

Time- and dose-dependent effects of methyl jasmonate on key flavonoid pathway genes in *Gentiana* root cultures

Original Article

Abstract:

This study investigated the impact of methyl jasmonate (MJ) on the expression of key flavonoid biosynthetic genes including chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*) and dihydroflavonol 4-reductase (*DFR*) in root cultures of *Gentiana lutea* and *G. boissieri*. Gene expression was assessed via RT-PCR on days 2, 4, 6, 8, and 10 following MJ treatment at the concentrations of 0.1, 0.5 and 1.0 mM. In *G. lutea*, *CHS* expression was markedly upregulated by 0.5 mM MJ on days 4 and 10, while the activity of *DFR* gene surged 25.46-fold at 0.1 mM MJ on day 8. The expression level of *F3H* exhibited a striking 33.20-fold increase at 0.1 mM MJ on day 2, though it declined at higher concentrations. In *G. boissieri*, gene expression levels of *CHS* peaked later, with the highest levels observed at 0.5 mM MJ on day 8 and 1.0 mM MJ on day 10. *DFR* activity reached a maximum 17.11-fold increase at 1 mM MJ on day 8, while *F3H* expression was highest at 1.0 mM MJ on day 10 and 0.5 mM MJ on day 4. These findings showed that MJ modulates flavonoid biosynthesis gene expression levels in *Gentiana* root cultures in a time-, dose-, and species-specific manner, providing valuable insights for manipulating secondary metabolite production through elicitor-based strategies.

Key words:

Gentiana boissieri, *Gentiana lutea*, chalcone synthase, dihydroflavonol 4-reductase, flavanone 3-hydroxylase, methyl jasmonate, root culture

Apstrakt:

Naslov na srpskom

U ovom istraživanju ispitivan je uticaj metil-jasmonata (MJ) na ekspresiju ključnih gena uključenih u biosintezu flavonoida, uključujući halkan sintazu (*CHS*), flavanon 3-hidroksilazu (*F3H*) i dihidroflavonol 4-reduktazu (*DFR*), u korenskim kulturama vrsta *Gentiana lutea* i *G. boissieri*. Ekspresija gena određivana je metodom RT-PCR drugog, četvrtog, šestog, osmog i desetog dana nakon tretmana MJ u koncentracijama od 0,1, 0,5 i 1,0 mM. Kod vrste *G. lutea*, ekspresija gena *CHS* bila je značajno povećana pri koncentraciji od 0,5 mM MJ četvrtog i desetog dana, dok je ekspresija gena *DFR* dostigla 25,46 puta viši nivo pri koncentraciji od 0,1 mM MJ osmog dana. Nivo ekspresije gena *F3H* pokazao je izrazito povećanje od 33,20 puta pri koncentraciji od 0,1 mM MJ drugog dana, ali je opadao pri višim koncentracijama. Kod vrste *G. boissieri*, nivoi ekspresije gena *CHS* dostigli su maksimum kasnije, pri čemu su najviše vrednosti zabeležene pri 0,5 mM MJ osmog dana i 1,0 mM MJ desetog dana. Ekspresija gena *DFR* dostigla je maksimalno povećanje od 17,11 puta pri koncentraciji od 1,0 mM MJ osmog dana, dok je ekspresija gena *F3H* bila najviša pri 1,0 mM MJ desetog dana i 0,5 mM MJ četvrtog dana. Dobijeni rezultati pokazuju da MJ modulise nivoe ekspresije gena uključenih u biosintezu flavonoida u korenskim kulturama roda *Gentiana* na način koji zavisi od vremena tretmana, koncentracije i vrste, pružajući značajne smernice za unapređenje proizvodnje sekundarnih metabolita primenom strategija zasnovanih na elicitorima.

Ključne reči:

Gentiana boissieri, *Gentiana lutea*, halkan sintaza, dihidroflavonol 4-reduktaza, flavanon 3-hidroksilaza, metil-jasmonat, kulture korena

Introduction

Plants synthesize secondary metabolites, essential components of the plant's defense system against various stress factors, in addition to primary

metabolites such as carbohydrates, proteins, and nucleic acids that are necessary for their growth and development. Secondary metabolites, including various compounds like phenolic compounds,

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terpenoids, and alkaloids are valuable compounds used in medicine, food, agriculture, cosmetics, perfumery, and textiles. However, because secondary metabolites are present at low levels in plants under normal conditions, various external applications are made to increase their synthesis (Selwal et al., 2023). Elicitors of biotic and abiotic origin induce chemical reactions known as elicitation, which stimulate the plant's stress response (Maqsood et al., 2024) and when applied at appropriate concentrations and durations, they enhance the production of secondary compounds in plants (Baenas et al., 2014). Methyl jasmonate (MJ) is a naturally occurring plant hormone and a derivative of jasmonic acid, which plays a critical role in regulating plant defense mechanisms and secondary metabolism (Luo et al., 2023). MJ influences various metabolic pathways, including those for alkaloids, flavonoids, and terpenoids, thereby enhancing the production of these valuable compounds (Jeyasri et al., 2023). Indeed, its external applications have become a valuable tool for enhancing the production of secondary metabolites in various plant species. MJ interacts with receptors in plant cells, triggering a cascade of biochemical and molecular events. This leads to the upregulation of genes responsible for secondary metabolite biosynthesis (Jeong et al., 2018; Yin et al., 2019; Yang et al., 2024).

Gentiana lutea L., commonly known as yellow gentian, is a prominent member of the Gentianaceae

family, renowned for its medicinal and therapeutic properties. Its rhizomes and roots have been used in both traditional and modern medicine for a variety of therapeutic purposes, including anti-inflammatory, antioxidant and antimicrobial effects, as well as digestive, liver and gallbladder disorders, strengthening the immune system, regulating blood circulation, chronic malaria poisoning, relieving mental and physical depression and general malaise (Mirzaee et al., 2017; Jiang et al., 2021). In addition to these pharmaceutical properties, *G. lutea* is used in the production of bitter liqueurs, tonics food, cosmetics and some anti-smoking products, and as an ornamental plant with its attractive yellow flowers (Akbar & Akbar, 2020) (**Fig 1**). For this reason, *G. lutea* is a highly demanded plant species, and this demand is met by harvesting plants from their natural habitats. *Gentiana lutea* thrives in rich, deep soils with high air capacity and good water permeability (Stepanovic & Radanovic, 2011), as well as in low to moderately humus, acidic loamy-sandy soils (Prakash et al., 2017). It has been determined that available water capacity is of great importance for *G. lutea*'s growth, development, dry matter content and vegetative characteristics such as plant height, number of leaves and total number of stems, and that it is very sensitive to summer heat, so it finds a habitat in high, sloping areas with limited sun exposure (Catorci et al., 2014). With this selectivity, *G. lutea* is a rare species that is native to

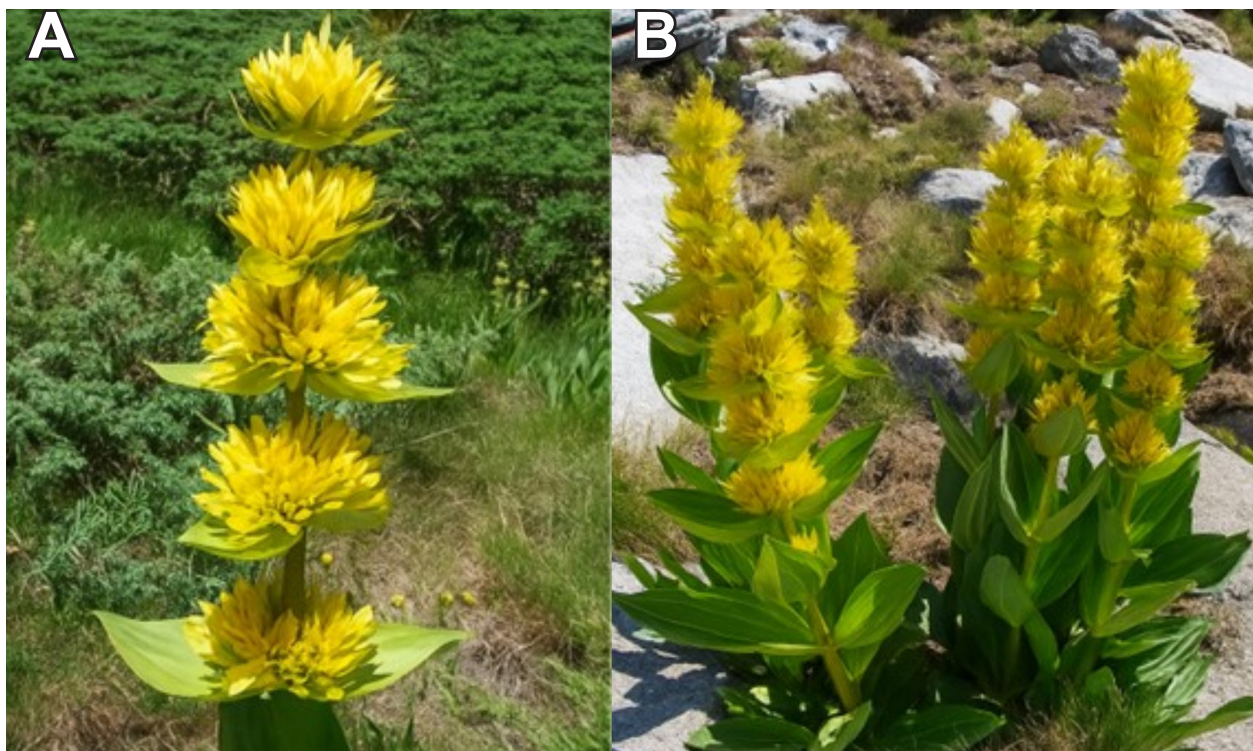


Fig. 1. *Gentiana lutea* plant (A) and inflorescence (B) (Erken & Kaleci, 2010)

the 800 to 2500 m high hilly regions of Central and Southern Europe (Cuenca-Lombrana et al., 2018). Both its rare distribution in the world flora due to its ecological selectivity and its uncontrolled harvesting (Fois et al., 2016) have caused it to become one of the endangered plant species and to become rapidly extinct (Catorci et al., 2014). For this reason, around 100 *Gentiana* species, including *G. lutea*, are protected by law in many European countries (Momcilovic et al., 1997).

Gentiana boissieri Schott & Kotschy ex Boiss., another *Gentiana* species is endemic to Turkey (Davis, 1988). It is distributed in the Karagöl region of Niğde Bolkar Mountain, in moist meadows with an altitude between 2400-2700 meters, and represents a very attractive plant species with its purple flowers (**Fig. 2**). Although it survives thanks to its rhizomes, its population is under anthropogenic pressure (Savran et al., 2015). As a matter of fact, according to the Red Data Book of Plants of Turkey, it is listed as VU (vulnerable: highly threatened in the medium term) (Ekim et al., 2000).

Gentiana lutea and *G. boissieri* are rich in iridoids, secoiridoids, and xanthenes, and they also contain small amounts of alkaloids, phytosterols, triterpenes, and essential oils (Vidari & Vita-Finzi, 2010; Aras Aşçı et al., 2022). However, it was determined that these species contain also a significant amount of phenolic acids and flavonoids (Aras Aşçı et al., 2022). Flavonoids represent a prominent class of natural compounds, classified as plant secondary metabolites with a characteristic polyphenolic structure, and are abundantly distributed throughout the plant kingdom (Panche et al., 2016). These molecules play essential roles in various aspects of plant physiology, including growth, development, and defense mechanisms under biotic and abiotic

stress conditions (Shomali et al., 2022). In addition to their functions in plants, flavonoids have attracted considerable scientific attention for their wide-ranging pharmacological properties including antioxidant, antimicrobial, antimutagenic, anti-carcinogenic, and cardioprotective activities (Karak, 2019; Ekalu & Habila, 2020; Khan et al., 2021). Due to these bioactive properties, flavonoids are recognized as promising therapeutic agents with potential applications in the prevention and treatment of numerous diseases. Their documented health-promoting effects have led to their extensive incorporation into nutraceuticals, pharmaceuticals, medical formulations, and cosmetic products (Panche et al., 2016).

In plants, secondary metabolite biosynthesis occurs through coordinated induction of genes encoding key enzymes in their metabolic pathways, typically triggered by environmental stimuli. Chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*) and dihydroflavonol 4-reductase (*DFR*) are key genes encoding enzymes in the flavonoid biosynthetic pathway, influencing the physiological processes and stress responses of plants. This study was carried out to investigate the effects of MJ applied to root cultures of *G. lutea* and *G. boissieri* species at different duration and concentrations on the expression levels of *CHS*, *F3H* and *DFR* genes in the biosynthesis pathway of flavonoids to evaluate its role in flavonoid accumulation.

Material and Methods

Surface sterilization and germination of seeds

The roots of the seedlings obtained by germinating the seeds of *G. lutea* and *G. boissieri* under *in vitro* conditions were used as plant materials. To increase germination rates, the seeds were initially treated with



Fig.2. *G. boissieri* plants and flowers

a 750 ppm GA₃ solution for 24 h (Erken & Kaleci, 2010; Erken & Özzambak, 2017). After being washed with sterile dH₂O, the seeds were first immersed in 70% ethanol for 45 sec and then shaken in a 0.1% mercuric chloride (HgCl₂) solution containing a few drops of Tween-20 for 3 min (Petrova et al., 2006). Following a surface sterilization process involving four 5-min washes, the seeds were transferred to a ½ MS (Murashige & Skoog, 1962) medium containing 0.01 mg/L naphthaleneacetic acid (NAA), 0.1 mg/L 6-benzyladenine (BA), 20 g/L sucrose, and 6 g/L agar, for germination. The seeds were cultured in the dark at 25 °C±1 until germination. Subsequently, they were placed in a climate-controlled chamber set to a photoperiod of 16 h of light and 8 h of darkness. When the seedlings reached the 3-4 leaf stage, the roots were separated from the shoots and used to initiate root culture.

Application of MJ

Propagation of roots in sufficient quantities for MJ treatments was carried out using the method previously described in detail by Aras Aşçı et al. (2022). For MJ applications approximately 0.5 g root pieces were cultured in 50 mL liquid ½ MS medium containing 1 mg/L NAA, 0.11mg/L BA, and 30 g/L sucrose for one week. Then filter sterilized MJ at 0.1, 0.5 and 1.0 mM was added to root cultures. The cultures were maintained on a shaker at 90 rpm under dark conditions and at 25 °C±1. Roots were harvested on days 2, 4, 6, 8 and 10 following MJ treatments. Harvested roots were washed, dried and made ready for analysis. The research was established according to the randomized plots experimental design with 3 replicates and 5 replicates in each replicate.

Determination of gene expression levels

After the MJ treatments, the roots harvested on the 2nd, 4th, 6th, 8th, and 10th days were washed with sterile distilled water, and after the excess water was absorbed with paper towels, approximately 100 mg of root tissue was crushed thoroughly using liquid nitrogen. RNA isolation was then performed using the PureZol (BioRAD, USA) solution, following the manufacturer's instructions. After isolation, DNase I (RNase-free Takara Bio Inc.) to prevent genomic DNA contamination and RNeasy MinElute Cleanup Kit (Qiagen) to purificate RNA were applied according to the manufacturers's recommendations. The concentration and purity of the total RNA obtained were determined using NanoDrop (Thermo, USA) and 1% agarose gel, and the samples were stored at -80 °C until further use. cDNA synthesis was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific,

USA), and the cDNA was stored at -20 °C until used for gene expression analysis.

In this study, the expression levels of *CHS*, *F3H* and *DFR* genes related to flavonoid biosynthesis were determined using RT-PCR analyses. *CHS* gene encodes a key enzyme in the flavonoid biosynthesis pathway, in plants, catalyzing the first committed step by condensing one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA to produce naringenin chalcone. This reaction initiates the production of flavonoids, a diverse group of secondary metabolites critical for plant defense and survival (Dao et al., 2011). The *F3H* gene encodes the enzyme flavanone 3-hydroxylase, which catalyzes the hydroxylation of flavanones at the 3-position to form dihydroflavonols. This reaction is a key step in the flavonoid biosynthetic pathway. By converting flavanones into dihydroflavonols, *F3H* helps set the stage to produce various flavonoid compounds (Pelletier & Shirley, 1996). The *DFR* gene encodes the enzyme dihydroflavonol 4-reductase. This enzyme converts dihydroflavonols to flavan-3,4-diols. It plays a critical role in one step of flavonoid biosynthesis, specifically converting dihydroflavonols such as dihydrokaempferol and dihydromyricetin to flavan-3,4-diol species (Shirley et al., 1992) (**Fig. 3**).

Specific primers for genes involved in the catechin biosynthesis pathway, *CHS*, *F3H* and *DFR* (**Tab.1**) were used in combination with a reference gene to amplify cDNA samples. The amplification was performed using Sso Advanced Universal SYBR Green Super Mix (Bio-Rad, USA) on the CFX Connect Real-Time Detection System (Bio-Rad, USA), and gene expression levels were determined. Each reaction consisted of 10 µL, containing 0.5 µl of each primer and 100 ng of target cDNA. The RT-PCR conditions were as follows: initial denaturation at 95 °C for 30 seconds, followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 55-58 °C for 10 seconds, and the final step was set as the melting curve. The experiments were conducted in 96-well transparent plates, with the *Gentiana actin* gene used as the reference gene. RNA was isolated in three biological replicates for each treatment, and each sample was subjected to duplicate RT-PCR reactions.

The expression levels of each gene obtained from the RT-PCR results were analyzed using the 2^{-ΔΔC_t} comparative CT method (Livak & Schmittge, 2001) in the CFX Manager™ program (Bio-Rad, USA). Changes in gene expression levels were normalized relative to the expression level of the reference gene and calculated as fold changes compared to the control.

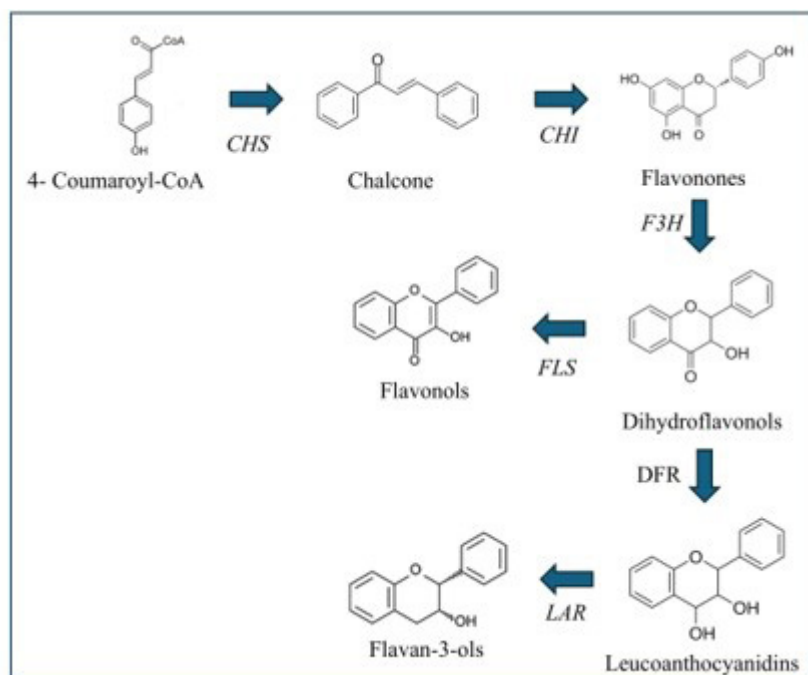


Fig. 3. Position of *CHS*, *F3H* and *DFR* genes in the flavonoid biosynthesis pathway (Zhu et al. 2015)

Statistical analyses

Statistical analyses were performed using JMP Pro version 17 (SAS Institute Inc., Cary, NC, USA). Differences between the applications were determined using the Least Significant Difference (LSD) test at $p \leq 0.05$.

Results

Following MJ treatments at different concentrations, the expression levels of *CHS*, *F3H* and *DFR* genes involved in the biosynthesis of phenolic compounds were determined by RT-PCR in the roots of *Gentiana lutea* and *G. boissieri* harvested on days 2, 4, 6, 8, and 10. The activities of the genes were found to vary significantly depending on MJ concentrations and harvest times ($p \leq 0.05$).

In *G. lutea*, *CHS* gene expression showed a marked increase in roots treated with 0.5 mM MJ for 4 and 10 days, whereas no significant differences were observed among other concentrations or harvest times (Fig. 4). *F3H* activity showed a

notable increase, especially in roots harvested on day 2 compared to the control (Fig. 5). In these roots, the highest gene activity was observed at 0.1 mM MJ with a 32.89-fold increase, while increased concentrations led to decreased gene expression. Compared to the control no notable differences were observed during later harvest times. The highest *DFR* gene expression was recorded in roots treated with 0.1 mM MJ and harvested on day 8. With this application, *DFR* gene activity increased 25.46-fold compared to the control (Fig. 6).

In *G. boissieri*, *CHS* activity was quite low during the early harvest periods including days 2 and 4. As the harvest period progressed, *CHS* gene activity increased depending

on MJ concentrations (Fig. 7). The greatest *CHS* activity was detected in roots exposed to 0.5 mM MJ for 8 days and in roots subjected to 1.0 mM MJ for 10 days, with the next highest activity seen in roots treated with 0.1 mM MJ and collected on day 6. *F3H* gene activity peaked in roots exposed to 1.0 mM MJ for 10 days and in those treated with 0.5 mM MJ for 4 days (Fig. 8). For *DFR*, the highest gene expression with a 17.11-fold increase was observed with 1 mM MJ on day 8, while the other treatment groups did not show any significant difference (Fig. 9).

These findings indicated that the expression levels of genes within the phenolic compound biosynthesis pathway varied considerably based on the MJ concentrations, harvest times, *Gentiana* species, and the genes.

Discussion

Gentiana species, in addition to iridoids, secoiridoids, and xanthonenes, also contain significant amounts of phenolic compounds (Mustafa et al., 2015; Aras Aşçı

Table 1. Sequences of primers of genes

Genes	Forward Primer	Reverse Primer
<i>CHS</i>	5'-AAAAACTGCGGTCTACGAGGC -3'	5'-ATCTTCCTTCTGCTTCTGGGT -3'
<i>DFR</i>	5'-GGGAATGAAGCACACTATGGT -3'	5'-CTTGCTCCATCTCTTGCTGAA -3'
<i>F3H</i>	5'-TGGTGGAGAGAAGCCTAATGT -3'	5'-AACGACTGAACTAACGAAGCC -3'
<i>actin</i>	5'-TCCATAATGAAGTGTGATGT -3'	5'-GACCTGACTCGTCATACTC -3'

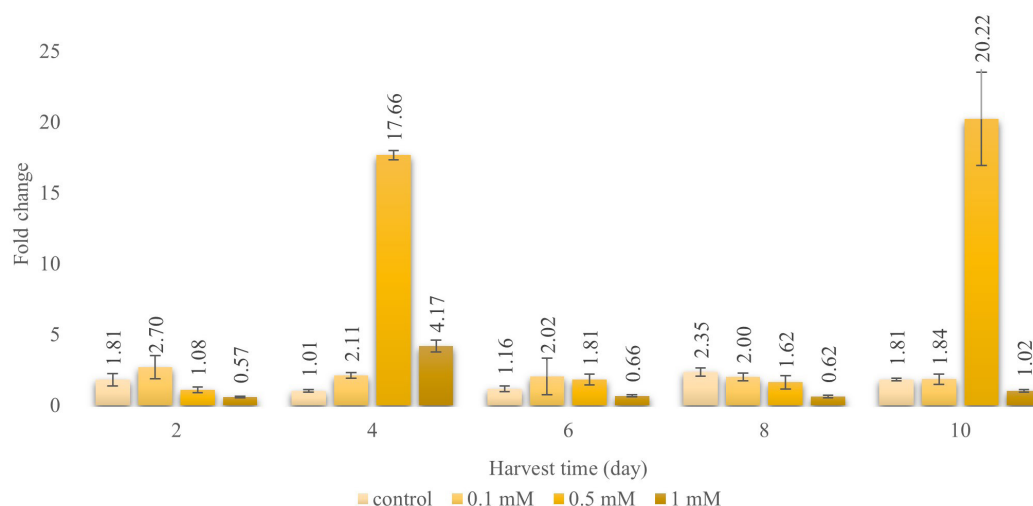


Fig 4. Time- and dose- dependent effects of MJ applications on the expression levels of CHS gene in *G. lutea* roots (LSD:3.24)

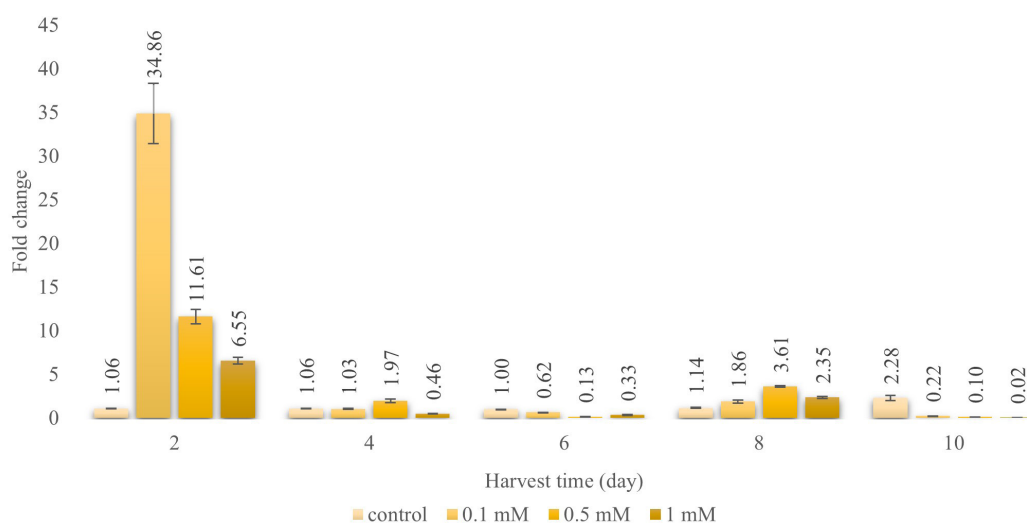


Fig 5. Time- and dose- dependent effects of MJ applications on the expression levels of *F3H* gene in *G. lutea* roots (LSD:2.69)

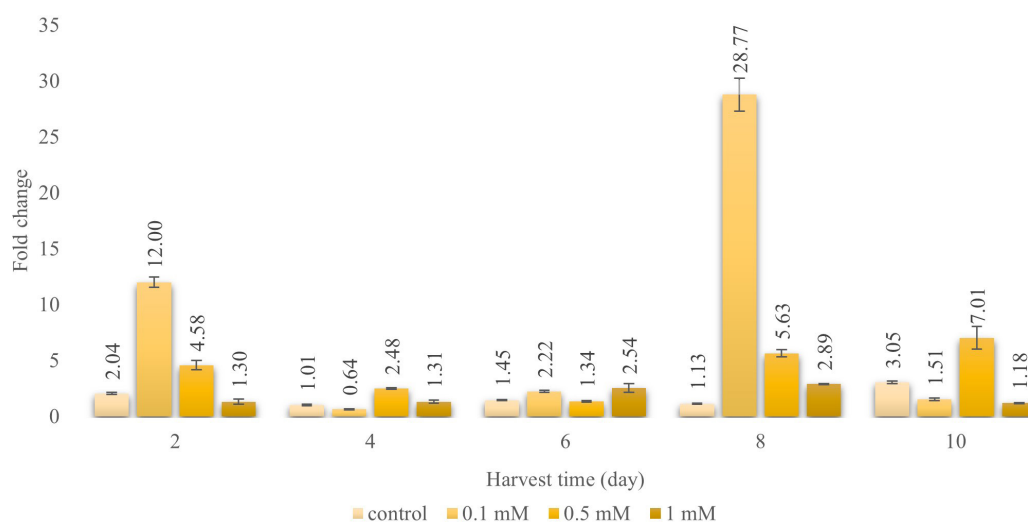


Fig 6. Time- and dose- dependent effects of MJ applications on the expression levels of *DFR* gene in *G. lutea* roots (LSD:1.98)

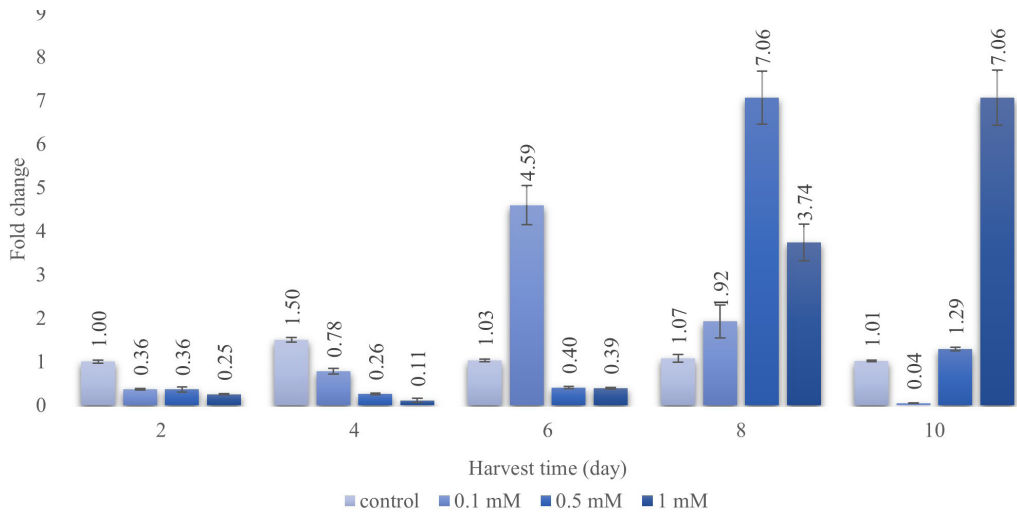


Fig 7. Time- and dose- dependent effects of MJ applications on the expression levels of *CHS* gene in *G. boissieri* roots (LSD:0.89)

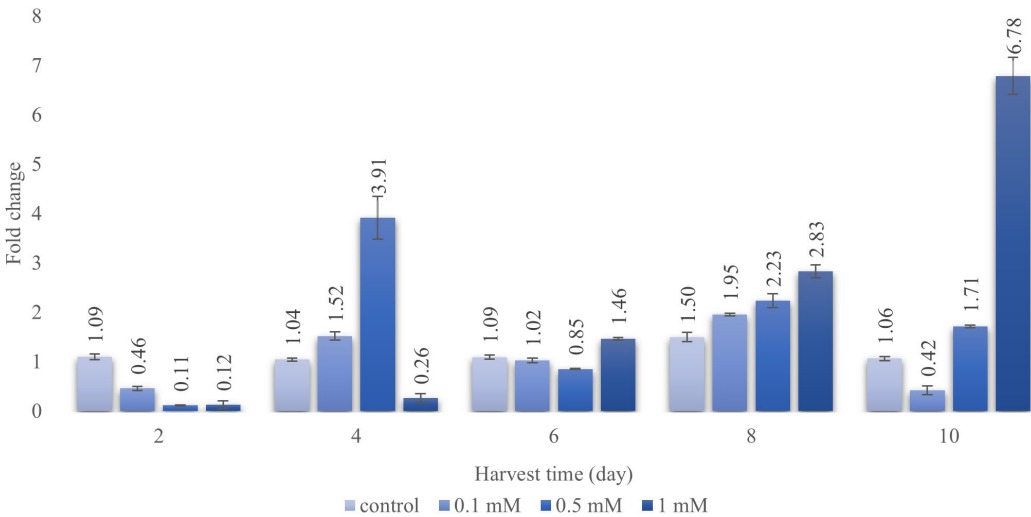


Fig 8. Time- and dose- dependent effects of MJ applications on the expression levels of *F3H* gene in *G. boissieri* roots (LSD:1.56)

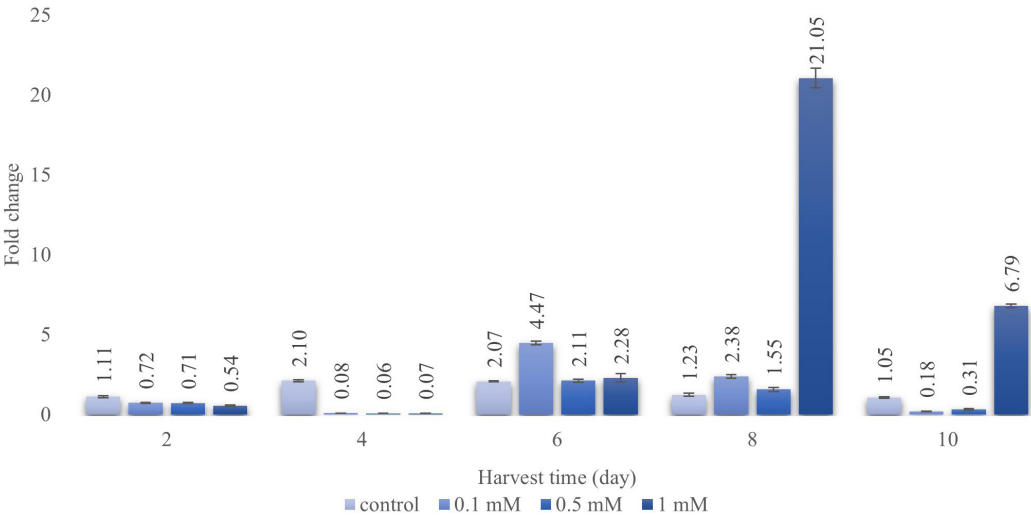


Fig 9. Time- and dose- dependent effects of MJ applications on the expression levels of *DFR* gene in *G. boissieri* roots (LSD:2.56)

et al., 2022). MJ, a lipid-derived phytohormone, serves as a critical signalling molecule regulating various physiological processes, including growth modulation, stress responses, and secondary metabolite biosynthesis (Wasternack & Hause, 2013). When applied at optimal concentrations and durations, acting as a signaling molecule in plant cells, MJ markedly boosted the accumulation of phenolic compounds (Gumerova et al., 2015; Rawat et al., 2020; Demirci, 2022). Khan et al. (2017) informed that MJ promoted phenolic compound biosynthesis by activating transcription factors that upregulate the expression of associated genes. This study investigated the effects of MJ treatments on the expression of *CHS*, *F3H* and *DFR* genes, which are critical for flavonoid biosynthesis, in root cultures of *G. lutea* and *G. boissieri*. RT-PCR analyses revealed that the expression levels of these genes exhibited significant variation depending on MJ concentrations, treatment durations, and the *Gentiana* species. Similarly, it was determined that the activities of *CHS*, *F3H* and *DFR* genes in Duke and Blueberry blueberry genotypes treated with 0.1 mM MJ for 3, 6, 9, and 24 hours significantly varied depending on the treatment duration and the cultivars (Cocetta et al., 2015).

MJ applications have been shown in various studies to significantly affect the expression of genes associated with phenolic compound synthesis in plants. For example, Jeong et al. (2018) reported that treatment with 100 µM MJ in soybean suspension cell cultures led to a dramatic upregulation of *CHS6* and *CHS7* genes, involved in isoflavonoid biosynthesis, by 790.02- and 50.60-fold, respectively. Similarly, Yang et al. (2024) found that MJ treatment in *Epimedium sagittatum* enhanced the expression of *CHS2*, *CHI2*, and *F3H* genes by 5.18-, 2.17-, and 2.80-fold, respectively, while downregulating the *CHS3* gene. The authors suggested that the upregulation of these genes in the flavonoid biosynthetic pathway contributed to increased accumulation of key flavonoids such as icariin, epimedin A, epimedin B, epimedin C, and icariside II. In another study, overexpression of the *CHS* gene in *Glycyrrhiza uralensis* hairy roots led to an increase in flavonoid content (Yin et al., 2019). Rubio-Rodríguez et al. (2021) observed that MJ application to *Castilleja tenuiflora* upregulated genes involved in metabolite biosynthesis, including *PAL1*, *CHS1*, *DXS1*, and *G10H*, with a stronger induction effect on phenolic compounds than on iridoids. Likewise, MJ treatments in *Salvia miltiorrhiza* hairy roots (Hao et al., 2015) and in *Prunella vulgaris* (Tang et al., 2022) were shown to significantly enhance the expression of genes critical to phenolic biosynthesis. Additionally, MJ application in *Mentha*

spicata hairy roots increased the expression of *PAL*, *C4H*, *HPPR*, and *4CL* genes, key players in phenolic acid biosynthesis, by 4.04-, 3.62-, 1.75-, and 1.45-fold, respectively, while suppressing *TAT* gene expression (Yousefian et al., 2020). Collectively, these findings suggest that MJ generally promotes the upregulation of genes involved in the biosynthesis of phenolic compounds in plants, although the expression levels of specific genes may vary depending on the plant species and experimental conditions.

Conclusion

This study demonstrates that MJ serves as a highly effective elicitor, markedly inducing the expression of pivotal genes associated with phenolic biosynthesis, notably *CHS*, *F3H* and *DFR*. The concentration- and time-dependent patterns of gene expression observed in *Gentiana lutea* and *G. boissieri* illustrate the intricate regulatory network linking MJ signalling to the phenylpropanoid pathway. Notably, MJ's elicitation effect is not constant but varies substantially based on factors such as concentration, exposure duration, plant species, and target gene specificity. These findings not only highlight MJ's significant potential for metabolic engineering but also underscore the necessity of meticulously optimizing experimental parameters to maximize its efficacy. Overall, this research highlights MJ as a valuable tool for increasing phenolic compound yields for agricultural and pharmaceutical use, underscoring the necessity of detailed, species-specific investigations to fully realize its biotechnological potential.

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