$14,273.01 \pm 956.95\text{ab}$	$2201.64 \pm 690.31\text{b}$	$2831.21 \pm 141.33\text{c}$	$1016.73 \pm 202.01\text{b}$	$0.219 \pm 0.016\text{a}$

Note: Different lowercase letters within each column indicate significant differences among treatment groups ($p < 0.05$); the same letter indicates no significant difference ($p > 0.05$). For POD and GOT (interaction $p < 0.05$), letters are based on comparisons among all 12 treatment combinations. For GPT, letters indicate hormone main effects (NAA vs. ABT, $p = 0.011$) and for SOD, letters indicate hormone main effects (NAA and IAA vs. ABT, $p < 0.001$). MDA showed no significant main effects or interaction ($p > 0.05$). The letter markings in the table represent only the relative differences among treatment groups and do not involve comparisons with the blank control.

SOD activities under NAA and IAA treatments were significantly higher than that under ABT, reaching the maximum value of $1553.51 \text{ U}\cdot\text{g}^{-1} \text{ FW}\cdot\text{h}^{-1}$ under the $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA treatment, while the minimum value occurred under $1.0 \text{ mg}\cdot\text{L}^{-1}$ ABT ($1016.73 \text{ U}\cdot\text{g}^{-1} \text{ FW}\cdot\text{h}^{-1}$) (Table 2). Within the IAA series, SOD activities at 0.3 and $0.5 \text{ mg}\cdot\text{L}^{-1}$ were significantly higher than that at $0.1 \text{ mg}\cdot\text{L}^{-1}$ ($p = 0.007$ and 0.003 , respectively); no significant differences in SOD activity were observed among the different concentrations within the NAA or ABT series.

GPT activity reached its maximum value of $3044.92 \text{ }\mu\text{g}\cdot\text{g}^{-1}\cdot 30 \text{ min}^{-1}$ under the $0.3 \text{ mg}\cdot\text{L}^{-1}$ NAA treatment, whereas GOT activity was highest under NAA treatment, with a maximum value of $4657.73 \text{ }\mu\text{g}\cdot\text{g}^{-1}\cdot 30 \text{ min}^{-1}$ under the $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA treatment (Table 2).

Soluble protein content was highly significantly affected by concentration ($p < 0.01$), with the $0.1 \text{ mg}\cdot\text{L}^{-1}$ treatment being significantly higher than the other concentrations, and reached the maximum value of $0.521 \text{ mg}\cdot\text{g}^{-1} \text{ FW}$ under the $0.1 \text{ mg}\cdot\text{L}^{-1}$ ABT treatment. Soluble sugar content was affected by PGR type, concentration, and their interaction, with the ABT treatment being highly significantly higher than the IAA and NAA treatment groups. The maximum value ($43.73 \text{ mg}\cdot\text{g}^{-1} \text{ FW}$) was observed under the $1.0 \text{ mg}\cdot\text{L}^{-1}$ ABT treatment (Table 3).

Table 3: Effects of different concentrations of NAA, IAA, and ABT on soluble protein and soluble sugar contents of *F. macrophylla*.

Treatment	Concentration ($\text{mg}\cdot\text{L}^{-1}$)	Soluble Protein ($\text{mg}\cdot\text{g}^{-1} \text{ FW}$)	Soluble Sugar ($\text{mg}\cdot\text{g}^{-1} \text{ FW}$)
NAA	0.1	$0.505 \pm 0.054\text{a}$	$17.93 \pm 11.30\text{c}$
	0.3	$0.485 \pm 0.027\text{b}$	$23.10 \pm 7.81\text{bc}$
	0.5	$0.408 \pm 0.047\text{b}$	$38.69 \pm 14.34\text{ab}$
	1	$0.426 \pm 0.070\text{b}$	$33.45 \pm 6.68\text{ab}$
IAA	0.1	$0.471 \pm 0.074\text{ab}$	$21.81 \pm 4.19\text{bc}$
	0.3	$0.362 \pm 0.030\text{b}$	$16.48 \pm 0.35\text{bc}$
	0.5	$0.418 \pm 0.016\text{b}$	$19.88 \pm 1.93\text{bc}$
	1	$0.416 \pm 0.091\text{b}$	$19.81 \pm 0.54\text{bc}$
ABT	0.1	$0.521 \pm 0.013\text{a}$	$33.18 \pm 4.98\text{ab}$
	0.3	$0.395 \pm 0.025\text{b}$	$37.98 \pm 11.35\text{ab}$
	0.5	$0.417 \pm 0.045\text{b}$	$37.79 \pm 5.99\text{ab}$
	1	$0.440 \pm 0.072\text{b}$	$43.73 \pm 6.02\text{a}$

Note: Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$); the same letter indicates no significant difference ($p > 0.05$). Soluble protein letters indicate the concentration main effect (0.1 vs. others, $p < 0.05$). Soluble sugar exhibited a significant interaction ($p < 0.05$); therefore, letters are based on comparisons among all 12 treatment combinations. The letter markings in the table represent only the relative differences among treatment groups and do not involve comparisons with the blank control.

3.3 Effects of Different Treatments on Endogenous Hormone Contents

ABA content was significantly affected by PGR type and the interaction. ABA content in the ABT treatment was generally higher than that in the IAA treatment. The highest ABA content was observed in the $0.1 \text{ mg}\cdot\text{L}^{-1}$ ABT treatment ($13.14 \text{ ng}\cdot\text{L}^{-1}$), and the lowest in the $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA group ($9.40 \text{ ng}\cdot\text{L}^{-1}$). Within-series pairwise comparisons showed that, in the NAA series, ABA content at $0.5 \text{ mg}\cdot\text{L}^{-1}$ was significantly lower than those at 0.1 , 0.3 , and $1.0 \text{ mg}\cdot\text{L}^{-1}$ ($p = 0.019$, 0.022 , and 0.008 , respectively); no significant differences in ABA content were observed among the different concentrations within the IAA or ABT series ($p > 0.05$).

BR content was significantly affected by PGR type and the interaction. The highest BR content was observed in the $1.0 \text{ mg}\cdot\text{L}^{-1}$ NAA group ($14.00 \text{ ng}\cdot\text{L}^{-1}$), and the lowest in the $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA group

(10.94 ng·L⁻¹). Within the NAA series, BR content at 1.0 mg·L⁻¹ was significantly higher than that at 0.5 mg·L⁻¹ ($p = 0.014$); no significant differences in BR content were observed among the different concentrations within the IAA or ABT series ($p > 0.05$).

MeJA content was highly significantly affected by PGR type, ABT treatment was significantly higher than those in the NAA and IAA treatments. Within the NAA series, MeJA content at 0.3 mg·L⁻¹ was significantly higher than that at 0.5 mg·L⁻¹ ($p = 0.005$), and MeJA content at 1.0 mg·L⁻¹ was also significantly higher than that at 0.5 mg·L⁻¹ ($p = 0.011$); no significant differences in MeJA content were observed among the different concentrations within the IAA or ABT series ($p > 0.05$).

The effect of PGR type on IAA content approached significance ($p = 0.043$). GA and ZT contents did not differ significantly among treatments (Table 4).

Table 4: Effects of different concentrations of NAA, IAA, and ABT on endogenous hormone contents of *F. macrophylla*.

Treatment	Concentration	ABA (ng·L ⁻¹)	BR (ng·L ⁻¹)	GA ₃ (pg·L ⁻¹)	IAA (pmol·L ⁻¹)	MeJA (pmol·L ⁻¹)	ZT (ng·L ⁻¹)
NAA	0.1	11.79 ± 0.59abc	12.84 ± 0.78abc	11.69 ± 1.02	10.67 ± 0.50a	7.74 ± 0.09b	9.81 ± 3.46
	0.3	11.73 ± 0.93abc	12.33 ± 0.36bc	12.16 ± 0.58	11.65 ± 1.00a	7.96 ± 0.13b	12.06 ± 0.31
	0.5	9.40 ± 0.50c	10.94 ± 1.02c	11.68 ± 0.52	10.40 ± 0.20a	7.37 ± 0.20b	11.01 ± 0.43
	1	12.15 ± 0.72ab	14.00 ± 1.07a	12.38 ± 0.89	11.25 ± 0.44a	7.88 ± 0.11b	11.22 ± 1.01
IAA	0.1	10.79 ± 0.31bc	12.11 ± 0.66bc	11.38 ± 0.77	10.63 ± 0.45a	7.66 ± 0.12b	11.22 ± 0.33
	0.3	9.96 ± 0.83bc	11.48 ± 0.31bc	11.69 ± 0.54	10.38 ± 0.58a	7.45 ± 0.23b	13.65 ± 4.23
	0.5	10.89 ± 1.83bc	12.71 ± 0.78abc	11.71 ± 0.42	9.90 ± 1.09a	7.51 ± 0.22b	11.50 ± 0.48
	1	10.87 ± 0.50bc	12.27 ± 0.69bc	11.40 ± 0.40	10.32 ± 0.62a	7.77 ± 0.22b	10.80 ± 0.34
ABT	0.1	13.14 ± 1.50a	12.72 ± 0.26abc	12.09 ± 0.53	11.27 ± 1.26a	8.23 ± 0.65a	11.95 ± 0.13
	0.3	12.98 ± 1.01ab	13.51 ± 0.61ab	11.96 ± 0.81	10.75 ± 0.48a	8.71 ± 0.65a	11.96 ± 0.71
	0.5	11.69 ± 0.79abc	13.37 ± 0.53ab	11.25 ± 1.05	10.49 ± 0.56a	8.01 ± 0.26a	11.58 ± 0.35
	1	11.05 ± 0.78abc	12.39 ± 0.43abc	11.84 ± 0.70	11.32 ± 0.47a	8.02 ± 0.22a	11.50 ± 0.95

Note: Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$); the same letter indicates no significant difference ($p > 0.05$). ABA and BR showed significant interaction effects ($p < 0.05$), so letters are based on all 12 treatment combinations. GA, IAA, JA and ZT showed no significant interaction; for IAA and JA, hormone main effects were significant (IAA: all groups share letter a because pairwise comparisons were non-significant; MeJA: NAA and IAA share b, ABT is a). GA and ZT showed no significant main effects or interaction ($p > 0.05$), hence no letters. The letter markings in the table represent only the relative differences among treatment groups and do not involve comparisons with the blank control.

3.4 Effects of Different PGR Treatments on the Accumulation of Bioactive Constituents

The contents of genistein and genistin varied considerably among different treatments (Table 5, Fig. 3). Among all treatments, for genistein, the 0.3 mg·L⁻¹ ABT treatment yielded the highest content (4.59 mg·g⁻¹), followed by 1.0 mg·L⁻¹ ABT (3.94 mg·g⁻¹) and 1.0 mg·L⁻¹ IAA (3.89 mg·g⁻¹). Within the ABT series, genistein content at 0.3 mg·L⁻¹ was significantly higher than that at 0.1, 0.5, and 1.0 mg·L⁻¹ (all $p < 0.05$). For genistin, the 1.0 mg·L⁻¹ ABT treatment gave the highest content (33.82 mg·g⁻¹), followed by 1.0 mg·L⁻¹ IAA (31.77 mg·g⁻¹) and 0.5 mg·L⁻¹ ABT (23.13 mg·g⁻¹). IAA treatments generally outperformed NAA treatments, with genistin contents in all IAA treatments exceeding 16.76 mg·g⁻¹. The combined content of genistein and genistin reached its maximum under the 1.0 mg·L⁻¹ ABT treatment (37.76 mg·g⁻¹).

Notably, under ABT treatment, the optimal concentrations for the accumulation of genistein and genistin were inconsistent: genistein peaked at 0.3 mg·L⁻¹ ABT, whereas genistin peaked at 1.0 mg·L⁻¹ ABT, suggesting that the regulatory mechanisms governing the aglycone and glycoside forms differ.

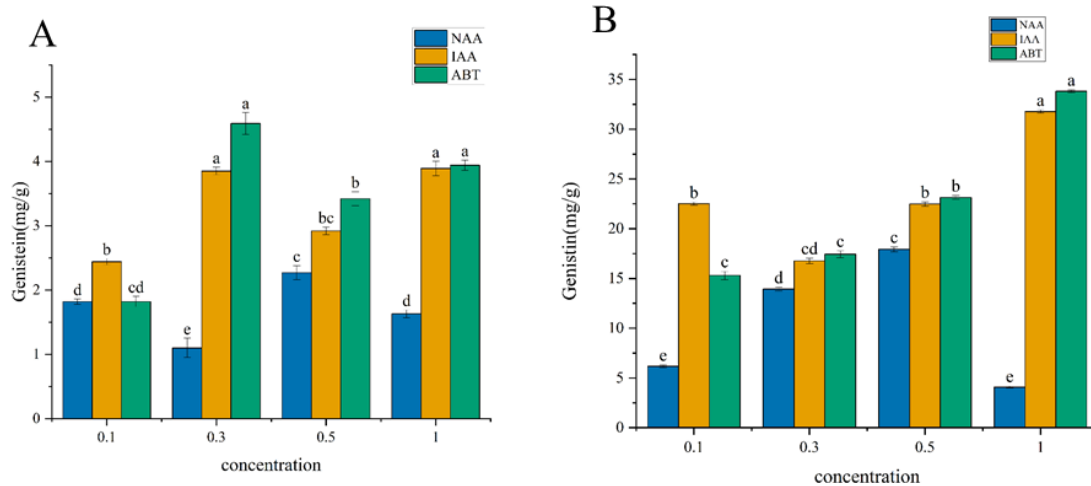


Figure 3: Effects of different concentrations of NAA, IAA, and ABT on the accumulation of bioactive constituents of *F. macrophylla*. (A): genistein; (B): genistin. Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$); the same letter indicates no significant difference ($p > 0.05$). Genistein and genistin exhibited significant interactions ($p < 0.05$); therefore, letters are based on comparisons among all 12 treatment combinations. All statistical comparisons are limited to treatment groups; no hormone-free control was included.

Table 5: Effects of different concentrations of NAA, IAA, and ABT on the accumulation of bioactive constituents of *F. macrophylla*.

Treatment	Concentration	Genistein (mg·g ⁻¹)	Genistin (mg·g ⁻¹)
NAA	0.1	1.82 ± 0.04d	6.18 ± 0.13e
	0.3	1.10 ± 0.15e	13.93 ± 0.18d
	0.5	2.27 ± 0.11c	17.93 ± 0.24c
	1	1.63 ± 0.06d	4.08 ± 0.10e
IAA	0.1	2.44 ± 0.05b	22.51 ± 0.15b
	0.3	3.85 ± 0.06a	16.76 ± 0.26cd
	0.5	2.92 ± 0.06bc	22.48 ± 0.21b
	1	3.89 ± 0.11a	31.77 ± 0.16a
ABT	0.1	1.82 ± 0.08cd	15.30 ± 0.41c
	0.3	4.59 ± 0.17a	17.43 ± 0.33c
	0.5	3.42 ± 0.11b	23.13 ± 0.19b
	1	3.94 ± 0.08a	33.82 ± 0.14a

Note: Different lowercase letters indicate significant differences among treatment groups ($p < 0.05$); the same letter indicates no significant difference ($p > 0.05$). Genistein and genistin exhibited significant interactions ($p < 0.05$); therefore, letters are based on comparisons among all 12 treatment combinations. The letter markings in the table represent only the relative differences among treatment groups and do not involve comparisons with the blank control.

3.5 Exploratory Analysis of Associations between Physiological Indicators and Bioactive Constituents

Spearman's rank correlation analysis preliminarily revealed the co-variation trends between various physiological factors and the two bioactive constituents under different PGR treatments (Table 6).

Under NAA treatment, genistin content was positively correlated with GPT activity ($\rho = 0.872$, $q < 0.001$), but was significantly negatively correlated with BR content ($\rho = -0.829$, $q = 0.001$); genistein was negatively correlated with ABA ($\rho = -0.608$, $q = 0.036$) and endogenous IAA ($\rho = -0.608$, $q = 0.036$). Under IAA treatment, genistein was significantly negatively correlated with GOT activity ($\rho = -0.734$,

$q = 0.026$); genistin showed a negative trends with ZT ($\rho = -0.605$, $q = 0.037$). Under ABT treatment, genistin was significantly negatively correlated with ABA content ($\rho = -0.725$, $q = 0.008$).

Table 6: Spearman rank correlation coefficients (ρ) between physiological parameters and genistein/genistin contents under different PGRs treatments.

Treatment	NAA		IAA		ABT	
	Genistein	Genistin	Genistein	Genistin	Genistein	Genistin
POD	0.294	0.764	-0.531	0.669	-0.126	-0.146
SOD	-0.007	0.093	0.196	-0.691	0	-0.348
MDA	0.259	-0.136	-0.028	-0.022	0.07	-0.21
GPT	0.014	0.872**	0.517	-0.734	-0.007	0.362
GOT	-0.077	0.029	-0.734*	0.389	0.13	0.714
Soluble protein	-0.28	-0.15	-0.259	0.259	-0.699	-0.483
Soluble Sugar	0.105	0.207	-0.552	0.561	0.392	0.505
ABA	-0.608*	-0.650*	-0.175	0.259	-0.07	-0.725**
BR	-0.497	-0.829**	-0.497	0.324	0.287	-0.16
GA	-0.378	-0.243	0.273	-0.367	0.028	-0.231
IAA	-0.608*	-0.357	0.119	0.13	-0.119	0.16
MEJA	-0.874**	-0.507	-0.189	0.518	0.399	-0.288
ZT	-0.35	-0.071	0.503	-0.605*	-0.07	-0.394

Note: Correlations were calculated separately for each treatment group (NAA, IAA, and ABT; $n = 12$ per group). Raw p -values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) method, with all 78 tests (3 groups $\times$ 13 parameters $\times$ 2 compounds) treated as a single family. * $q < 0.05$; ** $q < 0.01$.

4 Discussion

Rooting is a critical step for the successful establishment of tissue-cultured plantlets, directly affecting their survival rate upon transplanting and subsequent growth and development. Moreover, as an important organ for plant secondary metabolism, root development is often closely associated with the biosynthesis and accumulation of isoflavonoids.

In the study, IAA was markedly superior to NAA and ABT among the treatments tested in promoting root elongation, with the $0.5 \text{ mg}\cdot\text{L}^{-1}$ IAA treatment producing the longest roots (6.28 cm). This is consistent with the established understanding that IAA, as the primary endogenous auxin, promotes root growth by regulating cell elongation [11]. Similarly, Hu et al. reported that different concentrations of IAA can significantly alter root morphology in maize [32]. In contrast, ABT at $1.0 \text{ mg}\cdot\text{L}^{-1}$ induced the highest root number (6.57 roots), but its rooting percentage (95.71%) and root length (4.43 cm) were relatively low, suggesting that ABT may preferentially promote root primordium differentiation and lateral root formation rather than axial elongation [33]. NAA achieved a favorable balance between rooting percentage (reaching 100% at most concentrations) and root length (4.96 cm at $0.5 \text{ mg}\cdot\text{L}^{-1}$), suggesting that it may have an overall advantage as a broad-spectrum growth regulator in this experiment [34].

Under NAA treatment, POD activity exhibited a pattern of initial increase followed by a decrease with increasing concentration, reaching its maximum at $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA, while MDA content remained relatively low. This suggests that this treatment may have achieved a favorable balance between promoting growth and mitigating membrane lipid peroxidation damage [35]. The higher SOD activity in NAA-treated plantlets compared with those under ABT and IAA further supports the notion that NAA, as a synthetic auxin, may trigger a stronger antioxidant response. However, the relatively small variation in SOD activity among treatments indicates that SOD functions as a constitutively expressed first line of defense, whereas

POD acts as a more flexible, inducible enzyme that participates in the differential responses to various PGRs [36,37].

GPT and GOT are key enzymes in nitrogen and amino acid metabolism in plants. Under the 0.3 mg·L⁻¹ NAA treatment, GPT activity reached the highest value of 3044.92 µg·g⁻¹·30 min⁻¹. Spearman's rank correlation analysis revealed a highly significant positive correlation between genistin content and GPT activity under NAA treatment ($\rho = 0.872$, $q < 0.001$). This suggests that NAA may be associated with enhanced GPT-mediated transamination efficiency, thereby providing more nitrogen metabolic precursors for isoflavone biosynthesis; however, further verification through enzyme kinetics or inhibitor experiments is required. In contrast, under IAA treatment, GOT activity was significantly negatively correlated with genistein content ($\rho = -0.734$, $q = 0.026$). A possible explanation for this opposing regulatory pattern is that, when IAA promotes root elongation, carbon skeletons and reducing power are preferentially allocated to root growth, creating substrate competition with isoflavone biosynthesis; this finding is consistent with the regulation of isoflavone biosynthesis under growth and stress conditions.

Soluble proteins and soluble sugars serve as important material bases and energy sources [38]. The application of auxins during rooting enhances the accumulation of soluble sugars in plants [39]. In the present study, soluble protein content was relatively high under all low-concentration auxin treatments, indicating that low auxin concentrations are conducive to maintaining high metabolic activity. Soluble sugar content was significantly higher under ABT treatment than under NAA and IAA. Under ABT treatment, genistin showed a positive trend with soluble sugar content ($\rho = 0.505$, $q = 0.094$), but this did not reach statistical significance; therefore, whether the higher genistin content under ABT is directly related to sugar supply remains to be further verified [40].

The application of exogenous growth regulators does not merely produce a single rooting effect but may reshape the endogenous hormone balance, thereby influencing development [41,42]. For instance, melatonin has been shown to promote lateral root formation in onion by inducing ROS production, a process closely associated with endogenous IAA accumulation in the differentiation zone [43]; in maize, an antagonistic interaction between ABA and auxin during lateral root initiation has been reported [44]. These findings echo our observation that elevated ABA levels under ABT treatment were accompanied by changes in root phenotype, suggesting that multi-hormone coordinated regulation may also play an important role in root system establishment in *F. macrophylla*.

In the present study, the ABA and MEJA contents in the ABT treatment group were generally higher than those in the NAA and IAA groups, suggesting that ABA-mediated signaling may be involved in the ABT-induced accumulation of isoflavones.

Spearman's rank correlation analysis revealed a significant negative correlation between genistin and BR content under NAA treatment ($\rho = -0.829$, $q = 0.001$). Since BR is a hormone that promotes cell elongation, the decrease in its level may indicate a partial shift in metabolic flux from "growth" to "secondary metabolism"; however, this is only one of several possible explanations. Under NAA treatment, genistein content was significantly negatively correlated with endogenous IAA content ($\rho = -0.608$, $q = 0.036$), which may reflect the feedback inhibition of endogenous IAA synthesis by exogenous NAA. Under IAA treatment, genistin content was significantly negatively correlated with ZT content ($\rho = -0.605$, $q = 0.037$). The above results indicate that the correlation patterns between isoflavone accumulation and endogenous hormones vary among different exogenous auxin treatments, and the coordinated changes of multiple hormones may be one of the key factors determining the final content of bioactive constituents.

In terms of bioactive constituent accumulation, the ABT treatment performed prominently. The genistin content reached 33.82 mg·g⁻¹ under 1.0 mg·L⁻¹ ABT, and the genistein content reached 4.59 mg·g⁻¹

under $0.3 \text{ mg}\cdot\text{L}^{-1}$ ABT, both representing the highest values among all treatments. It is speculated that this may be attributable to the synergistic regulation of multiple key nodes in the isoflavone biosynthetic pathway by IAA and NAA [45]. Similar synergistic effects have been reported in callus culture of *Genista tinctoria*, where specific auxin combinations can lead to significantly higher isoflavone content than single-auxin treatments [46]. In the present study, ABT was more effective than IAA and NAA in promoting genistin accumulation, which may be related to the fact that ABT simultaneously provides two distinct auxin signals, thereby activating the isoflavone biosynthetic pathway more efficiently. However, the optimal concentrations for the accumulation of the two bioactive constituents were not entirely consistent. This may be because genistein, as the aglycone form, relies more on carbon flux partitioning through the upstream phenylpropanoid pathway [47,48], whereas genistin, as a glycosylation product, is additionally constrained by UDP-glucosyltransferase (UGT) activity and glycosyl donor availability [49–51]. This nonlinear concentration–response relationship under ABT treatment suggests that future studies should employ a denser concentration gradient to precisely pinpoint the optimal concentration range, and this should be further verified through experiments such as UGT enzyme activity assays and dynamic monitoring of sugar donors.

Under the present experimental conditions, $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA achieved a favorable balance among “rooting–stress resistance–metabolism,” preliminarily indicating its suitability for standardized seedling propagation. $1.0 \text{ mg}\cdot\text{L}^{-1}$ ABT provided a promising candidate for high isoflavone production (total isoflavones $37.76 \text{ mg}\cdot\text{g}^{-1}$), but its weaker root system necessitates corresponding transplanting management; $1.0 \text{ mg}\cdot\text{L}^{-1}$ IAA offered another balanced option between root elongation and bioactive constituent accumulation. It should be emphasized that the above conclusions are based on the preliminary screening results of this experiment, and the following limitations should be fully considered in their interpretation and application.

It should be noted that this study has several methodological limitations. First, the experimental design did not include a hormone-free blank control group, which may affect the assessment of effect baselines and the quantification of true effect sizes; moreover, the 60-day harvest time point was mainly based on previous static observations without systematic time-course kinetic validation [10]. In addition, although three biological replicates are acceptable in preliminary screening experiments, the relatively limited sample size somewhat restricts the robustness of statistical extrapolation. Second, this study did not design combinations of different IAA:NAA ratios or interactions with cytokinins, making it impossible to clearly distinguish whether the superiority of ABT arises from synergistic signaling effects or a specific ratio; meanwhile, Spearman correlation analysis only reflects co-variation trends and is insufficient to infer causal relationships, and the related inferences require further experimental verification.

These limitations indicate that the conclusions of this study should be regarded as preliminary findings and need to be carefully interpreted and verified in subsequent studies. Future studies should include a true blank control to establish effect baselines, conduct time-course kinetic experiments, and expand the sample size to validate the trends revealed in this study. Furthermore, a denser ABT concentration gradient within the $0.1\text{--}1.0 \text{ mg}\cdot\text{L}^{-1}$ range should be employed, and transcriptomic or metabolomic approaches should be integrated to elucidate the molecular regulatory network underlying auxin-mediated regulation of isoflavone accumulation in *F. macrophylla*.

5 Conclusion

This study systematically evaluated the effects of three auxin types (NAA, IAA, and ABT) at four concentration levels on root development, physiological indicators, endogenous hormone levels, and isoflavone accumulation in *F. macrophylla* tissue-cultured plantlets, thereby preliminarily revealing the

distinct patterns by which auxin type and concentration modulate the trade-offs among growth, stress resistance, and secondary metabolism.

Among the tested combinations, 0.5 mg·L⁻¹ NAA achieved a rooting percentage of 100%, and performed best in activating the antioxidant system and maintaining membrane integrity, striking a relatively favorable balance among “growth–stress resistance–metabolism,” and is therefore suitable as a preferred option for standardized seedling propagation. ABT treatment was the most effective in promoting isoflavone accumulation; under 1.0 mg·L⁻¹ ABT, genistin content reached 33.82 mg·g⁻¹, and under 0.3 mg·L⁻¹ ABT, genistein content reached 4.59 mg·g⁻¹, both representing the highest values among all treatments. The 1.0 mg·L⁻¹ ABT treatment achieved the maximum total bioactive constituent content (total isoflavones 37.76 mg·g⁻¹), but its root system was relatively short (average root length only 4.43 cm), necessitating appropriate transplanting management. 1.0 mg·L⁻¹ IAA offered another balanced strategy between root elongation and bioactive constituent accumulation. These findings collectively indicate that the selection of plant growth regulators should be based on specific production objectives rather than pursuing extreme values of a single indicator.

Furthermore, the optimal accumulation concentrations of genistein and genistin under ABT treatment were inconsistent (0.3 and 1.0 mg·L⁻¹, respectively), suggesting differences in the regulatory mechanisms governing the aglycone and glycoside forms. This observation provides a new entry point for research on the metabolic regulation at the branching point between isoflavone aglycones and glycosides. However, these observations are based on the preliminary screening results of this study, and their generality and mechanistic explanations remain to be further verified by subsequent experiments, especially under conditions with a blank control group and an expanded sample size. Future studies should employ a denser concentration gradient within the 0.1–1.0 mg·L⁻¹ range to precisely pinpoint the optimal range.

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