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Metabolic, Biochemical, Morpho-physiological and Antioxidant Responses of *Chrysanthemum indicum* L. Callus to Methyl Jasmonate Elicitation under *In Vitro* Conditions

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ABSTRACT

Chrysanthemum indicum L., a medicinally important member of the Asteraceae family, is valued for both ornamental and therapeutic uses. Methyl jasmonate (MeJA), a key signaling molecule, helps regulate its growth and defense responses. This study sought to refine callus induction protocols and assess how different MeJA concentrations (0, 50, 100, and 150 µM) influence various metabolic, biochemical, morpho-physiological and antioxidant traits in callus cultures of *Chrysanthemum indicum* L. Following the germination of *Chrysanthemum indicum* seeds, various explants were cultured on MS medium under different hormonal regimes and light conditions to optimize callus induction. The most effective response (96.66% callus formation) occurred with leaf explants under a 16 h light / 8 h dark photoperiod, using a combination of 2 mg L^{-1} BAP and 2 mg L^{-1} 2,4-D. This condition also minimized initiation time and significantly boosted biomass. The fresh biomass of callus treated with 100 µM MeJA (2.72 g) was 1.05 g higher than the control (1.67 g), and enhanced callus volume by approximately 2-fold compared to the control. At this concentration, chlorophyll a and b reached their highest levels, accompanied by elevated production of carotenoids, phenolics, flavonoids, and enhanced antioxidant activity. At 150 µM MeJA, the maximum carotenoid (3.30 mg g^{-1} FW), phenolic (68.42 mg g^{-1} FW), and flavonoid (46.19 mg g^{-1} FW) levels were observed, along with a 33.79% rise in antioxidant potential relative to untreated controls. Additionally, MeJA treatment led to reduced malondialdehyde (MDA) levels, suggesting lower lipid peroxidation, as well as elevated proline and anthocyanin contents, indicative of improved oxidative stress tolerance. In summary, MeJA application, particularly at 100 and 150 µM, favorably influenced callus growth and enhanced secondary metabolite biosynthesis. These outcomes highlight MeJA's utility as an effective elicitor to improve the *in vitro* production of medicinal metabolites.

Keywords: Anthocyanin, Flavonoid, Phenol, Plant tissue culture, Secondary metabolites

INTRODUCTION

Chrysanthemum (Asteraceae) is a major ornamental genus native to East Asia and northeastern Europe, with China as the primary center of diversity, comprising about 40 species, hybrids, and many cultivated varieties [1]. The name derives from the Greek krus (gold) and anthemon (flower), meaning “golden flower.” It is the second most commercially important floricultural crop after roses, mainly used as a cut flower, with billions sold worldwide each year. Its wide variation in color and floral forms arises from pigments such as anthocyanins, carotenoids, and chlorophylls [2]. In addition to ornamental value, *Chrysanthemum* has long been used in traditional medicine for treating ailments such as bruises, rhinitis, malaria, and fevers, and for its anti-inflammatory, antipyretic, and antihypertensive effects [3,4]. Bioactive compounds, including flavonoids, alkaloids, terpenoids, and tannins, contribute to antioxidant, anticancer, cardiovascular protective, and other therapeutic properties [5]. Given the increasing global demand for natural therapeutics, efforts in cultivation and breeding have focused on enhancing secondary metabolite production in this plant [6].

Medicinal plants are rich in bioactive compounds categorized as primary and secondary metabolites. While primary metabolites are vital for growth and metabolism, secondary metabolites function mainly in plant–environment interactions and defense [1]. They are synthesized through phenylpropanoid, alkaloid, and isoprenoid pathways, forming groups such as phenolics, alkaloids, and terpenes. Beyond ecological roles, many secondary metabolites serve as therapeutic agents, with several developed into pharmaceuticals [2]. According to the WHO, about 80% of the world’s population depends on herbal medicines for primary healthcare [3].

Plant tissue culture is a key biotechnological approach widely applied for secondary metabolite production under controlled in vitro conditions [1]. To enhance metabolite biosynthesis, various elicitors have been tested, among which methyl jasmonate (MeJA) is highly effective [2]. Acting as a signaling molecule, MeJA stimulates pathways of pharmacologically important compounds (flavonoids, terpenoids, alkaloids) by regulating biosynthetic genes and enzymes, thus improving biomass and metabolite yields [3]. Positive effects of MeJA have been documented in several species, including increased callus biomass in *Rhododendron* [4], enhanced callus volume in *Salvia tebesana* [5], modulation of chlorophyll and phenolics in *Solanum lycopersicum* [6-8], elevated flavonoid and phenolic contents in *Allium jesdianum* [9], *Teucrium polium* and *Dracocephalum polychaetum* [10, 11], upregulation of anthocyanins in *Vitis vinifera* [12], higher proline in *Salvia officinalis* [12], and increased antioxidant enzyme activities in tomato, *Portulaca oleracea* and *Nigella sativa* [13-16]. Enhanced antioxidant activity has also been reported in *Allium jesdianum* [18]. Given this background, the objective of the present study was to optimize the conditions for callus induction and to evaluate the influence of varying concentrations of MeJA on the metabolic, biochemical, antioxidant, and morpho-physiological characteristics of *Chrysanthemum indicum* L. callus grown under *in vitro* conditions.

MATERIALS AND METHODS

Plant Material Collection and Surface Sterilization

Seeds of *Chrysanthemum indicum* L. were collected from Pakan Bazr Company, located in Isfahan, Iran. For surface sterilization, seeds were initially soaked in a 10% benomyl fungicide solution for five minutes. This was followed by a brief exposure to 70% ethanol for one minute and then immersion in 1% sodium hypochlorite for fifteen minutes [17]. Post-treatment, seeds were washed thoroughly three times with sterile distilled water to eliminate residual disinfectants. These disinfected seeds were transferred to Murashige and Skoog (MS) medium [18] under aseptic conditions. After a month, stem and leaf segments were excised from the regenerated seedlings to serve as explants for subsequent experiments.

MS Medium Preparation and Experimental Setup for Callus Induction

The MS basal medium was formulated based on standard procedures, incorporating plant growth regulators (PGRs) to enhance callus formation. For hormonal treatments, stock solutions of 2,4-D and NAA (Sigma-Aldrich, USA) were prepared by dissolving 10 mg of each in several drops of 96% ethanol, and the final volume was adjusted to 10 mL using double-distilled water. Likewise, 10 mg of cytokinin (BAP) was dissolved in 500 µL of 1N NaOH and diluted to 10 mL with distilled water, then stored at 4°C until use.

A factorial experiment was arranged in a completely randomized design (CRD) with three replicates, each comprising two micro-samples. The variables under investigation included five hormonal treatments (H1–H5), two explant sources (E1 for leaf, E2 for stem), and two light regimes (L = light; 16 h light / 8 h dark, D = dark; continuous darkness). The hormonal compositions were as follows: H1: control (no PGRs); H2: 1 mg L^{-1} NAA + 2 mg L^{-1} BAP; H3: 2 mg L^{-1} 2,4-D; H4: 0.2 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D; and H5: 2 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D. This setup aimed to identify the most favorable combination of explant, hormone, and light for optimal callus induction in *C. indicum* under *in vitro* conditions.

Formulation of Methyl Jasmonate (MeJA) Elicitor Solution

The MeJA elicitor was prepared by dissolving 4.36 mL of MeJA (Sigma-Aldrich) in 50 mL of 99.9% ethanol in a 100 mL glass container. The solution was thoroughly mixed until it became clear. To this, sterile distilled water was added to make the final volume 100 mL, ensuring uniformity by further mixing. Filtering was performed using a 0.22 µm syringe-driven filter (Millipore, Sigma-Aldrich) under sterile conditions. The filtered elicitor was stored and later incorporated into the medium according to as the protocol [19].

Callus Development Parameters

Various parameters were assessed to monitor callus formation and growth. Callus induction rate was determined by calculating the percentage of explants that generated callus. The time taken for visible callus emergence was recorded in days post-inoculation. Four weeks later, fresh weight was measured using an analytical balance, and dry weight was determined by oven-drying at 60°C for 48 hours, ensuring a consistent mass was obtained. Callus volume was determined via the Archimedes method, involving immersion in liquid and recording the displaced volume [20].

Chlorophyll and Carotenoid Content Estimation

For pigment analysis, 200 mg of callus material was homogenized with 10 mL of 80% acetone and placed in a Falcon tube. The samples were kept on ice and stirred gently for 30–60 minutes. The extract was then filtered to remove particulates. Absorbance readings were taken using a spectrophotometer after calibration with methanol. Readings for chlorophyll a, chlorophyll b, and carotenoids were noted at 663, 645, and 480 nm, respectively, following the method of Hemphill and Venketeswaran (1978) [21].

Quantitative Analysis of Phenolics, Flavonoids and Antioxidant Activity

The total phenolic content was analyzed using the Folin–Ciocalteu method according to McDonald *et al*., (2001) [22]. An ethanolic extract (0.32 mg mL^{-1}) was mixed with distilled water and Folin–Ciocalteu reagent. After five minutes, 7% Na_2CO_3 was added and the mixture was incubated at room temperature for 60–90 minutes. Absorbance was measured at 765 nm, and results were expressed in mg GAE g^{-1} FW.

Flavonoid quantification was done using the method described by Chang (2002) [23], with minor adjustments. The assay was prepared by mixing the extract with potassium acetate, aluminum chloride, and distilled water, followed by 45 minutes of incubation in the dark. Absorbance was taken at 510 nm. Antioxidant capacity was determined via DPPH radical scavenging assay using the protocol by Materska and Perucka [24], with slight modifications. A solution containing 3.9 mL of DPPH and 0.1 mL of extract was incubated in darkness for 30 minutes. The absorbance was read at 517 nm, and antioxidant activity was reported as a percentage.

MDA and Anthocyanin Assessment

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content following Heath and Packer's method [25]. Fresh tissue was ground in 50 mM phosphate buffer (pH 7.8) and centrifuged. The supernatant was combined with 20% TCA containing 0.5% TBA and heated at 95°C for 10 minutes. After cooling, absorbance at 532 nm was recorded. For anthocyanin estimation, the method of Bürkle *et al*., [26] was used. Finely chopped callus was extracted with 80% methanol, refrigerated for 24 hours with occasional shaking, then filtered. One milliliter of 1 M HCl was added to stabilize pigments, and absorbance at 530 nm was recorded against a methanol blank.

Proline and Antioxidant Enzyme Activity Measurement

Proline content was assessed using the Bates method [27]. 0.04 g of callus was homogenized in 3% sulfosalicylic acid, filtered, and reacted with ninhydrin reagent (prepared with glacial acetic acid, phosphoric acid, and ninhydrin). After incubation, the absorbance was measured at 520 nm. Catalase (CAT) activity was measured according to Aebi [28], using a mixture of phosphate buffer, H_2O_2, and enzyme extract. Peroxidase (POD) activity was determined based on guaiacol oxidation as described by Serrano-Martínez *et al*., (2008) [29]. The reaction mixture consisted of phosphate buffer, H_2O_2, guaiacol, and enzyme extract, and absorbance at 470 nm was monitored every 10 seconds over a 1-minute duration.

Statistical Analysis

The effects of explant type, growth regulator combinations, and light conditions were evaluated using a factorial arrangement based on a completely randomized design (CRD) with three replications per treatment. For the experiment on different MeJA concentrations, a CRD with three replications was applied. Data were analyzed using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA), and mean comparisons were performed with Duncan's multiple range test at a significance level of $p \leq 0.01$.

RESULTS AND DISCUSSION

Influence of Hormonal Combinations, Explant Type, and Illumination on Callus Characteristics

The analysis of variance (ANOVA) demonstrated that the interactive effect among hormonal treatments, type of explant, and lighting conditions was statistically significant at the 1% probability level. Therefore, a three-way interaction graph was utilized to illustrate the outcomes.

Percentage of Callus Formation

The maximum rate of callus induction (96.66%) occurred under the E1H5L condition, comprising leaf explants cultured with 2 mg L^{-1} BAP and 2 mg L^{-1} 2,4-D under illuminated conditions (Fig. 1 and 2a). The balanced ratio of cytokinin and auxin in this treatment likely triggered regulatory networks associated with undifferentiated cell proliferation, thereby enhancing callus formation efficiency [30]. The superior responsiveness of leaf explants may be attributed to their abundance of meristematic cells and heightened sensitivity to growth hormones. Additionally, exposure to light likely facilitated better photosynthetic activity and increased synthesis of secondary metabolites, which could have promoted callus growth [31]. In prior research, callus formation in *Coffea arabica* leaves was efficiently induced within three weeks using 2 mg L^{-1} 2,4-D combined with 1 mg L^{-1} BAP [32]. Likewise, in *Momordica cochinchinensis*, optimal callus development was noted in leaf-derived tissues treated with a mixture of BAP and 2,4-D [33].

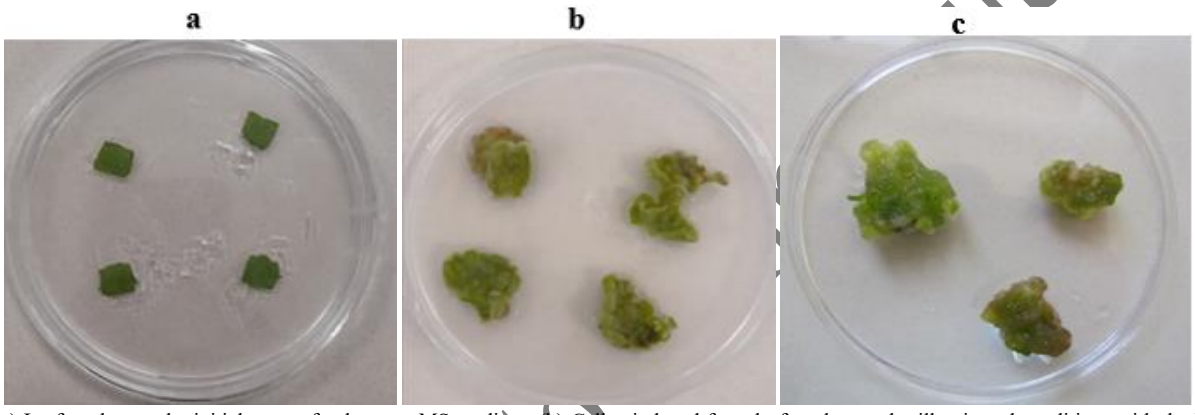
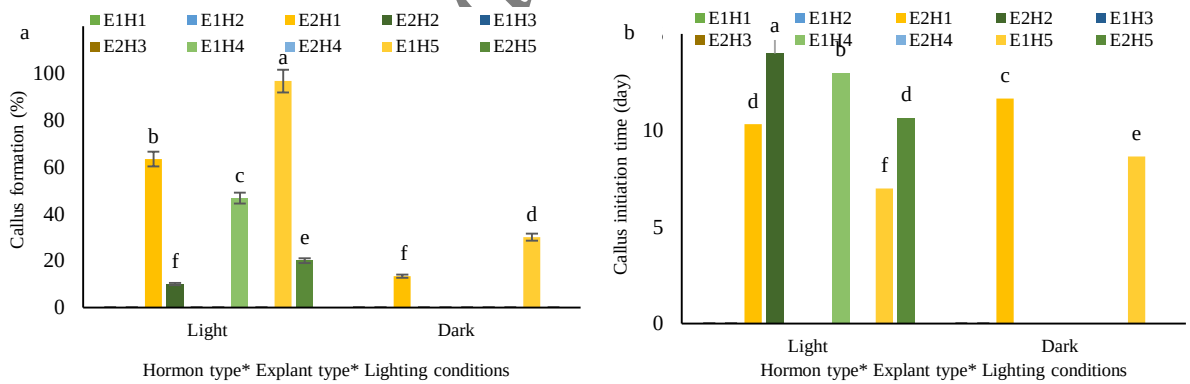


Fig. 1 a) Leaf explant at the initial stage of culture on MS medium; (b) Callus induced from leaf explant under illuminated conditions with the E1H5L hormone treatment (2 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D). c) Regenerated callus from the first round of culture.



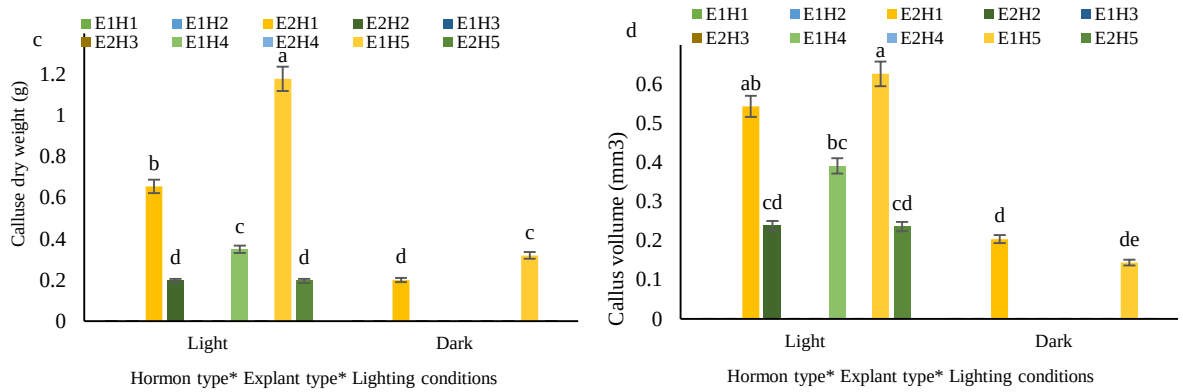


Fig. 2 Comparison of the mean interaction effects of different hormone combinations, explant types, and lighting conditions on callus formation percentage (a), callus initiation time (b), callus fresh weight (c) and volume callus (d) in *Chrysanthemum indicum* L. E1H1= leaf, control (no PGRs), E1H2; leaf, 1 mg L^{-1} NAA + 2 mg L^{-1} BAP, E2H1; stem, control (no PGRs), E2H2; stem, 1 mg L^{-1} NAA + 2 mg L^{-1} BAP, E1H3; leaf, 2 mg L^{-1} 2,4-D, E2H3; stem, 2 mg L^{-1} 2,4-D, E1H4; leaf, 0.2 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D, E2H4; stem, 0.2 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D, E1H5; leaf, 2 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D, E2H5; stem, 2 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D. Means sharing identical letters do not differ significantly at the 1% significance level ($p \leq 0.01$). Error bars indicate standard errors.

Time Required for Callus Initiation

The most rapid initiation of callus was observed in the E1H5L treatment. (Fig. 2b). Conversely, delayed callus emergence under the E2H2L (stem, 1 mg L^{-1} NAA + 2 mg L^{-1} BAP, light) condition can likely be attributed to the complex interplay between stem-derived explants, suboptimal hormone ratios, and light influence [34]. This suggests that the auxin-to-cytokinin balance in the latter treatment may have suppressed early cell division signals [35]. Similar findings were reported by Farhadi *et al.,* [36], who observed that in *Allium hirtifolium*, a combination of 1.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP led to the shortest initiation period for callus induction.

Callus Fresh Weight

Callus grown under the E1H5L treatment showed a notable increase in fresh biomass (Fig. 2c). The hormone combination likely promoted efficient cellular dedifferentiation and proliferation, contributing to greater fresh weight [37]. Light conditions may have further promoted cellular activity through hormonal regulation and enhancement of photosynthetic efficiency [38]. Light also fosters cell proliferation by energizing metabolic processes and modulating signaling pathways governed by auxin and cytokinin. The enhancement in energy availability and redox regulation under light exposure can explain the observed biomass increase. Additionally, leaf tissues inherently possess a greater density of active cell division zones than stems, supporting higher mass accumulation [39]. For instance, in *Justicia gendarussa*, treatment with 2 mg L^{-1} BAP and 1.5 mg L^{-1} 2,4-D increased fresh weight to 2.247 g [40].

Callus Volume

Significant augmentation in callus volume was evident in the E1H5L treatment (Fig. 2d). This result is likely a consequence of synergistic interactions between hormonal supplementation and environmental light conditions. The specific auxin and cytokinin concentrations presumably triggered enhanced cellular division and expansion, contributing to callus enlargement [41]. Moreover, light served as an important modulator of biochemical activities, improving metabolic efficiency and thereby enlarging tissue volume [42].

Effects of Methyl Jasmonate Elicitation on Callus Parameters

According to ANOVA, MeJA application exhibited a statistically significant effect ($p \leq 0.01$) on callus fresh weight, dry weight, and volume.

Callus Fresh Weight

Fresh biomass was significantly elevated with increasing MeJA concentration, peaking at 2.72 g under the 100 µM treatment, compared to 1.67 g in the control (Fig. 3a). Interestingly, no significant variation was noted between the 50 µM and 150 µM MeJA treatments. The improved growth at 100 µM may be due to MeJA's capacity to activate biosynthetic and defense-related pathways, thereby supporting enhanced metabolic function and tissue accumulation [43]. This concentration may have established an optimal balance between stress signaling and growth promotion, enabling greater metabolite production and callus expansion. Conversely, higher MeJA levels (150 µM) may have led to inhibitory feedback or excessive ethylene synthesis, negatively affecting biomass. A similar pattern was observed in *Rhododendron* species, where elevated MeJA levels positively correlated with callus biomass accumulation [4].

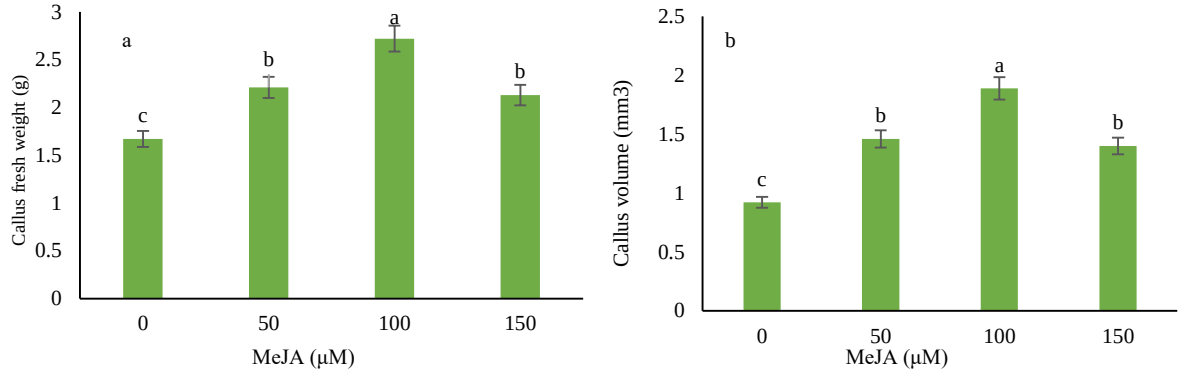


Fig. 3 Comparison of the mean effects of varying concentrations of the elicitor MeJA on callus fresh weight (a) and volume (b) in the *Chrysanthemum indicum* L. Means denoted by the same letters are not significantly different at the 5% significance level. Error bars indicate standard errors.

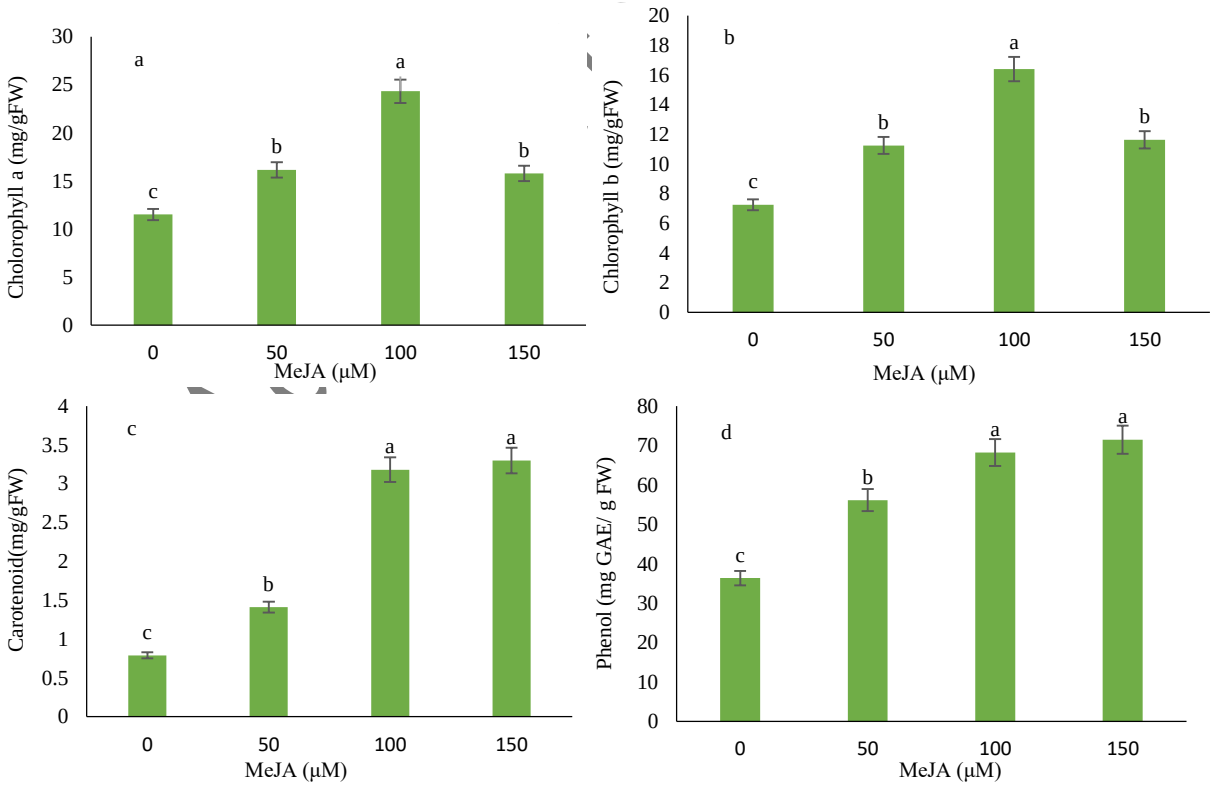
Callus Volume

A significant increase in callus volume was also evident under MeJA treatments, particularly at 100 µM, which resulted in a volume of 1.89 mm³, approximately double that of the control (0.923 mm³) (Fig. 3b). While the 150 µM concentration yielded 1.40 mm³, no statistical difference was found between this treatment and the 50 µM one. These results suggest that 100 µM MeJA optimally stimulated both cell expansion and division. The elicitor likely activated anabolic pathways that facilitated the accumulation of structural and storage compounds, thereby contributing to volumetric growth. However, exceeding this concentration may have triggered inhibitory stress responses [44]. Consistently, *Salvia tebesana* cultures displayed maximal callus volume at 100 µM MeJA, followed by a decline at 150 µM due to possible negative feedback effects [5].

Furthermore, statistical analyses indicated that MeJA significantly influenced physiological responses, metabolite profiles, and antioxidant traits at the 1% significance level ($p \leq 0.01$).

Chlorophyll a

The analysis revealed a significant effect of MeJA treatments on chlorophyll a concentration. The application of 100 µM MeJA resulted in the maximum increase, raising the chlorophyll a content to 16.39 mg g^{-1} fresh weight compared to the control group (Fig. 4a). There were no statistically significant differences between the 50 µM and 150 µM treatments (Fig. 4a). This enhancement at 100 µM might be attributed to MeJA's ability to promote photosynthetic processes and reinforce antioxidant defense [45]. Functioning as a regulatory molecule, MeJA upregulates genes responsible for chlorophyll biosynthesis and inhibits chlorophyll degradation under oxidative stress conditions [46]. In another investigation, *Solanum lycopersicum* treated with 50 µM MeJA exhibited a 27% rise in chlorophyll a, while a 200 µM concentration reduced it by 8%, indicating a dose-dependent effect [6].



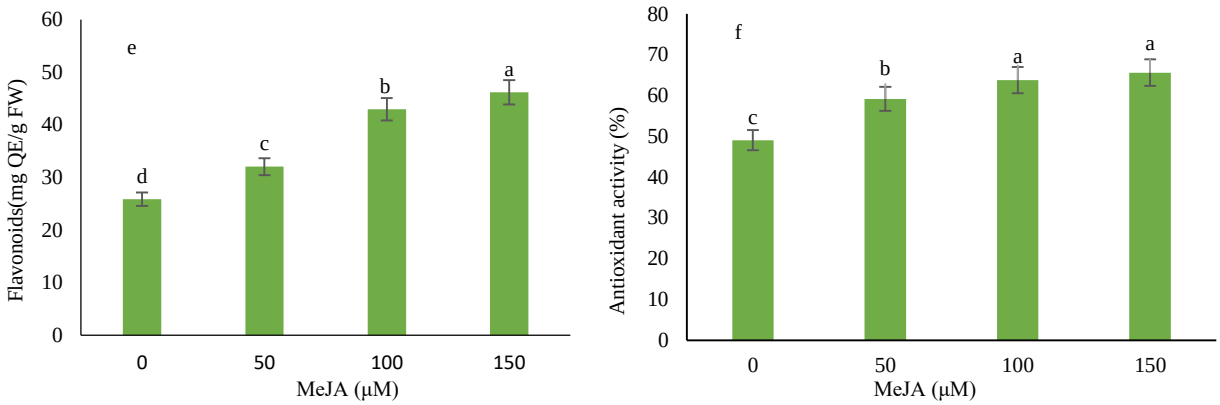


Fig. 4 Comparison of the mean effects of different MeJA elicitor concentrations on chlorophyll a (a), chlorophyll b (b), carotenoids (c), total phenolic content (d), flavonoids (e), and antioxidant activity (f) in the *Chrysanthemum indicum* L. Means sharing identical letters are not significantly different at the 1% probability level ($p \leq 0.01$). Error bars represent the standard errors of the means.

Chlorophyll b

The analysis revealed that MeJA treatments significantly influenced chlorophyll b level. Notably, the 100 µM concentration significantly elevated chlorophyll b levels to 16.39 mg g^{-1} fresh weight, surpassing other treatment groups (Fig. 4b). The observed rise is likely due to MeJA's influence on physiological regulation and improved photosynthetic efficiency [47]. This compound may stimulate the expression of chlorophyll-binding proteins that stabilize chlorophyll b and reduce oxidative degradation, thereby supporting pigment retention [47]. Similarly, previous findings showed that MeJA improved chlorophyll a, b, and carotenoid concentrations in tomato plants [7].

Carotenoids

Mean comparisons indicated that MeJA treatments significantly increased carotenoid levels. The 100 µM and 150 µM concentrations resulted in the highest carotenoid contents, reaching 3.18 and 3.30 mg g^{-1} fresh weight, respectively, with both values significantly exceeding the control. Even the 50 µM treatment boosted carotenoid levels to 1.41 mg g^{-1} fresh weight (Fig. 4c). This pattern suggests MeJA positively regulates carotenoid biosynthetic genes and suppresses carotenoid breakdown under stress. By enhancing stress resistance and metabolic activity, MeJA promotes carotenoid accumulation, particularly at elevated concentrations [8].

Total Phenolic Content

A substantial rise in phenolic content was observed with MeJA application. The highest levels, 68.21 and 68.42 mg g^{-1} fresh weight, were measured in the 100 µM and 150 µM treatments, respectively, with no significant difference between them. In contrast, the control recorded only 36.36 mg g^{-1} fresh weight. In comparison, the 50 µM treatment increased phenolic content to 50.92 mg g^{-1} fresh weight (Fig. 4d). This enhancement is due to MeJA's ability to activate the phenylpropanoid pathway, leading to phenolic compound biosynthesis. These secondary metabolites help neutralize reactive oxygen species (ROS), thereby protecting cellular structures. Moreover, MeJA modulates related enzyme activities and enhances the plant's overall defensive mechanisms [48, 49]. Research on *Allium jesdianum* demonstrated that all MeJA concentrations elevated phenolic levels compared to the untreated group [9], which aligns with studies on *Teucrium polium* callus cultures [11].

Flavonoids

Data showed that MeJA significantly enhanced flavonoid accumulation. The control yielded 25.87 mg g^{-1} fresh weight, whereas the MeJA treatments resulted in 32.06, 42.99, and 46.19 mg g^{-1} fresh weight, respectively. The flavonoid concentration increased progressively with rising MeJA concentrations (Fig. 4e). The highest increase (78.55%) was observed in the 150 µM treatment. MeJA acts as a signaling molecule that enhances the expression of flavonoid biosynthetic genes and associated regulatory pathways [50]. These results are supported by studies showing that MeJA elevated flavonoid levels in *Allium jesdianum* [9] and *Dracocephalum polychaetum* callus cultures [10].

Antioxidant Activity

A significant enhancement in antioxidant capacity was recorded under MeJA treatments. Compared to the control, the 50 µM, 100 µM, and 150 µM treatments increased antioxidant activity by 20.62%, 30.02%, and the highest amount, respectively (Fig. 4f). Although no significant differences were found among the three MeJA concentrations, all were statistically higher than the control. MeJA stimulates the biosynthesis of non-enzymatic antioxidants like flavonoids and phenolics, which neutralize ROS and safeguard cellular components such as membranes, proteins, and nucleic acids [27]. Additionally, this elicitor enhances the production of potent antioxidant compounds (e.g., chlorogenic acid, catechin, quercetin) via activation of the shikimate and phenylpropanoid pathways [51]. Similar results were reported by Yazdanian *et al*., [9], where *Allium jesdianum* callus cultures showed improved antioxidant performance upon MeJA treatment.

Malondialdehyde (MDA)

MDA content is commonly used to quantify lipid peroxidation and assess cellular oxidative damage [52]. In this study, control samples displayed an MDA level of 20.05 nmol g^{-1} fresh weight. The application of MeJA significantly reduced MDA levels, particularly at 100 µM, which brought the value down to 14.85 nmol g^{-1} fresh weight (Fig. 5a). No significant differences were noted between the 50 µM and 150 µM treatments. MeJA supports the antioxidant defense by promoting the expression of ROS-scavenging enzymes such as catalase, peroxidase, and ascorbate peroxidase. Additionally, it boosts the synthesis of phenolics and flavonoids, which act as ROS-neutralizing agents [53]. The marked reduction in MDA at the 100 µM level indicates that this concentration optimally protects cells against oxidative

stress. However, a slight increase in MDA at 150 µM might reflect overstimulation of stress responses or ethylene accumulation, resulting in mild oxidative effects. A similar trend was observed in other studies, where MDA content decreased with moderate MeJA levels but slightly rose at higher concentrations [13].

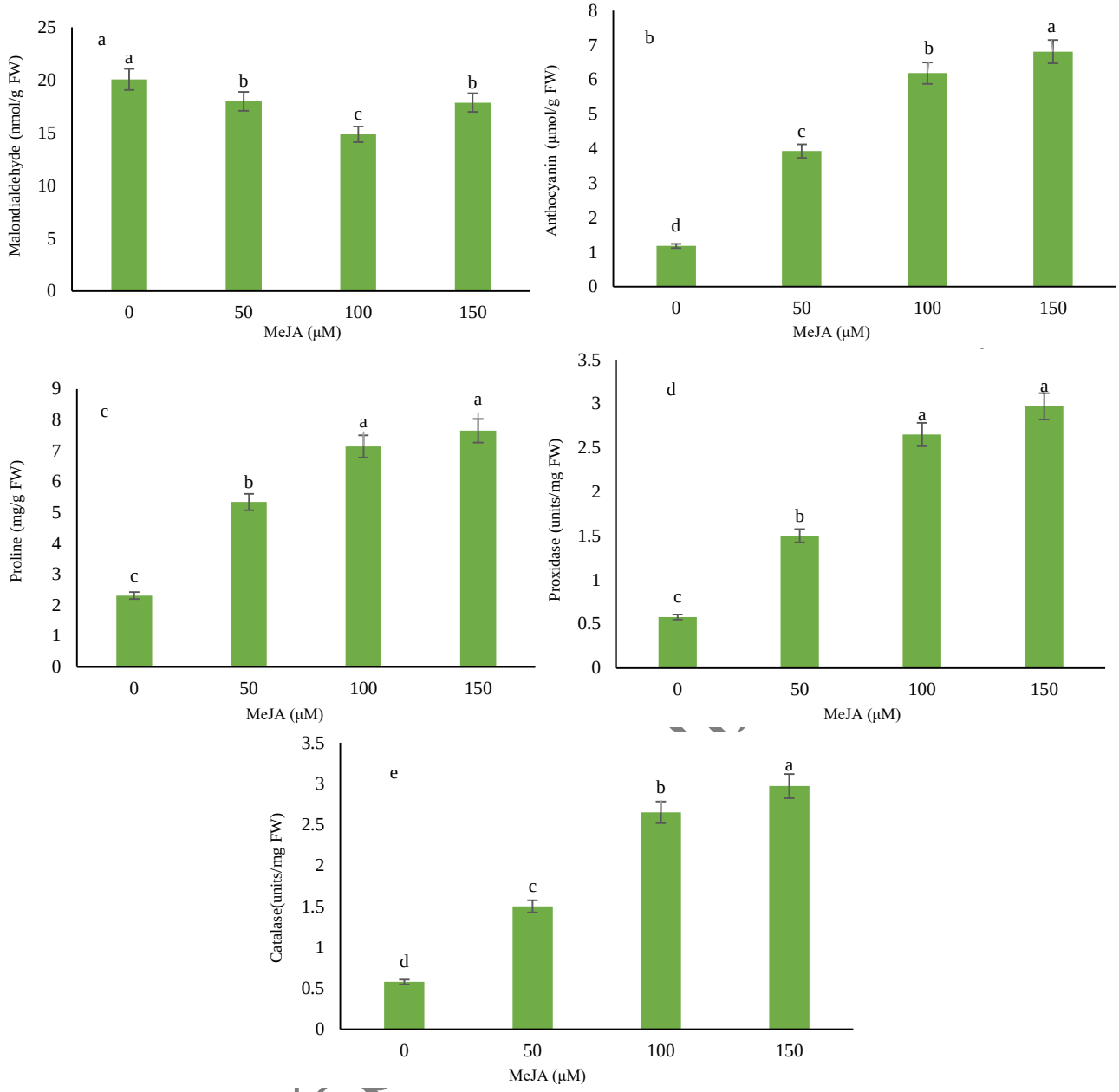


Fig. 5 Comparison of the mean effects of various MeJA concentrations on the contents of malondialdehyde (a), anthocyanin (b), proline (c), catalase (d), and peroxidase (e) in the *Chrysanthemum indicum* L. Means marked with the same letters are not significantly different at the 5% probability level based on the appropriate statistical test. Error bars indicate the standard errors of the mean.

Anthocyanin

The application of MeJA significantly influenced anthocyanin accumulation. In untreated control samples, the anthocyanin content was determined to be 1.18 µmol/g FW. However, all MeJA treatments led to considerable increases in this parameter. Specifically, 50 µM MeJA resulted in a rise to 3.93 µmol/g FW. The treatment with 100 µM MeJA led to a more pronounced increase, reaching 16.9 µmol/g FW. The highest value, 6.81 µmol/g FW, was recorded under the 150 µM MeJA treatment, which was 10% greater than that observed in 100 µM MeJA. Although 50 µM MeJA significantly elevated anthocyanin levels compared to the control, its effect was less pronounced than that observed with 100 µM and 150 µM MeJA (Fig. 5b). The rise in anthocyanin content following MeJA application is associated with its role in regulating secondary metabolic processes, particularly by promoting the expression of genes involved in anthocyanin biosynthesis [54]. Furthermore, MeJA aids in the buildup of these pigments by reducing oxidative stress and triggering plant defense systems, thereby improving resistance to environmental and biological stress factors [54]. Comparable results have been reported in *Vitis vinifera*, where MeJA application notably elevated anthocyanin levels due to upregulation of critical biosynthetic genes [55].

Proline

Data analysis revealed that control samples contained 31.2 mg/g FW of proline. A notable increase in proline content was evident under MeJA treatments. In the 50 µM MeJA treatment, the level rose to 34.5 mg/g FW. The application of 100 µM MeJA resulted in a proline concentration of 17.14 mg/g FW, reflecting an increase of 7.65 mg/g FW compared to the control (2.31 mg/g FW). Similarly, MeJA3 also

led to a proline content of 7.65 mg/g FW. These results suggest that elevated concentrations of MeJA induced more substantial proline accumulation, although the difference between 100 µM MeJA and 150 µM MeJA was minimal, only about 1.7% (Fig. 5c). The increase in proline levels following MeJA treatment can be linked to its function as an elicitor, which activates stress-related metabolic pathways. MeJA promotes proline biosynthesis through gene regulation and suppression of proline-catabolizing enzymes [56]. Peymaei *et al*., [12] similarly observed a marked increase in proline content in *Salvia officinalis* in response to MeJA, with higher MeJA levels eliciting greater accumulation.

Catalase

Experimental data revealed that MeJA significantly enhanced catalase activity compared to the control. The control group exhibited catalase activity of 0.578 units/mg FW. In the 50 µM MeJA treatment, catalase activity rose to 1.5 units/mg FW; for 100 µM MeJA, it increased to 2.65 units/mg FW; and in 150 µM MeJA, the activity reached 2.97 units/mg FW. A positive correlation was observed between MeJA concentration and enzyme activity, with the maximum value recorded under 150 µM MeJA (Fig. 5d). These observations support the role of MeJA as a potent elicitor that stimulates antioxidant enzyme activities and enhances defense responses. As an elicitor, MeJA induces catalase synthesis by triggering intracellular signaling pathways [14]. Catalase plays a pivotal role in neutralizing reactive oxygen species and minimizing oxidative stress. Therefore, increased catalase activity in MeJA-treated samples reflects an adaptive mechanism aimed at improving stress resilience [14]. Correspondingly, MeJA-induced elevation in catalase activity was also documented in tomato plants [15] and *Portulaca oleracea* [23].

Peroxidase

Results showed that MeJA treatment significantly increased peroxidase activity across all concentrations. In control plants, the peroxidase activity was 0.578 units/mg FW. Upon treatment with 50 µM MeJA, enzyme activity reached 1.5 units/mg FW. Application of 100 µM MeJA raised the activity to 2.65 units/mg FW, while 150 µM MeJA further increased it to 2.97 units/mg FW. This progressive rise in peroxidase activity with increasing MeJA concentrations reflects the compound's stimulating and eliciting properties (Fig. 5e). The observed upregulation in peroxidase activity suggests an enhanced antioxidant defense mechanism in response to MeJA. Peroxidase helps counter oxidative damage by detoxifying free radicals and reactive oxygen species [57]. Similar outcomes were reported in *Nigella sativa*, where MeJA exposure led to a gradual increase in peroxidase activity, thereby improving oxidative stress tolerance [16]. The results indicate that MeJA treatment simultaneously enhanced callus growth, secondary metabolite accumulation (phenolics, flavonoids, anthocyanins), and antioxidant defense (catalase and peroxidase activities), while reducing lipid peroxidation, demonstrating a coordinated interaction between metabolic, biochemical, and antioxidant responses in *Chrysanthemum indicum* callus.

CONCLUSION

This investigation established that the combination of 2 mg BAP and 2 mg 2,4-D under appropriate lighting conditions produced optimal results for callus induction, including the highest callus formation rate, the shortest initiation period, and the greatest callus fresh weight. Furthermore, the study demonstrated that MeJA functions effectively as an elicitor, enhancing the biosynthesis of secondary metabolites and the antioxidant system. Elevated levels of total phenolics, flavonoids, and anthocyanins, along with reduced lipid peroxidation, underscored MeJA's contribution to strengthening cellular defense. Additionally, increased proline content, serving as an osmo-protectant, suggests a role in augmenting stress resilience. Collectively, these findings highlight the utility of MeJA in improving the *in vitro* production of bioactive compounds. Future research should explore the molecular pathways underpinning these effects and investigate broader applications of MeJA in plant biotechnology.

Author Contributions

Younes Mahmoudi and Mojgan Soleimanizadeh conducted the experiments and collected the data. Younes Mahmoudi and Mojgan Soleimanizadeh also performed data analysis and manuscript preparation. Alireza Yavari supervised and guided the research process. All authors reviewed and approved the final manuscript.

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