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Original Research Paper

Influence of plant growth regulators and chitosan elicitation on callus induction and improvement of morphophysiological, metabolic and antioxidant traits in *Chrysanthemum indicum* L.

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ABSTRACT

Chitosan, as a natural elicitor, can enhance plant defense mechanisms and modulate physiological, biochemical, metabolic, and antioxidant pathways. Its application *in vitro* offers a promising strategy to improve the production of valuable secondary metabolites in *Chrysanthemum indicum* L. In this study, Murashige and Skoog (MS) medium was supplemented with various hormonal combinations *viz*., H1 (control), H2 (1 mgL^{-1} NAA + 2 mgL^{-1} BAP), H3 (0.2 mgL^{-1} 2,4-D), H4 (0.2 mgL^{-1} BAP + 0.5 mgL^{-1} 2,4-D), and H5 (2 mgL^{-1} BAP + 2 mgL^{-1} 2,4-D) and applied to different explants (leaf and stem) to optimize callus induction. Optimization was performed by evaluating callus induction percentage, initiation time, fresh weights, and callus volume. Following chitosan elicitation, several metabolic and biochemical traits were evaluated, including carotenoids, total phenolics, flavonoids, anthocyanins, proline content, antioxidant capacity, and the enzymatic activities of catalase and peroxidase. Among the treatments, the combination of 2 mg/L BAP + 2 mg/L 2,4-D applied to leaf explants under light conditions, produced the highest callus induction rate (96.66%), minimum days to response, and significantly increased fresh weights and callus volume. Chitosan treatments (25, 50, and 75 mgL^{-1}) were applied to calluses derived from the optimized treatment (2 mg/L BAP+2 mg/L 2,4-D applied to leaf explants). Among the tested concentrations, chitosan at 50 mgL^{-1} was the most effective, leading to the maximum increases in fresh weight (2.63 g), callus volume (1.81 mm^3), carotenoids (2.93 mg/g FW), total phenolics (67.40 mg/g FW), flavonoids (41.32 mg/g FW), and antioxidant activity (62.75%). Additionally, this treatment enhanced anthocyanins (5.19 mg/g FW), proline (6.14 mg/g FW), catalase (14.37 U/mg FW), and peroxidase (2.45 U/mg FW) activities. Overall, the combined use of plant growth regulators (BAP+2,4-D) and chitosan, particularly at 50 mgL^{-1}, presents an efficient approach to enhance callus development and improve physiological, metabolic, and antioxidant responses in *Chrysanthemum indicum* L. under *in vitro* conditions.

Keywords: Antioxidant activity, chitosan, *Chrysanthemum*, elicitor, *in vitro* culture

INTRODUCTION

The genus *Chrysanthemum*, family Asteraceae, is one of the oldest and most economically important ornamental and medicinal plant groups worldwide. It includes approximately 41 species, mainly distributed in East Asia and Northeastern Europe, with China recognized as the primary center of origin for most modern cultivars (Hadizadeh et al., 2022). Owing to their wide diversity in flower color and form, long postharvest longevity, and adaptability to diverse climatic conditions, *Chrysanthemum* species are extensively used in floriculture, landscaping, traditional medicine, and the pharmaceutical industry. In the global floriculture market, chrysanthemum ranks second after rose, accounting for nearly 9.77% of the total cut flower trade. Its cultivation area exceeds 4,600 ha, reflecting its increasing economic significance (Pan et al., 2025).

Among the species, *Chrysanthemum morifolium* and *Chrysanthemum indicum* L. are the most widely cultivated for ornamental and medicinal purposes. *C. indicum* L., a diploid species (2n = 18), has been used for centuries in traditional Chinese and Korean medicine due to its strong anti-inflammatory, detoxifying, and cardioprotective properties (Yan et al. 2019). More than 190 chemical constituents have been

identified in *C. indicum*, including flavonoids, phenolics, tannins, terpenoids, and volatile oils, which collectively exhibit antioxidant, antimicrobial, anticancer, and immunomodulatory activities (Hadizadeh et al. 2022). Beyond medicinal use, *C. indicum* is also utilized in cosmetics, nutraceuticals, and functional foods, further highlighting its pharmacological and commercial potential.

Major fungal, bacterial, viral, and insect pests significantly reduce productivity and ornamental value, while also suppressing metabolite accumulation (Chen et al., 2025). These challenges have encouraged the development of alternative approaches, particularly *in vitro* culture systems, which enable the production of disease-free, genetically uniform plant material under controlled conditions (Espinosa-Leal et al., 2018).

Elicitors play a critical role in stimulating the biosynthesis of defense-related secondary metabolites with high pharmacological value. Among them, chitosan-a biodegradable, biocompatible, and low-toxicity derivative of chitin-has received considerable attention for its ability to enhance plant growth, stress tolerance, and metabolite accumulation (Fenice & Gorrasi, 2021). Chitosan has been shown to increase the production of phenolics, flavonoids, and other bioactive compounds by activating defense-related signaling pathways, while also stimulating antioxidant enzymes such as catalase, peroxidase, polyphenol oxidase, and ascorbate peroxidase (Sree Rayanoothala et al., 2024). Additionally, chitosan promotes callus biomass accumulation and cellular proliferation under in vitro conditions (Elshahawy et al., 2022). Despite these promising effects, limited studies have simultaneously examined the influence of chitosan on callus growth, morphophysiological traits, antioxidant responses, and secondary metabolite biosynthesis in *C. indicum*. Therefore, the present study aims to develop an optimized *in vitro* protocol by integrating PGR composition, explant type, light conditions, and chitosan elicitation to enhance callus induction, growth, and metabolic performance. The role of elicitors such as chitosan in enhancing callus morphogenesis and metabolic performance has already been demonstrated in several medicinal and ornamental plants, indicating their strong potential to stimulate bioactive compound production under *in vitro* culture conditions (Ahmed et al., 2023).

MATERIALS AND METHODS

This study was carried out at the Plant Tissue Culture and Plant Physiology Laboratories, Faculty of Agriculture, University of Hormozgan, Bandar Abbas, Iran. The university is located in the southern coastal region of Iran (27°11′ N latitude and 56°16′ E longitude), characterized by a hot and humid coastal climate, with a mean annual temperature of approximately 27–30°C, an average annual rainfall of about 170 mm. All *in vitro* experiments were conducted under controlled laboratory conditions (25±2°C, 16/8 h light/dark photoperiod), independent of external weather conditions.

Preparation and sterilization of plant material

Seeds of *C. indicum* L. var. aromaticum obtained from Pakan Bazr Company, Isfahan, Iran. For surface sterilization, seeds were immersed in 10% benomyl for 5 minutes, 70% ethanol for 1 minute, and 1% sodium hypochlorite for 15 minutes, followed by three rinses with sterile distilled water. The seeds were cultured on MS medium, and after one month, leaf and stem explants were excised from young seedlings for further experiments.

Statistical analysis

To optimize callus induction in *C. indicum* L. var. *aromaticum*, a factorial experiment (5×2×2) was conducted using a completely randomized design with three replications. The experiment involved three factors: hormonal treatment (five levels), explant type (two levels: leaf and stem), and light condition (two levels: light and dark). This factorial arrangement resulted in a total of 20 treatment combinations, which were randomly assigned to the experimental units. Data were analyzed using SAS software (version 9.4), and mean separation was performed using Duncan's Multiple Range Test at $P \leq 0.05$ (Pradeepkumara et al., 2024; Pradeepkumara et al., 2023). The details of the 20 treatment combinations are presented in Table 1.

Assessment of callus formation

Callus induction was evaluated by calculating the percentage of explants that formed callus relative to the total number of cultured explants. After four weeks of incubation, the fresh weight (FW) of callus were recorded using a precision balance. Callus volume was measured using the liquid displacement method based on Archimedes' principle.

Table 1 : Summary of the 20 treatment combinations used for callus induction in *C. indicum* L.

Treatment	Hormone code	Hormone description	Explant type	Light condition
T_1	H_1	Control	Leaf	Light
T_2	H_2	1 mg L^{-1} NAA + 2 mg L^{-1} BAP	Leaf	Light
T_3	H_3	0.2 mg L^{-1} 2,4-D	Leaf	Light
T_4	H_4	0.2 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D	Leaf	Light
T_5	H_5	2 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D	Leaf	Light
T_6	H_1	Control	Stem	Light
T_7	H_2	1 mg L^{-1} NAA + 2 mg L^{-1} BAP	Stem	Light
T_8	H_3	0.2 mg L^{-1} 2,4-D	Stem	Light
T_9	H_4	0.2 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D	Stem	Light
T_{10}	H_5	2 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D	Stem	Light
T_{11}	H_1	Control	Leaf	Dark
T_{12}	H_2	1 mg L^{-1} NAA + 2 mg L^{-1} BAP	Leaf	Dark
T_{13}	H_3	0.2 mg L^{-1} 2,4-D	Leaf	Dark
T_{14}	H_4	0.2 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D	Leaf	Dark
T_{15}	H_5	2 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D	Leaf	Dark
T_{16}	H_1	Control	Stem	Dark
T_{17}	H_2	1 mg L^{-1} NAA + 2 mg L^{-1} BAP	Stem	Dark
T_{18}	H_3	0.2 mg L^{-1} 2,4-D	Stem	Dark
T_{19}	H_4	0.2 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D	Stem	Dark
T_{20}	H_5	2 mg L^{-1} BAP + 2 mg L^{-1} 2,4-D	Stem	Dark

Measurement of carotenoids

For pigment extraction, 200 mg of callus tissue was homogenized in 10 mL of 80% acetone. The extract was filtered, and absorbance was measured at 480 nm using a spectrophotometer, according to the method of (Hemphill & Venketeswaran, 1978).

Total phenolic content was measured using the Folin–Ciocalteu method by mixing ethanolic extract with Folin–Ciocalteu reagent and sodium carbonate, incubating for 1.5 hours, and measuring absorbance at 765 nm; results were expressed as mg GAE/g FW. Total flavonoid content was determined using the Chang et al. (2002) method by mixing the extract with potassium acetate and aluminum chloride, incubating for 45 minutes in the dark, and measuring absorbance at 510 nm.

Antioxidant activity measurement

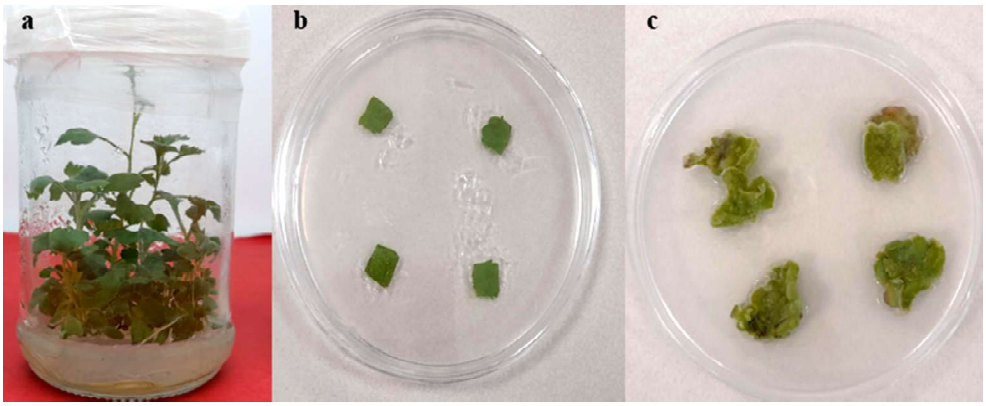
Antioxidant activity was evaluated using the DPPH radical scavenging assay by mixing DPPH solution with the extract, incubating in the dark at room temperature for 30 minutes, and measuring absorbance at 517 nm.

To determine anthocyanin content, callus tissue was finely chopped and placed in a Falcon tube. Approximately 10 mL of 80% methanol was added, and the mixture was stored at 4°C for 24 hours with intermittent shaking. After the extraction period, the solution was filtered. To stabilize the anthocyanin pigments, 1 mL of 1 M hydrochloric acid was added. The acidified extract was then analyzed spectrophotometrically at 530 nm after calibration with 80% methanol (Bürkle et al., 2018).

Proline content was determined according to Bates et al. (1973) by homogenizing callus tissue in salicylic acid, reacting the extract with ninhydrin reagent, and measuring the absorbance at 520 nm.

Catalase (CAT) activity was assayed using the method of (Aebi, 1984). The reaction mixture contained 2.5 mL of 50 mM phosphate buffer (pH 7.0), 0.2 mL of 1% hydrogen peroxide, and 0.3 mL of enzyme extract. The decrease in absorbance was recorded at 240 nm over 1 minute to calculate enzyme activity.

Peroxidase (POD) activity was measured based on the method of (Serrano-Martínez et al., 2008).



(a) *In vitro*-grown plantlets maintained on MS basal medium, b) Leaf explants placed on callus-induction medium at the start of the experiment, c) Induced callus tissues formed on MS medium supplemented with 2 mg/L BAP + 2 mg/L 2,4-D after 7 days of incubation

Fig. 1 : Different stages of *in vitro* culture and callus induction in *C. indicum* L.

The reaction mixture included 3 mL of 50 mM phosphate buffer (pH 7.0), 10 µL of 30% hydrogen peroxide, 3 µL of 200 mM guaiacol solution, and 100 µL of enzyme extract. Absorbance was measured at 470 nm every 20 seconds for a total duration of one minute. Enzyme activity was expressed as the amount of H, O, decomposed per minute per milligram of protein.

RESULTS AND DISCUSSION

Effect of hormones, explant type, and light

After seed germination on MS medium (Fig. 1a, b), leaf and stem explants from *in vitro* grown plants were cultured on media containing different hormonal combinations. ANOVA showed a highly significant three-way interaction among hormone treatment, explant type, and light condition for all callus induction parameters, and three-way interaction plots were used to illustrate these combined effects on callus formation.

A highly significant variation in callus induction response was observed among different hormonal combinations, explant types, and light conditions (Fig. 2a). The highest callus formation percentage (96.66%) was obtained from leaf explants cultured on MS medium supplemented with 2 mgL^{-1} BAP and 2 mgL^{-1} 2,4-D under light conditions (E1H5L). This result indicates that an equimolar balance between auxin and cytokinin is critical for promoting optimal cell division and dedifferentiation, thereby activating signaling pathways associated with totipotency and morphogenesis (Su et al., 2014). A significant difference was also observed in the time required for callus initiation among treatments (Fig. 2b). The earliest callus initiation (14 days) occurred in the E1H5L treatment, indicating that balanced concentrations of 2,4-D and BAP accelerated cellular dedifferentiation and reprogramming.

Callus fresh weight was significantly influenced by hormonal treatment and light conditions. The highest fresh weight was recorded in leaf explants cultured under the E1H5L treatment, which can be attributed to the synergistic effect of 2,4-D and BAP in stimulating cell division and undifferentiated growth (Bano et al., 2022). Consistent with fresh weight results, the greatest callus volume was also observed in the E1H5L treatment (Fig. 3b).

Effect of chitosan elicitor on various callus characteristics

Analysis of variance revealed that chitosan elicitation had a highly significant effect ($p \leq 0.01$) on all evaluated callus traits of *C. indicum* L., indicating a strong and concentration-dependent response. Overall, chitosan treatments enhanced callus growth compared to the control, although the magnitude of the response varied with concentration.

Callus fresh weight increased significantly under all chitosan treatments (Fig. 4a). The control showed an average fresh weight of 1.67 g, which increased to 2.11 g at 25 mgL^{-1} chitosan and reached a maximum at 50 mgL^{-1}, indicating optimal biomass accumulation. At 75 mgL^{-1}, fresh weight declined to 2.07 g but remained higher than the control, suggesting a slight inhibitory effect at higher concentrations. These effects

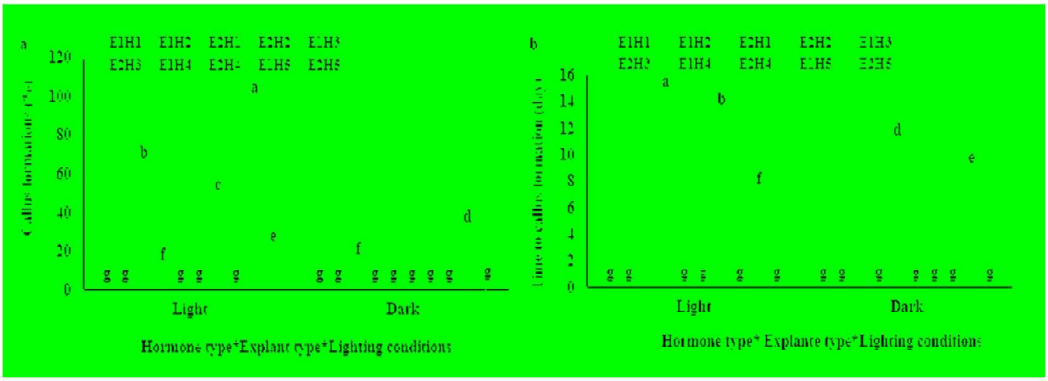


Fig. 2 : Comparison of the mean interaction effect of hormone, explant type, and light conditions on callus percentage (a) and time to callus initiation (b) in the *Chrysanthemum*

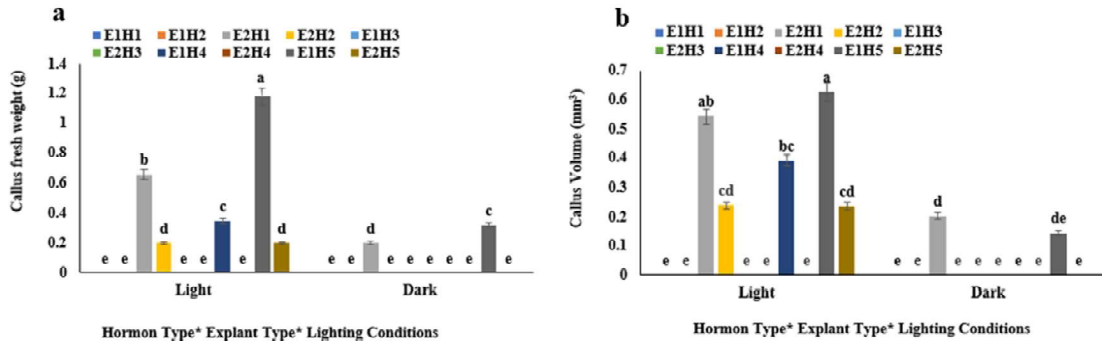


Fig. 3 : Comparison of the mean interaction effects of hormone, explant type, and light conditions on callus fresh weight (a), and callus volume (b) of the *Chrysanthemum*

may be related to chitosan-mediated regulation of auxin and cytokinin signaling, nutrient uptake, antioxidant defense, and cellular homeostasis (Suarez-Fernandez et al., 2020). A similar trend was observed for callus volume (Fig. 4b). All chitosan treatments increased callus volume compared with the control, reaching 1.39 mm³ at 25 mgL^{-1} and a maximum of 1.81 mm³ at 50 mgL^{-1}. Increasing the concentration to 75 mgL^{-1} reduced callus volume to 1.32 mm³, although it remained higher than the control. Enhanced callus expansion at optimal chitosan levels may result from increased cell division, improved antioxidant enzyme activity, and nutrient uptake (Elateeq et al., 2021).

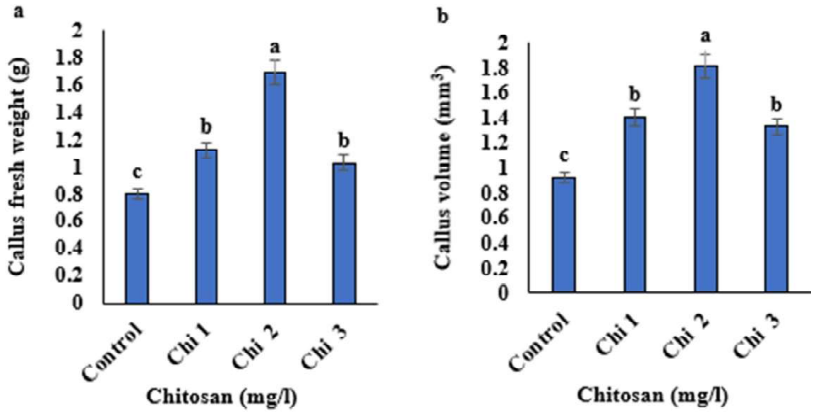


Fig. 4 : Comparison of the mean effects of different concentrations of chitosan elicitor on fresh weight (a), and callus volume (b) of the *Chrysanthemum*.

Effect of chitosan elicitors on metabolic traits

Analysis of variance (ANOVA) showed that chitosan elicitors had a highly significant effect ($p \leq 0.01$) on the metabolic traits of *C. indicum* L., indicating a strong influence of chitosan on plant metabolic responses.

Carotenoids

Carotenoid content increased significantly under all chitosan treatments compared to the control (Fig. 5a). The control plants exhibited a carotenoid level of 0.790 mgg^{-1} FW, whereas application of 25 mgL^{-1} chitosan increased carotenoid content to 1.308 mgg^{-1} FW. The highest carotenoid accumulation (2.93 mgg^{-1} FW) was recorded at 50 mgL^{-1} chitosan, identifying this concentration as optimal for carotenoid biosynthesis. The enhancement of carotenoid accumulation under chitosan elicitation may be attributed to the activation of carotenoid biosynthetic enzymes and the reduction of oxidative stress through the induction of antioxidant enzymes. Similar increases in carotenoid content following chitosan application have been reported in *Salvia abrotanoides* (Attaran Dowom et al., 2022).

Phenol and flavonoids

Chitosan treatments significantly increased total phenol and flavonoid contents compared to the control (Fig. 5b, c). The control showed the lowest phenolic and flavonoid levels. At 25 mgL^{-1} chitosan (Chi1), phenolic content increased to 55.63 mgg^{-1} FW, while flavonoids reached 31.03 mgg^{-1} FW. The highest accumulation of both phenols (67.40 mgg^{-1} FW) and flavonoids (41.32 mgg^{-1} FW) was observed at 50 mgL^{-1} chitosan (Chi2), indicating this concentration as optimal for their biosynthesis. At 75 mgL^{-1} chitosan (Chi3), phenolic and flavonoid contents decreased to 51.14 and 30.86 mgg^{-1} FW, respectively, suggesting mild stress or feedback inhibition at higher concentrations. The stimulatory effect of chitosan on phenolic and flavonoid accumulation is attributed to activation of the phenylpropanoid and shikimate pathways, and similar results have been reported in *Salvia abrotanoides* and *Linum usitatissimum* (Attaran Dowom et al., 2022).

Antioxidant activity

Chitosan treatments significantly enhanced antioxidant activity in *C. indicum* L. compared with the control

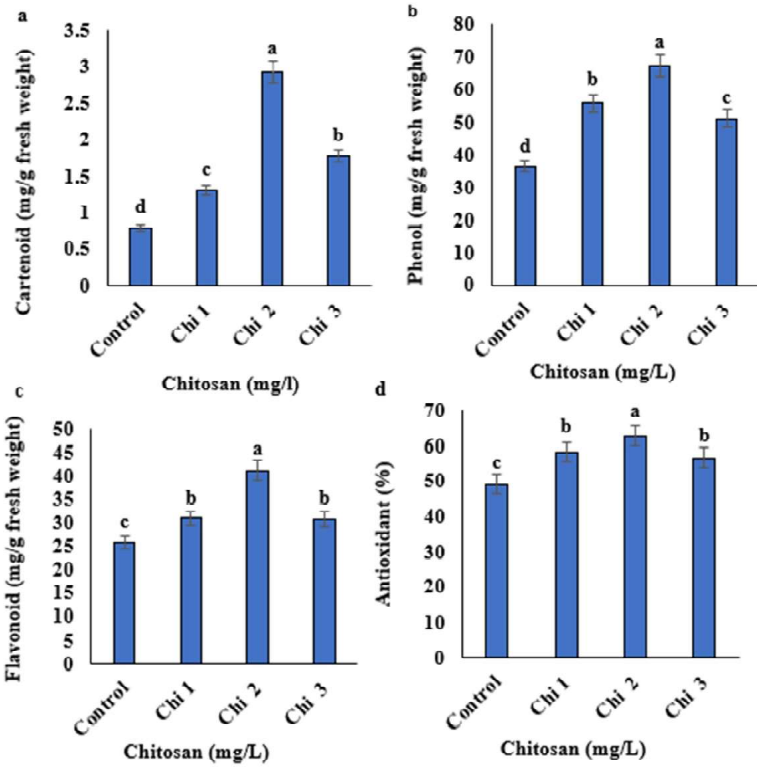


Fig. 5 : Comparison of the mean effect of different concentrations of chitosan elicitor on carotenoids (a), phenols (b), flavonoids (c), and antioxidants (d) of the plant *Chrysanthemum*

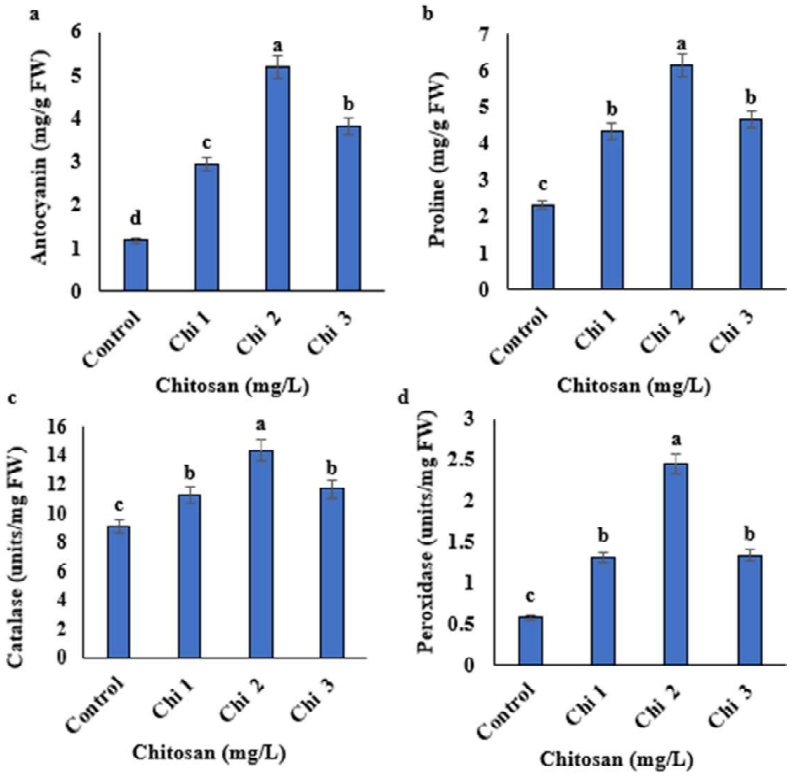


Fig. 6 : Comparative analysis of the mean effects of varying concentrations of chitosan elicitor on malondialdehyde (a), anthocyanin (b), proline (c), catalase (d), and peroxidase (f) levels in the *Chrysanthemum*.

(Fig. 5d). Application of 25 mgL^{-1} chitosan (Chi1) increased activity to 58.18%, while the highest activity (62.75%) was observed at 50 mgL^{-1} (Chi2), indicating this as the optimal concentration. Chitosan enhances antioxidant activity by activating defense signaling pathways, and ethylene cascades, and by inducing ROS-scavenging enzymes. Similar improvements in antioxidant activity following chitosan application have been reported in *Linum usitatissimum* (Attaran Dowom et al., 2022).

Effect of chitosan elicitor on biochemical traits

The analysis of variance (ANOVA) showed that the application of chitosan elicitor had a highly significant effect ($p \leq 0.01$) on all evaluated biochemical traits of *C indicum* L.

Anthocyanin

Chitosan significantly and concentration-dependently increased anthocyanin content in *C. indicum* L. (Fig. 6a). The control contained 1.18 mgg^{-1} FW, which increased to 2.93 mgg^{-1} FW at 25 mgL^{-1} chitosan (Chi1) and reached a maximum of 5.19 mgg^{-1} FW at 50 mgL^{-1} (Chi2). Enhanced anthocyanin accumulation may result from chitosan-induced activation of phytohormonal pathways, improved membrane stability, and nutrient uptake, reducing oxidative stress and favoring pigment biosynthesis, consistent with findings in *Carthamus tinctorius* (Golkar et al., 2019).

Proline

Chitosan significantly increased proline content in *C. indicum* L. compared with the control (Fig. 6b). Control plants contained 2.31 mgg^{-1} FW, which increased to 3.34 mgg^{-1} FW at 25 mgL^{-1} chitosan (Chi1) and reached a maximum of 6.14 mgg^{-1} FW at 50 mgL^{-1} (Chi2). At 75 mgL^{-1} (Chi3), proline decreased to 4.65 mgg^{-1} FW, indicating partial inhibition or stress effects. Chitosan likely enhances proline accumulation by activating hormonal pathways, including salicylic acid, jasmonic acid, and abscisic acid, and by regulating genes involved in osmoprotectant biosynthesis under stress (Sree Rayanoothala et al., 2024).

Effect of chitosan on antioxidant enzymes

Chitosan elicitation significantly enhanced the activities of key antioxidant enzymes, CAT and peroxidase (POD), in *C. indicum* L. (Fig. 6c, d). Control plants exhibited baseline activities of

9.15 units mg^{-1} FW for CAT and 0.578 units mg^{-1} FW for POD. Treatment with 25 mgL^{-1} chitosan (Chi1) increased CAT to 11.25 units mg^{-1} FW and POD to 1.306 units mg^{-1} FW. The highest activities were observed at 50 mgL^{-1} (Chi2), with CAT reaching 14.37 units mg^{-1} FW and POD 2.45 units mg^{-1} FW, indicating this concentration as optimal for stimulating enzymatic antioxidant defenses. Chitosan enhances antioxidant enzyme activities by activating defense signaling pathway, and by inducing ROS-scavenging mechanisms that detoxify hydrogen peroxide and protect cellular components from oxidative damage (Arif et al., 2022). Similar enhancements in CAT and POD activities following chitosan application have been reported in *Momordica charantia* L. (Sharifi-Rad et al., 2020).

CONCLUSION

This study demonstrates that chitosan application as an elicitor in *C. indicum* L. callus culture significantly enhances morpho-physiological, biochemical, metabolic, and antioxidant traits. Among the tested hormonal combinations, 2 mgL^{-1} BAP + 2 mgL^{-1} 2,4-D under light conditions (E1H5L) was the most effective, yielding the highest callus induction, shortest initiation time, and greatest fresh weight and callus volume. This hormonal balance supported efficient callogenesis and early tissue proliferation. Chitosan at 25, 50, and 75 mgL^{-1} significantly improved growth and biochemical parameters, with 50 mgL^{-1} being the most effective. This concentration maximized callus fresh weight and volume and increased carotenoids, total phenolics, flavonoids, antioxidant activity, anthocyanins, and proline, while enhancing CAT and POD activities. These results indicate that moderate chitosan levels stimulate cellular metabolism, activate defense responses, and improve physiological performance *in vitro*. Overall, combining the E1H5L hormonal treatment with 50 mgL^{-1} chitosan is an effective approach for enhancing callogenesis, biomass production, and stress-related biochemical responses in *C. indicum* L.

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