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Effects of Methyl Jasmonate on Phenolic Acid Production in *in vitro* Root Cultures of *Gentiana lutea* and *Gentiana boissieri*

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Abstract: This research examines the impact of varying durations and concentrations of methyl jasmonate (MeJA) treatments on the phenolic acid production in *in vitro* roots of *Gentiana lutea* and *Gentiana boissieri*. These species are highly valued for their bioactive metabolites in the pharmaceutical and food industries, as well as their ornamental appeal in horticulture. Nevertheless, both species face threats from overharvesting and human-induced pressures. The study utilized root cultures grown in liquid media, followed by MeJA elicitation at concentrations of 0.1, 0.5, and 1.0 mM for durations of 2, 4, 6, 8, and 10 days. Phenolic acids, including gallic acid (GA), chlorogenic acid (CGA), caffeic acid (CA), *p*-coumaric acid (*p*-QA), *o*-coumaric acid (*o*-QA), ferulic acid (FA), cinnamic acid (CNA), and rosmarinic acid (RA), were quantified in the harvested roots using HPLC. The results revealed that phenolic acid levels were influenced by both MeJA concentration and application duration. The findings highlight that MeJA, when optimally applied, can enhance phenolic acid production in *in vitro* root cultures of *G. lutea* and *G. boissieri* effectively.

Key words: Plant Biotechnology, Secondary Metabolite Production, *In vitro* Techniques, Antioxidant Activity, HPLC

1. Introduction

Gentiana lutea L. (Yellow Gentian), belonging to the Gentianaceae family, is a notable plant species widely utilized in both traditional and modern medicine. Its roots and rhizomes are known for their diverse pharmacological properties, including anti-inflammatory, antioxidant, and antimicrobial effects. They are also employed in the treatment of digestive, cardiac, liver, and gallbladder disorders, as well as in immune system enhancement and circulatory regulation [1-6]. Beyond its medicinal applications, *G. lutea* serves as a key ingredient in the production of liqueurs, tonics, cosmetics, and smoking cessation products, and is valued as an ornamental plant due to its striking yellow flowers [3, 7]. However, increasing demand for these applications has resulted in the extensive harvesting of the plant from its natural habitats.

G. lutea thrives in nutrient-rich, well-draining, humus-rich, acidic, sandy-loamy soils [8, 9]. It is also highly sensitive to heat, restricting its distribution to specific climatic conditions [10]. In Turkey, *G. lutea* is primarily found in high-altitude regions, such as the Western and Eastern Black Sea mountain ranges, including the Uludağ and Rize mountains, at altitudes ranging from 1000 to 2500 meters [11-14]. The species prefers cool and humid climatic conditions and is limited to specific ecological niches due to these habitat-specific requirements. However, overharvesting and habitat degradation further threaten its limited distribution in Turkey [15, 16]. Consequently, several European countries and Turkey have implemented legal protection measures for *G. lutea*, and *in vitro* propagation techniques have been utilized to support its conservation and sustainable use [17, 18].

G. boissieri Schott & Kotschy ex Boiss., another species of the same genus, is endemic to Türkiye [19]. This visually appealing plant, characterized by its vibrant purple flowers, is found in moist pastures at altitudes of 2400–2700 meters in the Karagöl region of the Niğde Bolkar Mountains. Although its population has persisted due to its rhizomes, it remains under intense anthropogenic pressure [20]. The *Red Data Book of Plants of Turkey* lists this species as "VU" (vulnerable), indicating a high risk of extinction in the medium term [21].

Given the ecological selectivity and endangerment of *Gentiana* species due to overharvesting, *in vitro* secondary metabolite production has gained prominence as an alternative method for obtaining these valuable compounds [22]. These techniques facilitate the development of pilot-scale production systems, akin to "plant factories," for synthesizing secondary metabolites without impacting natural populations [22, 23]. Various *in vitro* methods, including callus, cell suspension, shoot, embryo, and root cultures, have been employed. Among these, root cultures are particularly effective due to their high productivity, genetic and biochemical stability, and rapid growth under *in vitro* conditions [24, 25]. Numerous studies have demonstrated the successful production of valuable metabolites in significant quantities using root cultures [26-28].

Moreover, *in vitro* secondary metabolite production can be further enhanced by applying biotic or abiotic elicitors. Methyl jasmonate (MeJA), a methyl ester of jasmonic acid, is a widely used elicitor that regulates plant defense mechanisms and secondary metabolite synthesis. MeJA activates specific receptors in plant cells, initiating biochemical and molecular pathways that enhance the expression of genes involved in the biosynthesis of secondary metabolites [29-31]. This elicitor has been used successfully to increase the production of compounds such as alkaloids, flavonoids, and terpenoids.

Both *G. lutea* and *G. boissieri* are rich in iridoids, secoiridoids, xanthones, and significant amounts of phenolic acids [11]. Phenolic acids are organic compounds with a phenolic structure that play an important role in plant defense and contribute significantly to human health. With potent antioxidant properties, these compounds combat oxidative stress by neutralizing free radicals. They also possess anti-inflammatory, anticancer, and antimicrobial effects, making them highly valuable for health applications [32-33].

This study aimed to evaluate the effects of MeJA treatments of varying concentrations and durations on the synthesis of phenolic acids in *in vitro* root cultures of *G. lutea* and *G. boissieri*. Additionally, it sought to establish an optimized protocol for producing these important secondary metabolites.

2. Material and Method

2.1. Materials

This study utilized seeds of *G. lutea* and *G. boissieri*, which were germinated under *in vitro* conditions. Roots of the obtained seedlings served as the starting material. The seeds of *G. lutea* were obtained from the Kütahya Regional Forestry Directorate after obtaining the necessary permits from the Ministry of Agriculture and Forestry, General Directorate of Forestry. The seeds of *G. boissieri* were collected from the Bolkar Mountains in Niğde during the seed ripening period in cooperation with the Yalova Atatürk Horticultural Cultures Central Research Institute.

2.2. Germination of Seeds

To improve the low germination rates typical of *Gentiana* species, seeds were treated with 750 ppm gibberellic acid (GA_3) for 24 h [12, 13]. Following GA_3 treatment, seeds were rinsed with sterilized distilled water ($_{dd}H_2O$), then sterilized using 70% ethanol for 45 sec. Subsequently, they were treated with a 0.1% mercuric chloride ($HgCl_2$) solution with a few drops of Tween-20 for 3 min [18]. The seeds were rinsed four times with sterile $_{dd}H_2O$, with each rinse lasting at least 5 minutes. They were then transferred to ½ MS medium [34] supplemented with 0.1 mg/L benzyl adenine (BA), 0.01 mg/L naphthalene acetic acid (NAA), 20 g/L sucrose, and 6 g/L agar. The cultures were maintained in the dark at 25°C in a climate-controlled room [35]. After germination, the cultures were exposed to a 16-hour light/8-hour dark photoperiod (50 µmol/m²s light intensity) until seedlings developed 3–4 nodes.

2.3. Establishment and Propagation of in vitro Roots

Roots from germinated seeds were propagated using the protocol by Drobky et al. [36] for various *Gentiana* species (Fig. 1). Root tips measuring 1.5 cm were initially cultured in 50 mL of agar-free ½ MS media containing 30 g/L sucrose, 0.1 mg/L BA, and 1 mg/L NAA. Roots were maintained in this medium for three weeks before being transferred to liquid ½ MS medium containing only 30 g/L sucrose without growth regulators. For *G. lutea*, root growth was slow; thus, the original medium (½ MS with 30 g/L sucrose, 0.1 mg/L BA, and 1 mg/L NAA) was used for propagation. Cultures were maintained in a climate-controlled room at 25°C ± 1°C in darkness on a shaker set to 90 rpm. Roots were subcultured every three weeks for a total of three subcultures to provide sufficient material for the experiments.

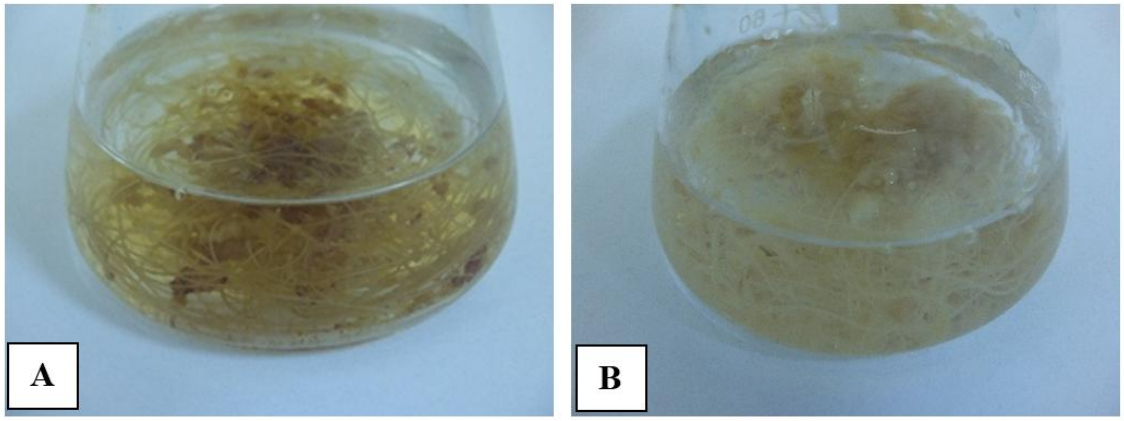

Figure 1. *G. lutea* (A) and *G. boissieri* (B) root cultures in 250 ml Erlenmeyers

2.4. Methyl Jasmonate (MeJA) Elicitation

For MeJA elicitation, *G. lutea* roots were cultured in ½ MS medium supplemented with 30 g/L sucrose, 0.1 mg/L BA, and 1 mg/L NAA, while *G. boissieri* roots were grown in ½ MS medium without growth regulators containing only 30 g/L sucrose. Inoculation densities were set at 20 g/L for *G. lutea* and 15 g/L for *G. boissieri*, as recommended by Aras Aşcı et al. [11]. MeJA was dissolved in methanol, prepared in distilled water, sterilized by filtration, and applied to 1-week-old root cultures at 0.1, 0.5, and 1.0 mM concentrations. Control groups were treated with $_{dd}H_2O$ containing the same methanol volume as the MeJA solution. Cultures were maintained at 25°C ± 1°C in darkness on a shaker set at 90 rpm. A randomized plot design was used, with three replicates per treatment, each consisting of five Erlenmeyer flasks. Roots were harvested on days 2, 4, 6, 8, and 10 post-treatment, rinsed with sterile $_{dd}H_2O$, blotted dry, and prepared for analyses.

2.5. Root Yield Assessment

Fresh root weight (FW) was measured post-harvest using an analytical balance, with results expressed in grams per Erlenmeyer. Dry root weight (DW) was obtained by drying the roots at 40°C until a constant weight was achieved, with results similarly recorded as grams per Erlenmeyer.

2.6. Extraction of in vitro Roots

Harvested roots were entirely dried, finely powdered using a mortar and pestle, and 100 mg of the material was extracted three times with 5 mL methanol in an ultrasonic water bath at room temperature for 15 min each. The supernatants were collected after centrifugation at 4000 rpm for 10 min and concentrated under vacuum at 45°C using a rotary evaporator. The dried extract was dissolved in 2 mL HPLC-grade methanol, filtered through a 0.45 µm filter, and analyzed via HPLC in triplicate.

2.7. Determination of Phenolic Acids by HPLC

Phenolic acid levels (GA, CGA, CA, *p*-CA, *o*-CA, FA, CNA, and RA) were determined using the method of Göktürk Baydar et al. [37]. A Shimadzu HPLC system with a Diode Array Detector (DAD) and an Agilent-Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 µm) was employed. The mobile phase consisted of acetic acid-ultrapure water (2:98, v/v) (A) and methanol (B), with a flow rate of 0.8 mL/min at 40°C. The gradient program ranged from 0% to 100% (B) over 117 min. Detection wavelengths were set to 278 nm for GA, RA, CNA, and *o*-CA; 309 nm for *p*-CA; 320 nm for CA and FA; and 325 nm for CGA. HPLC data were analyzed using Shimadzu Class-VP Chromatography Laboratory Software, and results were expressed as µg/g dry weight of roots.

2.8. Statistical Analysis

Data were analyzed using SPSS 16.0 statistical software, with differences among treatments determined using Duncan's multiple range test ($p \leq 0.05$).

3. Results

In order to determine the effects of 0, 0.1, 0.5 and 1.0 mM MeJA applied to *G. lutea* and *G. boissieri* root cultures on days 2, 4, 6, 8 and 10, *in vitro* root biomass (FW and DW) were determined and phenolic compound contents were calculated by HPLC.

The effects of MeJA on the FW of *G. lutea* and *G. boissieri* root cultures were evaluated over 10 days. In *G. lutea*, the control group consistently exhibited the highest FW across

time, reaching 0.8904 g (Aa) on day 10. The 0.5 mM MeJA treatment produced a FW of 0.7359 g (ABc) on day 8, showing significant growth compared to higher doses, and increased further to 0.7785 g (Ac) by day 10. The 1.0 mM MeJA treatment resulted in consistently lower FW values, ending at 0.7146 g (Ad) on day 10 (Fig. 2A).

In *G. boissieri*, FW values were lower overall (Fig. 2B). The control group achieved the highest FW of 0.5763 g (Aa) on day 10. The 0.5 mM MeJA treatment resulted in a FW of 0.4719 g (Ac), while the 1.0 mM MeJA treatment produced the lowest FW (0.465 g Ac). These results demonstrate that 0.5 mM MeJA was most effective in promoting root growth at intermediate stages for both species, whereas higher concentrations inhibited growth.

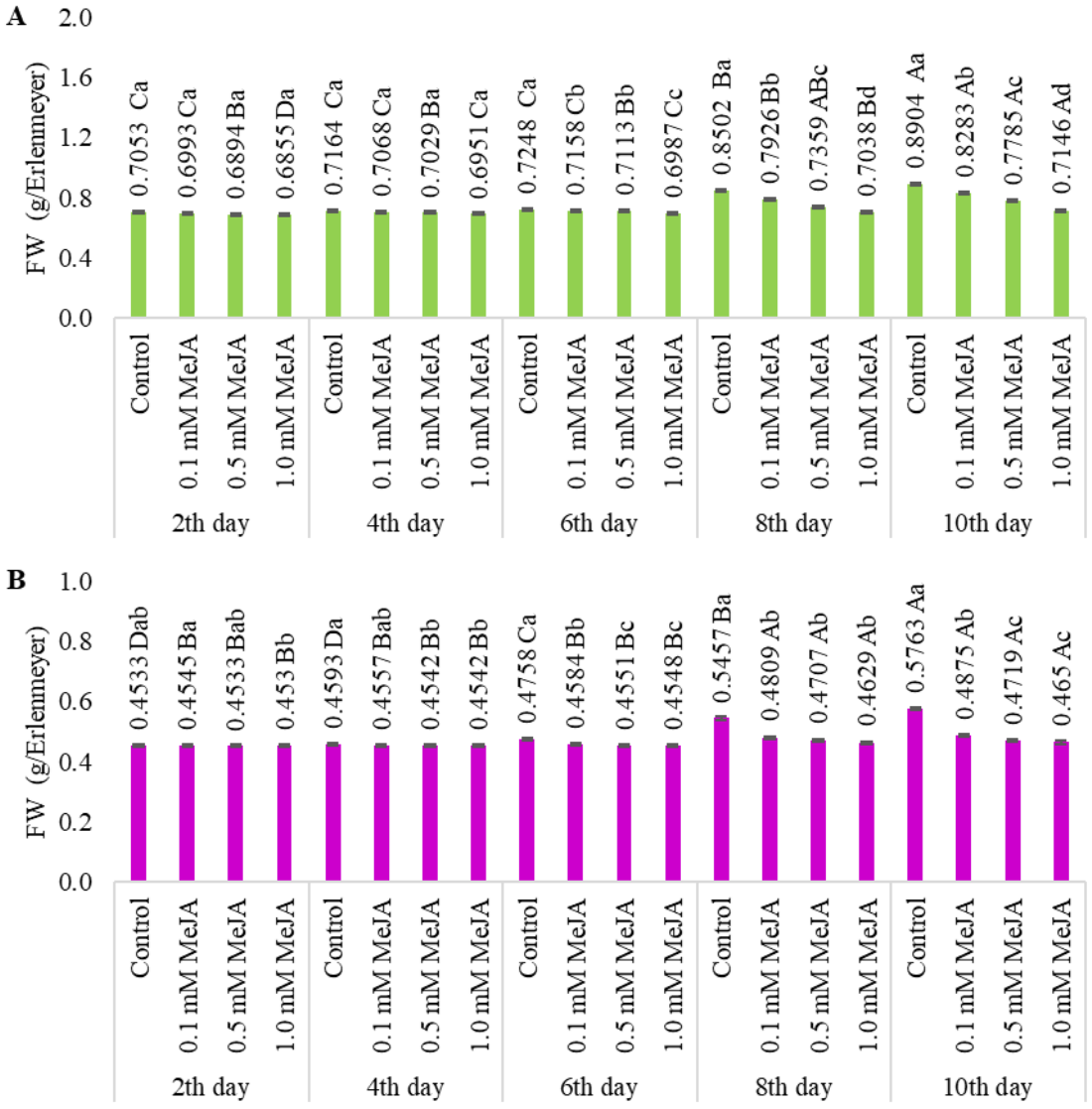

Figure 2. Fresh weights per Erlenmeyer of *G. lutea* (A) and *G. boissieri* (B) root cultures

The effects of methyl jasmonate (MeJA) on root dry weight (DW) in *G. lutea* and *G. boissieri* were evaluated over 10 days. In *G. lutea*, the control group consistently exhibited the highest DW, peaking at 0.144 g on day 8. While DW values generally decreased with increasing MeJA concentrations, no statistically significant differences were observed among treatments or across days (Fig. 3A). Conversely, in *G. boissieri*, statistically significant differences were observed both among MeJA treatments and

across days. The control group showed the highest DW on day 10 (0.0714 g), whereas 1.0 mM MeJA consistently resulted in the lowest DW, reaching 0.0546 g on day 10 (Fig. 3B). These results demonstrate that *G. boissieri* is more sensitive to MeJA treatments than *G. lutea*, with higher MeJA concentrations suppressing DW accumulation in both species.

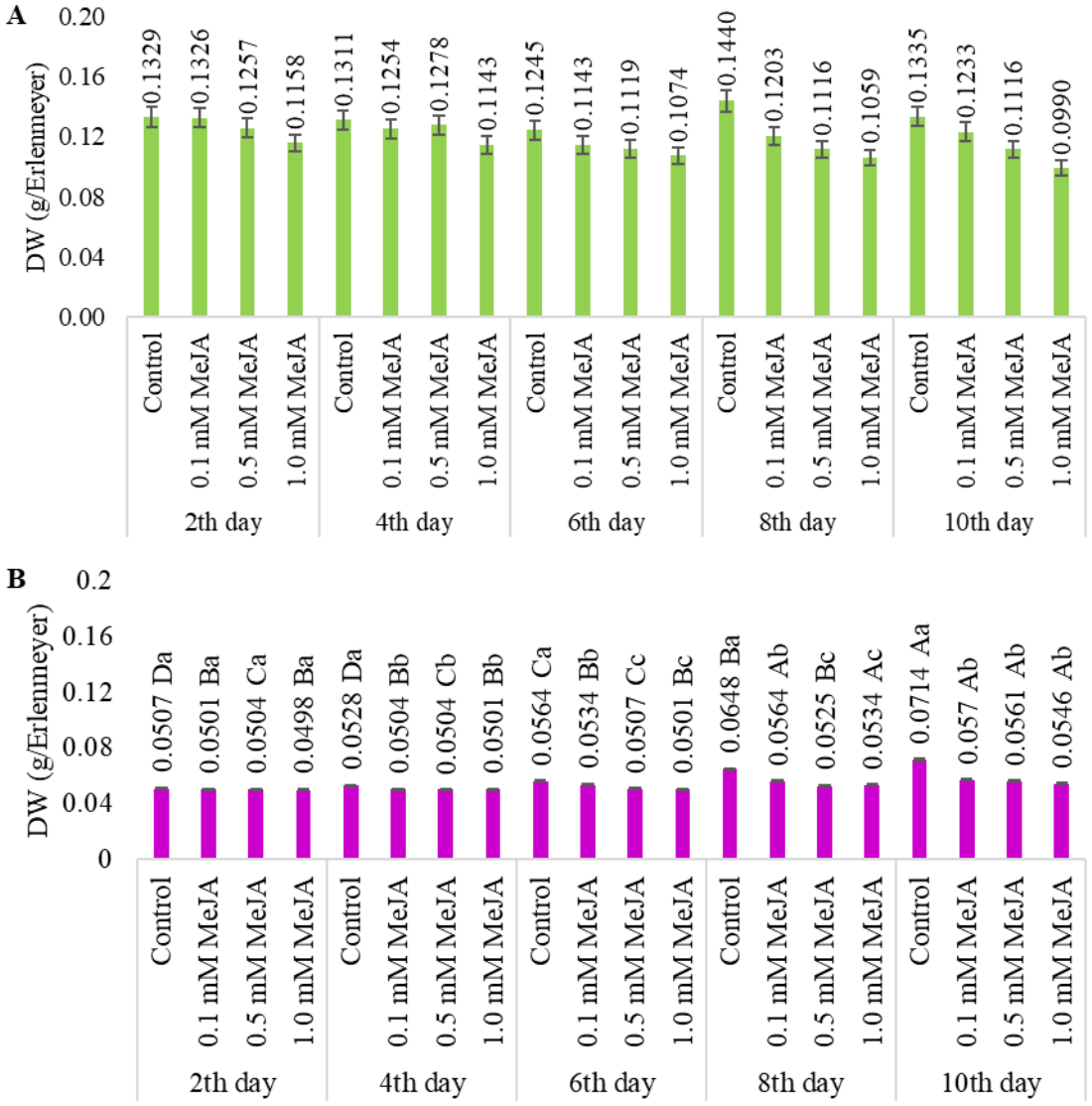

Figure 3. Dry weights per Erlenmeyer of *G. lutea* (A) and *G. boissieri* (B) root cultures

In this study, the influence of concentration and application time of MeJA on phenolic acid accumulation in root cultures of *G. lutea* and *G. boissieri* were investigated. HPLC analyses revealed that GA, CA, and *p*-CA were undetectable in *G. lutea* roots, while GA, *p*-CA, and CA were below detection limits in *G. boissieri* roots. The study showed statistically significant differences ($p \leq 0.05$) in the interaction between MeJA concentration and application time in terms of phenolic acid levels detected in the roots of both *G. lutea* and *G. boissieri*.

The initial phenolic acid subjected to analysis in this study was FA, with the resulting data presented in Figure 4. On days 2, 4, 6 and 10, the highest levels of FA in *G. lutea* roots were detected in control group. On day 8, the highest concentration of FA was observed in roots treated with 0.1 mM MeJA. The lowest amount was observed in roots

harvested on day 8 following a 1.0 mM MeJA treatment (Figure 4A). In *G. boissieri* roots, MeJA treatments had a stimulating effect on FA accumulation, with the 0.5 mM concentration for 6 days resulting in a significant increase compared to the control group (22.86 µg/g; Figure 4B). Additionally, it was determined that *G. lutea roots* contained higher amounts of FA than *G. boissieri* roots.

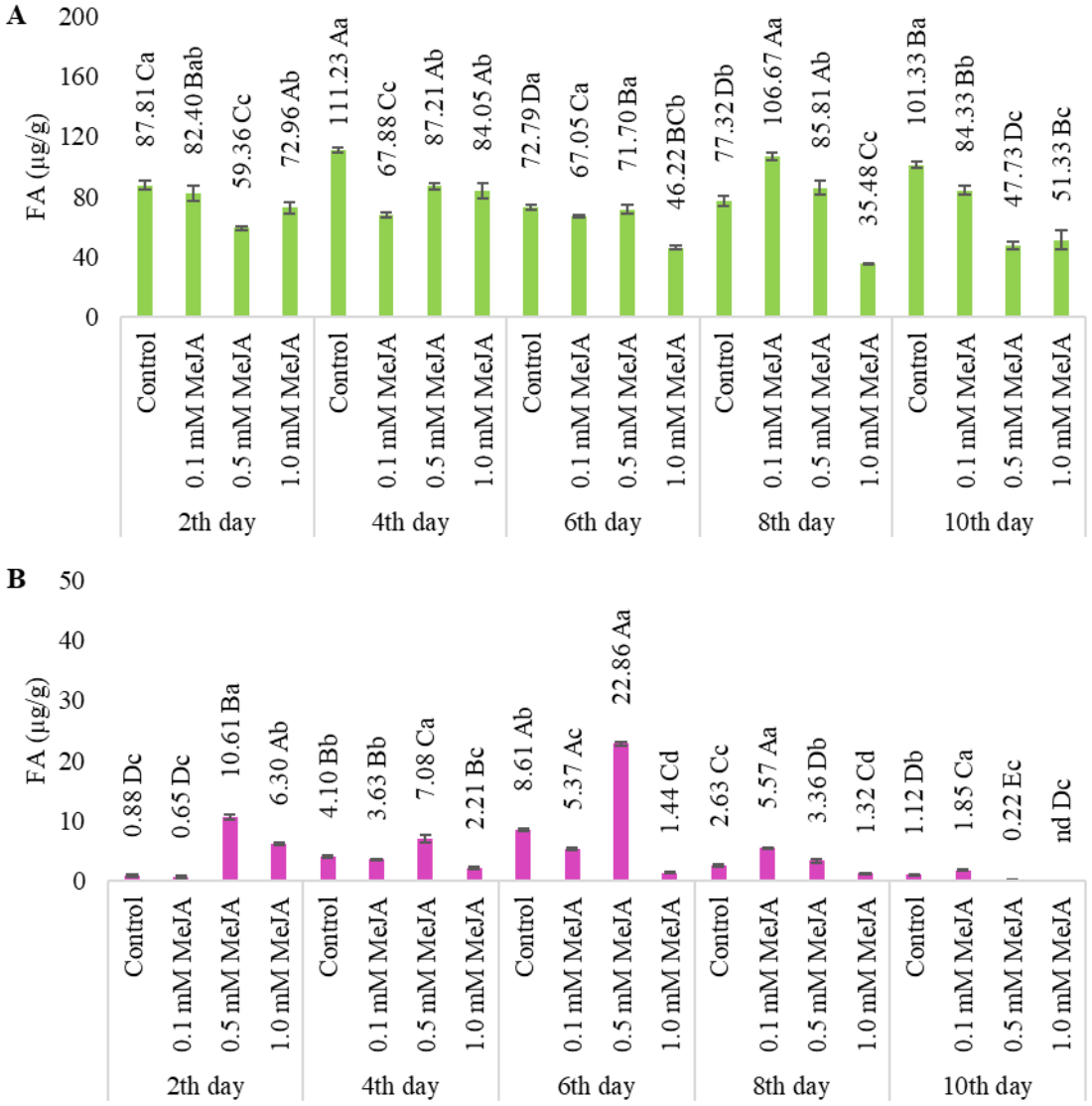

Figure 4. Variation in ferulic acid (FA) content in root cultures of *G. lutea* (A) and *G. boissieri* (B) depending on MeJA treatments and application times (Uppercase indicates day differences, lowercase indicates treatment differences ($p \leq 0.05$), nd: not detected).

The analysis of CGA data revealed that the highest levels of this compound were present in the roots of *G. lutea* and *G. boissieri* harvested on days 8 and 10 following the application of 0.1 mM MeJA. However, CGA accumulation was found to be significantly reduced at 0.5 and 1 mM MeJA concentration, and on days 8 and 10. (Figure 5A). In particular, extending the application time to 6, 8, and 10 days after treatment with 1.0 mM MeJA in *G. boissieri* roots resulted in the detection of CGA levels below the limit of quantification (Figure 5B).

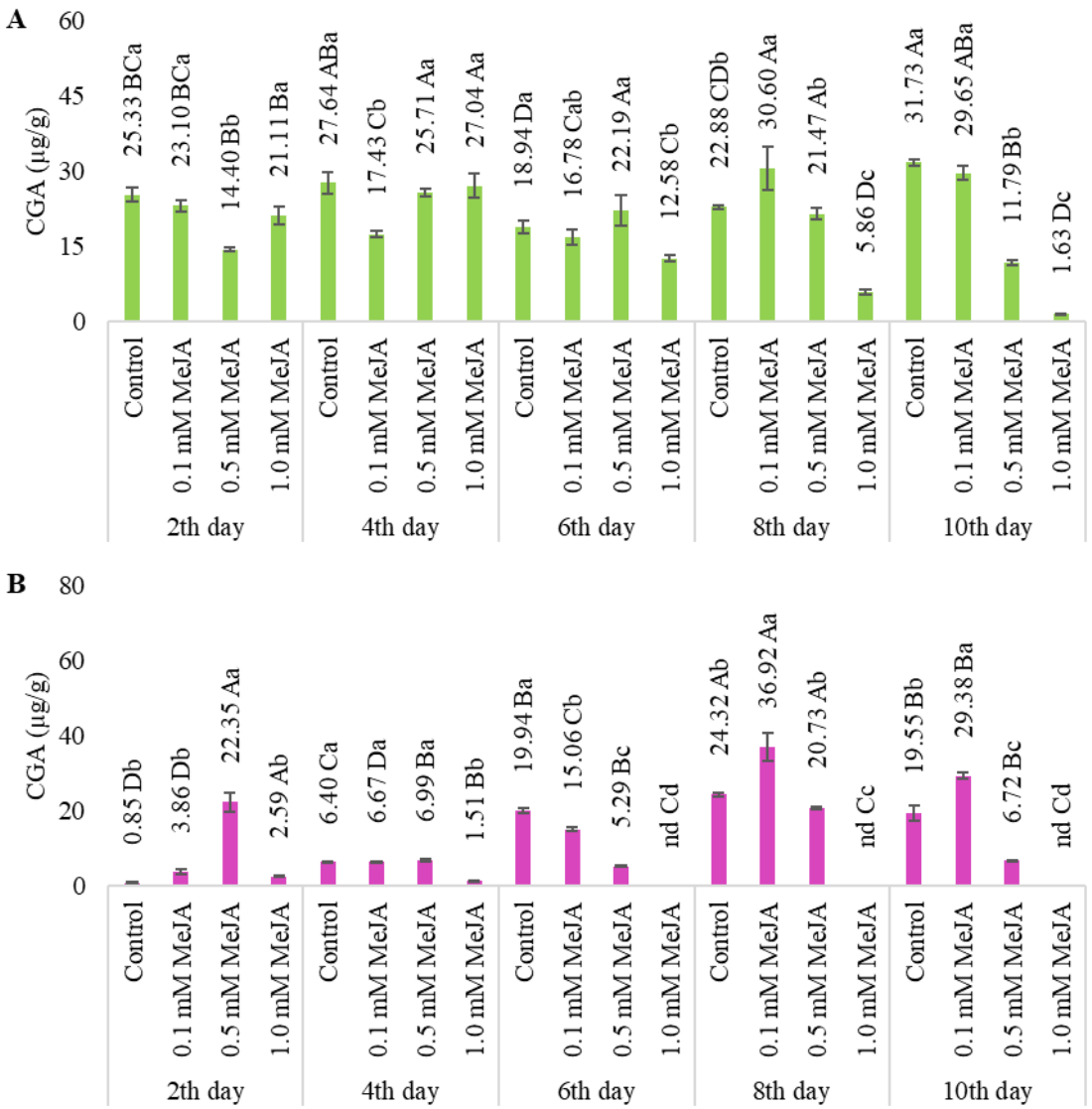

Figure 5. Variation in chlorogenic acid (CGA) content in root cultures of *G. lutea* (A) and *G. boissieri* (B) depending on MeJA treatments and application times (Uppercase indicates day differences, lowercase indicates treatment differences ($p \leq 0.05$), nd: not detected).

The study also examined the effects of RA, another phenolic acid. In *G. lutea*, it was observed that the content of rosmarinic acid decreased as the application time was extended and the concentration of MeJA increased (Figure 6A). The highest level of rosmarinic acid was recorded in the control group, which was harvested on day 4, with a value of 33.53 µg/g. In *G. boissieri*, an extension of the application time resulted in a reduction in rosmarinic acid accumulation, whereas MeJA treatments led to an increase in rosmarinic acid content (Figure 6B). The highest concentration of rosmarinic acid was observed in roots harvested on the second day following treatment with 0.5 mM MeJA.

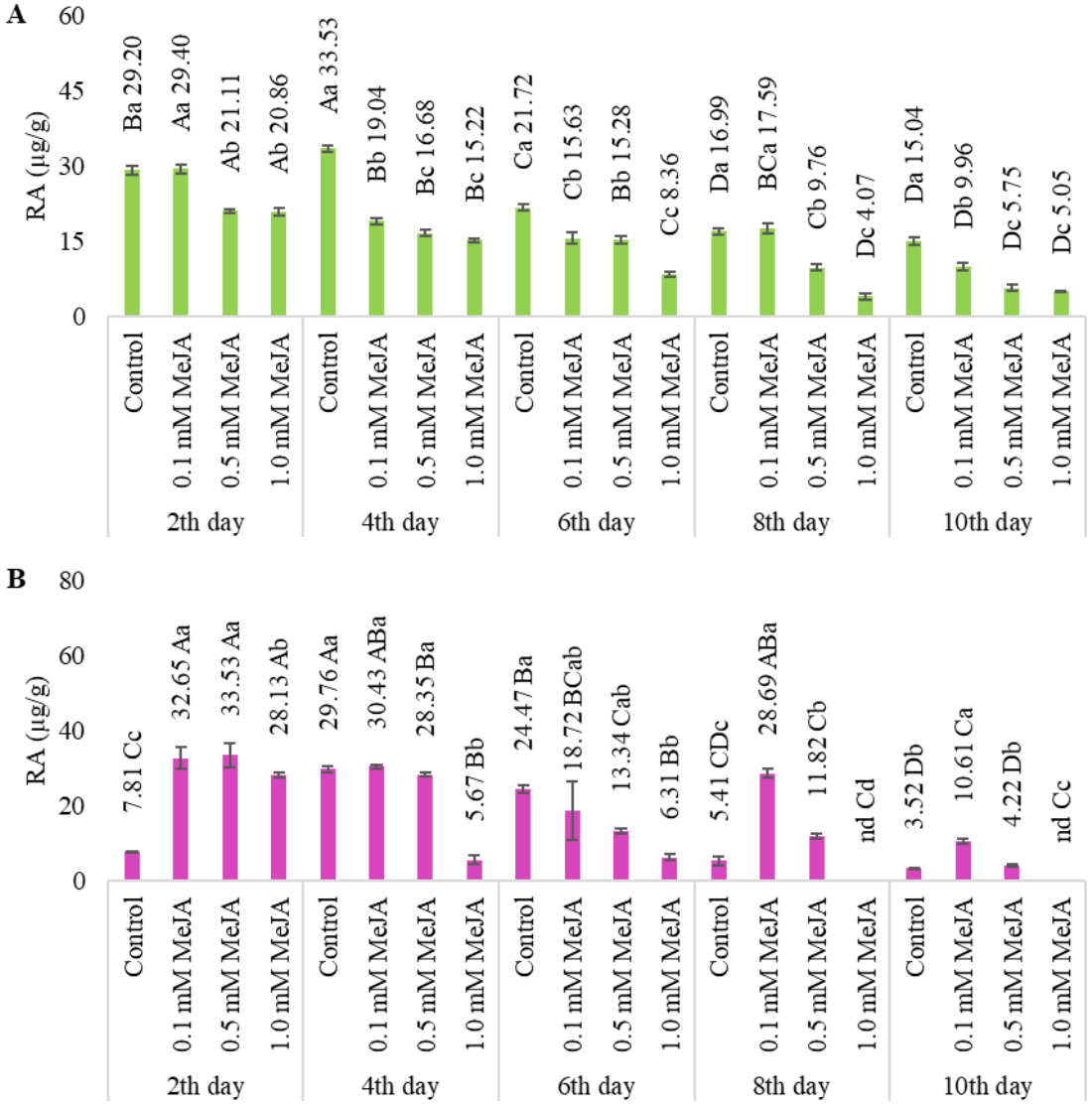

Figure 6. Variation in rosmarinic acid (RA) content in root cultures of *G. lutea* (A) and *G. boissieri* (B) depending on MeJA treatments and application times (Uppercase indicates day differences, lowercase indicates treatment differences ($p \leq 0.05$), nd: not detected).

Another phenolic acid analyzed in this study was *o*-QA. In *G. lutea* roots, *o*-QA content decreased with increasing application time. MeJA treatments were found to increase *o*-QA accumulation when applied at the appropriate concentration and duration. The most suitable treatment with the highest value of 181.93 µg/g was 0.1 mM MeJA for 2 days. (Figure 7A). In *G. boissieri*, the highest *o*-QA content was obtained with a 2-day treatment of 0.5 mM MeJA, reaching 116.98 µg/g, which was five times higher than the control (Figure 7B). However, extending the application time led to *o*-QA levels dropping below the detection limit in MeJA-treated samples.

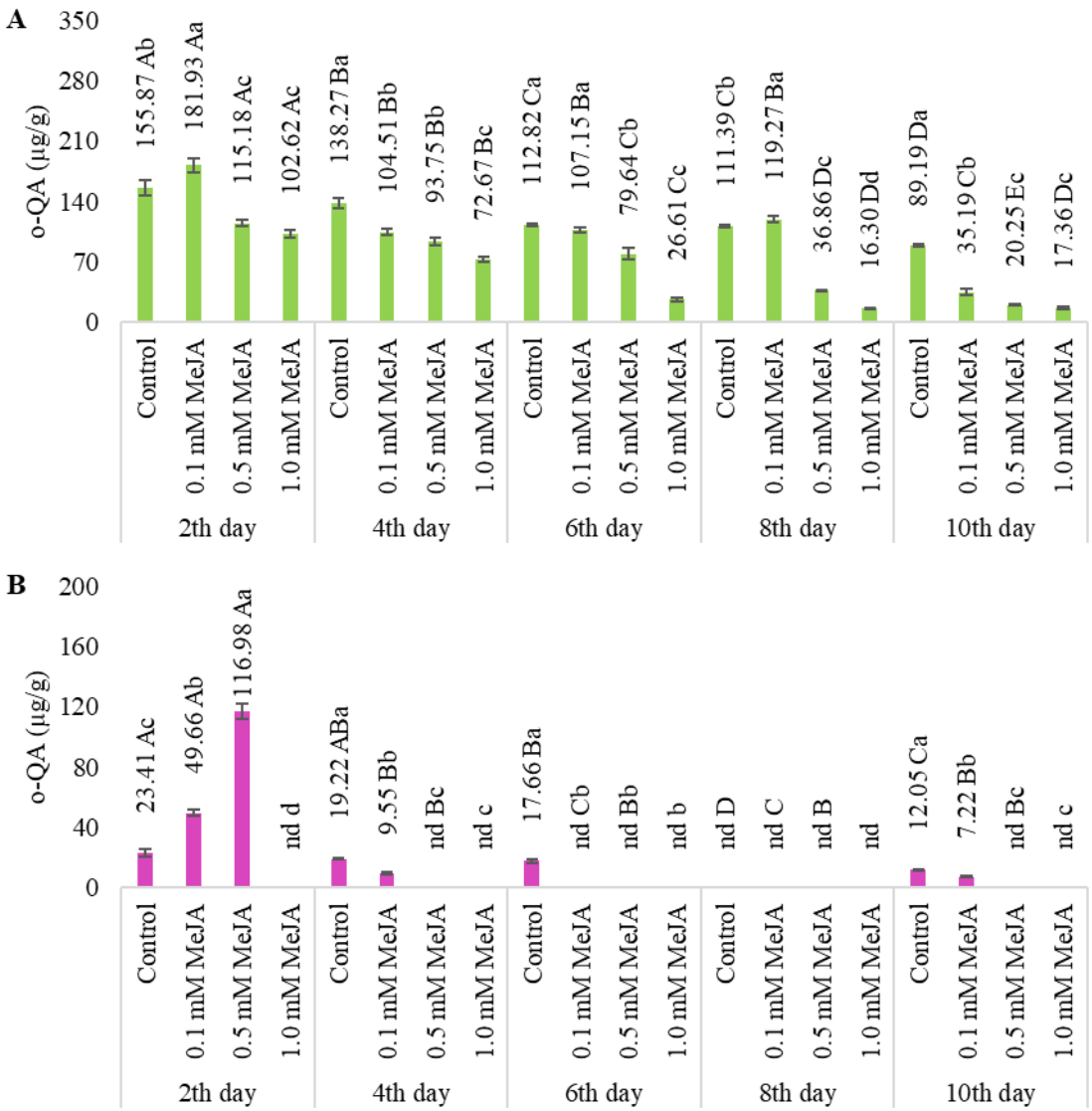

Figure 7. Variation in *o*-coumaric acid (*o*-QA) content in root cultures of *G. lutea* (A) and *G. boissieri* (B) depending on MeJA treatments and application times (Uppercase indicates day differences, lowercase indicates treatment differences ($p \leq 0.05$), nd: not detected).

CNA, another phenolic acid that was the subject of investigation, was not detected in root cultures of *G. boissieri*. In *G. lutea* roots, CNA levels were significantly diminished by MeJA treatments, with the highest CNA levels observed in control roots (Figure 8).

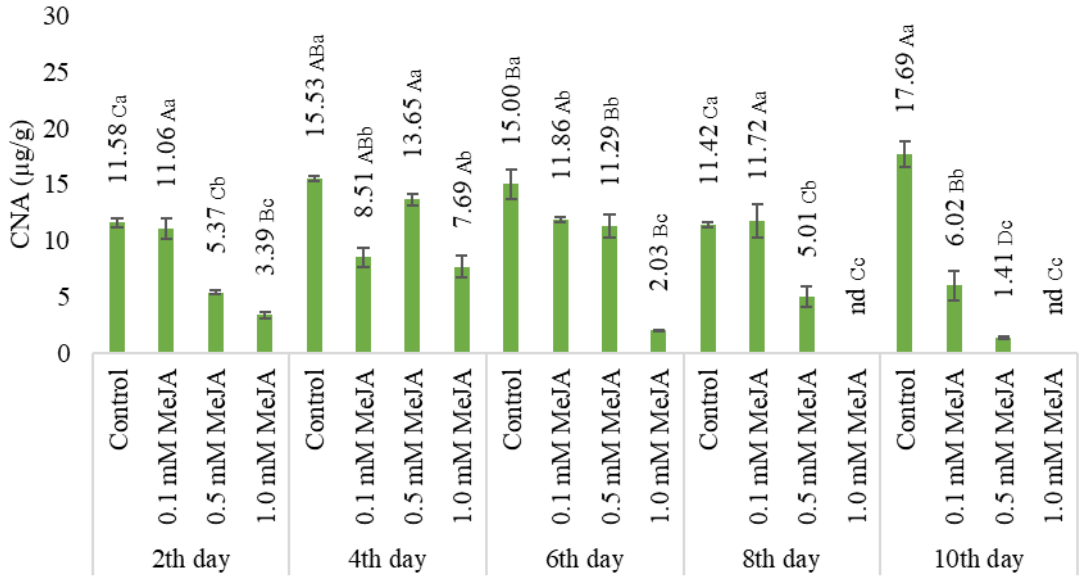

Figure 8. Variation in cinnamic acid (CNA) content in root cultures of *G. lutea* depending on MeJA treatments and application times (Uppercase indicates day differences, lowercase indicates treatment differences ($p \leq 0.05$), nd: not detected).

Additionally, CA was investigated as part of the phenolic acid, yet it remained underneath the detection limit in *G. lutea* roots. On the other hand, CA levels in *G. boissieri* roots showed significant variation depending on application times and MeJA concentrations (Figure 9). In the control roots harvested on day 2, CA was undetectable, while 0.1 and 0.5 mM MeJA treatments were found to stimulate CA accumulation. The highest value was recorded at 30.89 µg/g in roots collected on day 8 after 0.1 mM MeJA treatment.

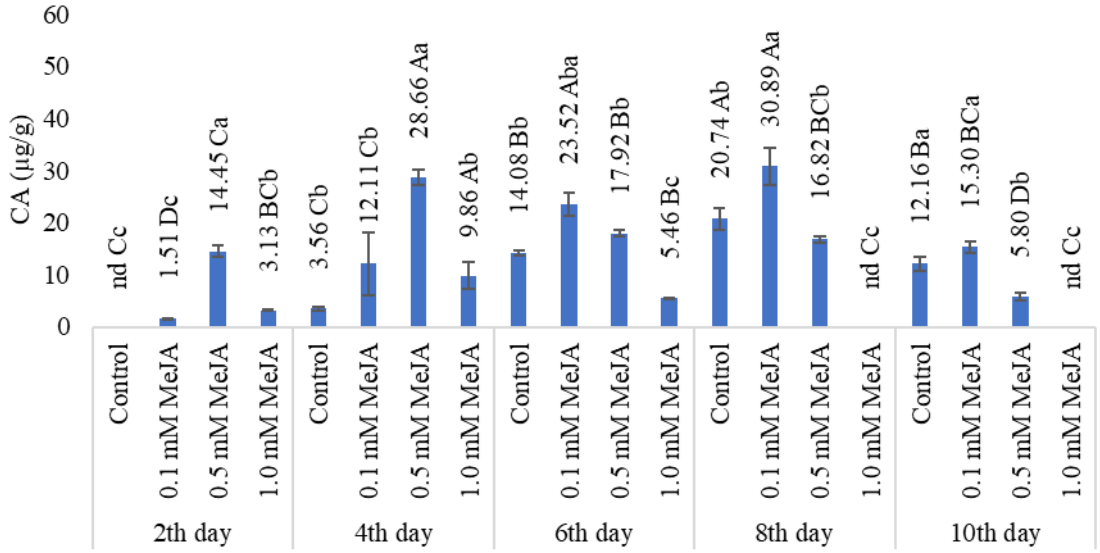

Figure 9. Variation in caffeic acid (CA) content in root cultures of *G. boissieri* depending on MeJA treatments and application times (Uppercase indicates day differences, lowercase indicates treatment differences ($p \leq 0.05$), nd: not detected).

4. Discussion

Gentiana species are well-known for their rich content of valuable metabolites, including iridoids, secoiridoids, and xanthones [38-40]. Additionally, these species contain

significant amounts of phenolic compounds [11], making them highly desirable in the pharmaceutical and food industries. However, their ecological specificity limits their distribution to certain areas, rendering them vulnerable to habitat destruction caused by uncontrolled harvesting. Despite ongoing conservation efforts, these species remain at risk of extinction [13, 21]. To address these challenges, *in vitro* secondary metabolite production techniques have gained importance, as they enable the sustainable production of high-value metabolites in consistent quality and quantity without harming natural habitats.

This study aimed to evaluate the effects of varying concentrations and durations of MeJA elicitation on the accumulation of phenolic acids, which are known for their antioxidant, anticancer, and antimicrobial properties, in the roots of *G. lutea* and *G. boissieri* under *in vitro* conditions. MeJA is a well-established elicitor for stimulating secondary metabolite biosynthesis, as supported by numerous studies [26, 29-31]. Its application has been shown to trigger the production of pathogenesis-related proteins and reactive oxygen species, as well as activate protective enzymes against oxidative stress. These processes are mediated by MeJA binding to specific cell membrane receptors, which initiate the expression of genes responsible for synthesizing these enzymes [41-43]. As a result, MeJA induces significant changes in the levels of plant secondary metabolites [29-31]. While studies on MeJA elicitation in *Gentiana* species under *in vitro* conditions are limited, notable effects on triterpenoid accumulation have been reported, highlighting its potential as an elicitor [44-45].

In this study, FW and DW of control roots increased progressively with the harvest period in both species. However, higher concentrations of MeJA significantly inhibited root growth in *G. lutea* and *G. boissieri*, consistent with previous reports of MeJA's growth-inhibitory effects on plant roots [29, 46-48]. This inhibitory effect is attributed to MeJA's interference with the mitotic cycle, particularly by preventing the transition from the G1 phase to the S-phase, thereby reducing the number of actively dividing cells and negatively affecting growth [49]. Furthermore, Noir et al. [50] suggested that MeJA delays the progression from the mitotic cycle to the endoreduplication cycle, amplifying its growth-inhibitory effects.

Distinct responses to MeJA elicitation were observed between *G. lutea* and *G. boissieri*. In *G. lutea*, no statistically significant differences were detected between MeJA concentrations or across days, whereas *G. boissieri* showed significant variations both between treatments and across harvest durations. Lower MeJA concentrations (0.1 mM) and shorter application times generally favored the accumulation of specific phenolic acids, such as CGA in *G. lutea*. In contrast, 0.5 mM MeJA proved optimal for FA accumulation in *G. boissieri*. These findings align with previous studies on other plant species, such as *Hyoscyamus niger*, where MeJA enhanced phenolic acid accumulation under similar conditions [51]. However, the inhibitory effects of higher MeJA concentrations on phenolic acid production underscore the importance of maintaining a balance between stimulation and stress-induced inhibition. A similar pattern has been observed in *Dendropanax morbifera*, where moderate MeJA concentrations enhanced phenolic compound production, while excessive levels disrupted cellular function, emphasizing the need for optimized elicitor applications [52].

The variability in MeJA's effects on secondary metabolite accumulation is strongly influenced by genotype, metabolite type, MeJA concentration, and application duration. Similar variability has been documented in other studies involving different plant species and metabolites [4, 29-31]. In this study, prolonged harvest durations, particularly at higher MeJA concentrations, negatively impacted phenolic acid accumulation. The

optimal condition for maximizing metabolite yield was identified as 0.1 mM MeJA. The reduction in metabolite production at higher concentrations may be attributed to MeJA's adverse effects on cell division and viability. Elevated MeJA levels have been shown to inhibit mitosis by blocking the G1 to S-phase transition, thereby reducing cell proliferation and ultimately affecting growth [50]. Furthermore, MeJA's negative impact on the transition from the mitotic cell cycle to the endoreduplication cycle further contributes to its growth-suppressing effects [49].

Although studies specifically investigating the effects of MeJA on phenolic acid accumulation in *Gentiana* species under *in vitro* conditions are limited, research on other plant species supports these findings. For example, in *Mentha spicata* hairy root cultures, 100 µM MeJA significantly increased the accumulation of CA, CGA, CNA, and RA, with the extent of accumulation dependent on harvest time. However, MeJA negatively impacted the accumulation of lithospermic acid [53]. Similarly, MeJA enhanced the production of rosmarinic acid and lithospermic acid B in *Salvia miltiorrhiza* hairy root cultures and rosmarinic acid in *Lithospermum erythrorhizon* cells [54-56]. Khan et al. [57] proposed that MeJA's stimulatory effects are linked to its activation of transcription factors that upregulate the biosynthesis of phenolic compounds.

This study demonstrated that MeJA is an effective elicitor for phenolic acid production but also revealed that optimal concentrations and application durations vary among *Gentiana* species. Specifically, the differences in phenolic acid production between *G. lutea* and *G. boissieri* highlight the impact of interspecies genetic diversity on this process. Similar variations have been reported in other plant species, such as *Mentha spicata*, where low MeJA concentrations enhanced phenolic acid production while higher concentrations resulted in inhibitory effects [53]. These findings underscore the need for individual optimization of MeJA treatments for each species [50, 52].

This study also emphasized the importance of translating laboratory-scale findings to large-scale bioreactor systems required for commercial production. Future studies evaluating the applicability of MeJA treatments at commercial scales could contribute to the development of sustainable metabolite production systems by determining the optimal concentrations and application methods of this elicitor [30]. In particular, detailed investigations into the hydrodynamic and metabolite stability aspects of MeJA application in bioreactor-based systems are essential [29].

5. Conclusion

This study investigated the effects of different MeJA concentrations and application durations on phenolic acid accumulation in *G. lutea* and *G. boissieri* root cultures under *in vitro* conditions. The results indicated that 0.1 mM MeJA was the most effective concentration for phenolic acid production, although the optimal duration varied depending on the metabolite. These findings highlight the potential of MeJA as an elicitor for enhancing secondary metabolite production in *Gentiana* species under controlled conditions. Optimized MeJA applications could serve as an effective strategy for the sustainable *in vitro* production of economically valuable metabolites.

Authorship contribution statement

İ. Albayrak: Conceptualization, Methodology, Analysis, Data Improvement. **A. Cessur:** Conceptualization, Methodology, Analysis, Data Improvement, Writing. **T. Demirci:** Conceptualization, Methodology, Data Improvement. **N. Göktürk Baydar:** Conceptualization, Methodology, Analysis, Writing - Review and Editing, Supervision, Project Administration, Funding Acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics Committee Approval and/or Informed Consent Information

As the authors of this study, we declare that we do not have any ethics committee approval and/or informed consent statement.

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