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Chitosan Nanoparticles' Biphasic Impact on *Salvia nemorosa*: Optimizing Resilience and Secondary Metabolism

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Abstract

Nanotechnology offers innovative approaches to enhance crop resilience against environmental stresses. Chitosan nanoparticles (ChNPs) enhance plant defense mechanisms and stimulate secondary metabolite production. *Salvia nemorosa*, a medicinal plant rich in bioactive compounds, is sensitive to stressors impacting its growth and metabolite accumulation. This study explored the dose-dependent effects of ChNPs on *S. nemorosa*'s physiological and biochemical parameters, including photosynthetic pigments, oxidative stress markers, antioxidant enzyme activities, and secondary metabolites. Two-month-old plants were foliar-sprayed with ChNPs (0–160 ppm), and their responses were assessed two weeks later. Lower doses (10–40 ppm, with an optimum at 20 ppm) improved growth, phenolic compound accumulation, and the activities of enzymes such as phenylalanine ammonia-lyase (PAL) and tyrosine aminotransferase (TAT), whereas higher doses (80–160 ppm) triggered oxidative stress, reduced chlorophyll and carotenoid contents, and decreased metabolite production. Antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD)—increased significantly at higher concentrations, reflecting a robust defense against oxidative damage. These findings highlight ChNPs' dual role in stress tolerance and metabolite biosynthesis, offering insights for optimizing medicinal plant cultivation via nanotechnology. This study provides comprehensive insights into the biphasic effects of ChNPs on *S. nemorosa*, contributing to sustainable cultivation strategies.

Keywords: Antioxidant enzymes, Chitosan nanoparticles, Oxidative stress, *Salvia nemorosa*, Phenolic compounds.

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Introduction

The genus *Salvia*, with over 900 species, is one of the largest and most significant groups of medicinal plants in the family Lamiaceae (Askari et al., 2021). *Salvia nemorosa* (wood sage) is a perennial herb valued for its ornamental beauty and medicinal properties, particularly its high content of phenolic acids, flavonoids, phytosterols, omega-3 fatty acids, and essential oils, which exhibit antioxidant, antimicrobial, and anti-inflammatory activities (Bahadori et al., 2017; Mirza & Sefidkon, 1999; Moazzami Farida & Radjabian, 2019; Moazzami Farida et al., 2016). These bioactive compounds are critical in plant defense against abiotic and biotic stresses and hold immense potential for pharmaceutical applications (Bahadori et al., 2017). However, environmental challenges, including soil degradation, climate change, and limited agricultural inputs, have driven the need to explore novel strategies for improving the production and quality of these medicinal plants.

In recent years, nanoparticles have offered innovative solutions for enhancing crop resilience under environmental stresses (Shoukat et al., 2024). Among these materials, chitosan nanoparticles (ChNPs) have gained significant attention due to their biocompatibility, biodegradability, and remarkable ability to stimulate plant defense mechanisms (Riseh et al., 2024). Derived from chitin, chitosan is a natural polysaccharide with unique properties, such as a positive surface charge, which enhances interactions with plant cell walls and improves bioactive molecule uptake (Sreelakshmi et al., 2024). ChNPs not only act as carriers for agrochemicals but also serve as elicitors, triggering the production of secondary metabolites and enhancing the antioxidant capacity of plants (Riseh et al., 2024).

Nanoparticles, particularly ChNPs, have demonstrated promising effects on plant growth, development, and physiology (Marslin et al., 2017). Studies have shown that ChNPs can enhance photosynthetic efficiency, stimulate secondary metabolite production, and improve stress tolerance by modulating the antioxidant defense system (Arif et al., 2021; Balusamy et al., 2022). Despite recent advances, the specific effects of ChNPs on *S. nemorosa* remain underexplored, and no studies have examined their dose-dependent impacts on this species' physiological and biochemical indices.

The impact of NPs on plants depends on factors like particle size, concentration, and exposure method (Rastogi et al., 2017). Depending on the applied doses, moderate concentrations of NPs can promote plant growth and mitigate oxidative stress (Ferrari et al., 2021; Moazzami Farida et al., 2020). However, higher concentrations of NPs may induce phytotoxicity by generating reactive oxygen species (ROS) (Dhiman et al., 2021). While excessive ROS can cause irreversible damage and cell death, balanced ROS formation can potentially ameliorate abiotic stress and enhance crop productivity (Sachdev et al., 2021). NPs can also alter the content of secondary metabolites in plants by influencing gene expression and the activity of essential enzymes in the biosynthesis pathways of these metabolites (Holghoomi & Colagar, 2024). For instance, different doses of TiO_2NPs can increase rosmarinic acid (RA) content in Dragonhead by influencing the expression of genes involved in its biosynthesis (Salar et al., 2021). Additionally, the RA amounts in *Melissa officinalis* varied with different concentrations of graphene and Ag nanoparticles (Soraki et al., 2021). Key enzymes such as phenylalanine ammonia-lyase (PAL) and tyrosine aminotransferase (TAT), involved in the phenylpropanoid pathway, play pivotal roles in the biosynthesis of phenolic compounds. Nevertheless, their response to ChNP treatment in *S. nemorosa* has not been comprehensively studied.

The current study addresses these knowledge gaps by systematically evaluating the dose-dependent effects of ChNPs on the physiological and biochemical parameters of *S. nemorosa*. This research investigates how different concentrations of ChNPs influence photosynthetic pigments, oxidative stress markers, antioxidant enzyme activities, and the accumulation of secondary metabolites, including phenolics, flavonoids, and anthocyanins. This study aims to provide novel insights into the mechanisms underlying

nanoparticle-plant interactions by focusing on the interplay between antioxidant defense systems and metabolic pathways. The findings contribute to the growing body of knowledge on nanotechnology applications in agriculture and offer practical implications for optimizing the cultivation of medicinal plants. This study offers comprehensive insights into the biphasic effects of ChNPs on *S. nemorosa*, advancing sustainable cultivation strategies and strengthening the application of plant nanobiotechnology.

Materials and Methods

Plant materials

Mature seeds of *S. nemorosa* were collected directly from Shahin Dej, West Azaribijan, Iran (36°37′41″ N and 46°34′16″ E). Seed dormancy was broken by soaking the seeds in distilled water for 10 minutes, placing them on a pre-wetted Whatman No. 1 filter paper in Petri dishes, and storing them at 4°C for 7 days. Germinated seedlings were transferred into plastic pots filled with a mixture of garden soil, coco peat, and perlite (3:1:1). The pots were kept in a greenhouse with a 16:8 h light (600 µmol photons m^{-2} s^{-1})-dark cycle and 65 ± 5% relative humidity, with a temperature maintained between 22-25 °C. Pots were rotated every 5 days to minimize spatial variation in the growing conditions. Plants were allowed to grow for two months before treatment with ChNPs.

ChNPs characterization and treatment

ChNPs were purchased as a ready-to-use suspension from the Iranian Nanomaterials Pioneers Company (NANOSANY, Mashhad, Iran), with no additional surfactants (e.g., Tween-20) or solvents required for preparation. The size of ChNPs was estimated to be 50 nm in diameter, metal basis, and spherical. Further morphological characteristics of ChNPs were confirmed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Fig. 1). Two-month-old plants were subjected to a single foliar spray treatment with ChNP solutions at 0, 10, 20, 40, 80, and 160 ppm, selected based on preliminary tests of ChNP interactions with *S. nemorosa*. Each treatment included three biological replicates. After two weeks, young leaves from both treated and untreated plants were harvested for subsequent physiological and biochemical analyses.

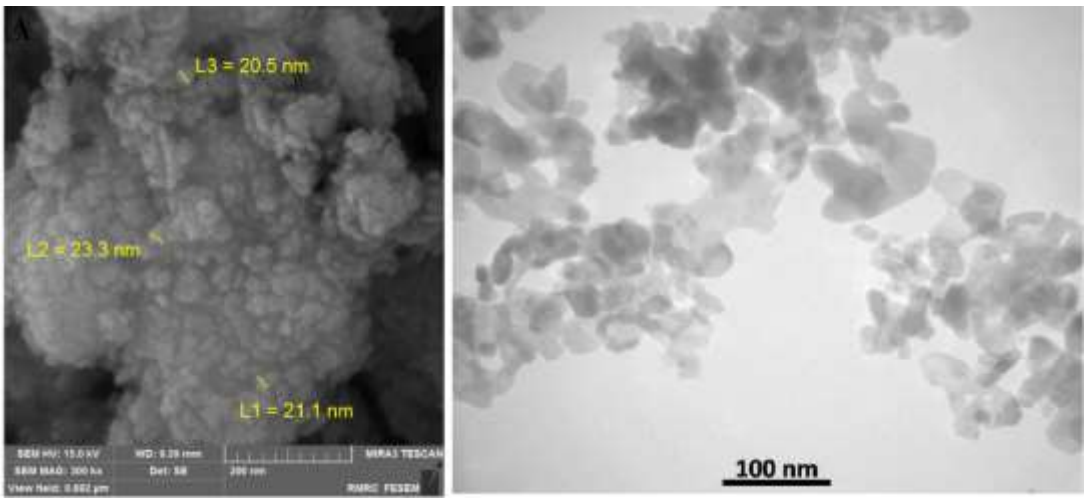

Fig. 1. a) Scanning Electron Microscope (SEM) and b) Transmission Electron Microscope (TEM) image of chitosan nanoparticles. Images provided by the Iranian Nanomaterials Pioneers Company, NANOSANY (Mashhad, Iran).

Photosynthetic Pigment Content:

Leaf samples (100 mg) were extracted in 80% acetone (v/v), and absorbance was measured at 647 and 663 nm for chlorophylls and 470 nm for carotenoids using a UV/visible spectrophotometer. Chlorophylls (*a* and *b*) and total carotenoid content were calculated based on (Lichtenthaler, 1987) and expressed as mg chlorophyll g^{-1} fresh weight (FW) and mg carotenoid g^{-1} FW, respectively.

MDA and H_2O_2 contents:
Malondialdehyde (MDA) content, indicative of lipid peroxidation, was determined following the method of (Heath & Packer, 1968), expressed as µM g^{-1} FW. Hydrogen peroxide (H_2O_2) levels were measured using the potassium iodide method (Sergiev et al., 1997), described as µM g^{-1} FW.

Proline and total protein contents:
Proline content was measured following (Bates et al., 1973) with standard proline solutions for calculation, expressed as µg g^{-1} FW. Protein extraction was performed by homogenizing 0.1 g leaf tissue in liquid nitrogen and extracting proteins using a 100 mM phosphate buffer (pH 6.0), 2 mM EDTA, 4 mM dithiothreitol, and 2% polyvinyl pyrrolidone, followed by centrifugation at 13,000 rpm and 4°C for 25 minutes. Protein concentration was quantified using (Bradford, 1976), with bovine serum albumin as the standard.

Antioxidant enzyme activities:
Superoxide dismutase (SOD) activity was measured as the enzyme's ability to prevent the photoreduction of nitro blue tetrazolium (NBT) according to (Giannopolitis & Ries, 1977), expressed as units mg^{-1} protein. Peroxidase (POD) activity was determined by (Abeles & Biles, 1991), described as µkat mg^{-1} protein. Catalase (CAT) activity was measured using the method of (Cakmak & Horst, 1991), described as µkat mg^{-1} protein.

Phenolic Compounds:
Leaf samples (1 g) were ground and extracted in 5 mL methanol for 24 h at 25°C. After centrifugation, supernatants were analyzed for phenolic compounds. Total phenol content (TPC) measured by the Folin-Ciocalteu method (Singleton et al., 1999), TPC was quantified using a standard curve of gallic acid (GAE) at 750 nm and expressed as mg GAE g^{-1} dry weight (DW). Total flavonoid content (TFC) was determined by the method of (Zhishen et al., 1999) using $AlCl_3$ and quantified against a quercetin standard curve at 415 nm, expressed as mg QE g^{-1} DW. Total anthocyanin content (TAC) was extracted with acidic methanol; TAC was quantified at 511 nm using a standard extinction coefficient of 33,000 M^{-1} cm^{-1} (Hara et al., 2003).

Phenolic Biosynthesis Enzyme Activities:
L-Phenylalanine ammonia-lyase (PAL) activity was assessed using the method of (Heide et al., 1989), using the molar absorption coefficient of trans-cinnamic acid (10,900 l mol^{-1} cm^{-1}), and expressed as nkat mg^{-1} protein. Tyrosine aminotransferase (TAT) activity was determined by the method of (Diamondstone, 1966), using the extinction coefficient of 4-hydroxybenzaldehyde (24,900 l mol^{-1} cm^{-1}), and enzyme-specific activity was expressed as nkat mg^{-1} protein.

Statistical Analysis
The experiments were arranged in a completely randomized design (CRD) with three replicates per treatment. Statistical analysis was performed using one-way analysis of variance (ANOVA), and mean comparisons were carried out using Duncan's post hoc test at a significance level of $P \leq 0.05$. Pearson correlation coefficients were computed to evaluate the relationships among the measured parameters, and a heatmap was generated using R software (https://www.r-project.org/). To uncover patterns and relationships among treatments and the physiological and biochemical data, principal component analysis (PCA) was performed with PAST software v4.17, with eigenvalues >1 retained for analysis (Hammer & Harper, 2005). Results are expressed as mean ± standard deviation (S.D.) based on three replicates.

Results

Photosynthetic Pigments
The levels of Chl *a*, and Chl *b*, and total chlorophyll showed a dose-dependent decrease with increasing ChNP concentrations (Fig. 2). In the control group, the content of Chl *a*, -*b*, and total chlorophyll were recorded as 5.59, 2.20, and 7.79 mg g^{-1} FW, respectively. At a low ChNP dose (10 ppm), Chl *a* levels (5.62 mg g^{-1} FW) were statistically similar to the control, whereas Chl b and total chlorophyll decreased slightly to 1.26 and 6.88 mg g^{-1} FW, respectively. At moderate concentrations, a pronounced reduction

was observed. For instance, at 40 ppm, Chl *a*, -*b*, and total chlorophyll amounts dropped to 4.64, 0.93, and 5.57 mg g^{-1} FW, respectively. The most significant decline occurred at 160 ppm, with Chl *a* at 3.08 mg g^{-1} FW, Chl *b* at 0.87 mg g^{-1} FW, and total chlorophyll at 3.95 mg g^{-1} FW. This trend highlights the dual role of ChNPs as low doses had negligible effects, whereas higher doses caused significant stress, likely affecting pigment synthesis.

Carotenoid content (Car) followed a similar pattern to chlorophyll, showing a marked reduction as ChNP concentration increased (Fig. 2). In the control plants, the carotenoid level was 1.56 mg g^{-1} FW. At 10 ppm of ChNPs, a moderate reduction to 1.33 mg g^{-1} FW was observed, while at 40 ppm, Car dropped to 0.90 mg g^{-1} FW. The lowest level of carotenoids was recorded at 160 ppm (0.58 mg g^{-1} FW), indicating the severe disruption in Car biosynthesis or degradation under high oxidative stress.

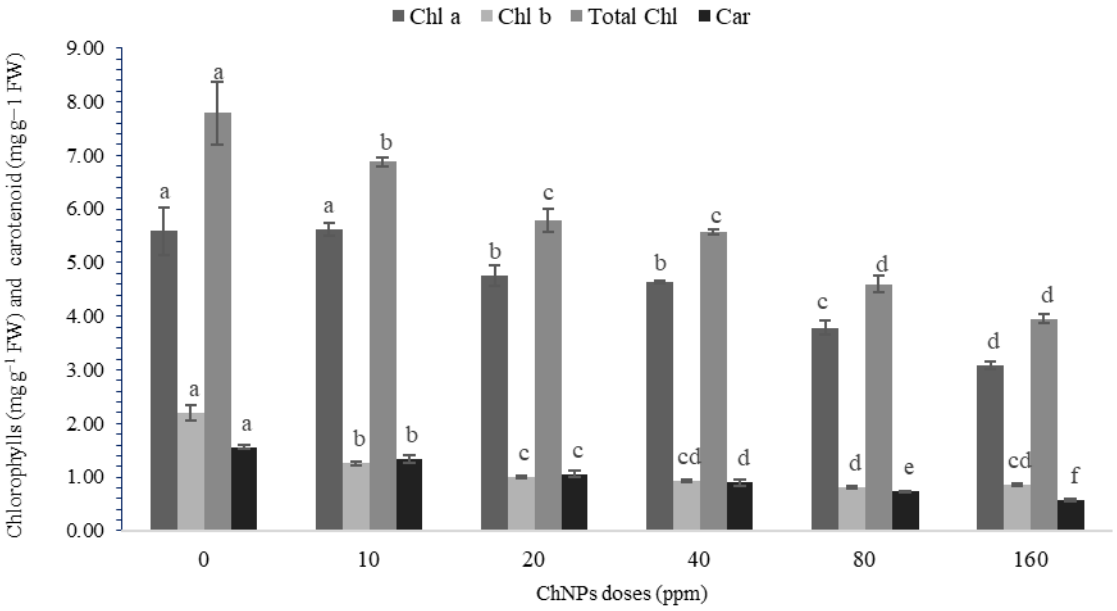

Fig. 2. The effect of different concentrations of ChNPs on photosynthetic pigments, including chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (total Chl), and Carotenoid (car) in the leaves of *Salvia nemorosa* after two weeks of exposure. Values are given as means ± standard deviation (S.D.) from three biological replicates. Columns with different letters indicate significant differences at $P \leq 0.05$ (Duncan's test).

Oxidative Stress Markers

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels, oxidative stress markers, increased significantly with higher ChNP concentrations (Fig. 3.). H_2O_2 content in control was 0.14 µmol g^{-1} FW but increased steadily to 0.39 µmol g^{-1} FW at 160 ppm (2.8-fold). Similarly, MDA levels, indicative of lipid peroxidation, were 11.72 nmol g^{-1} FW in control and rose to 14.38 nmol g^{-1} FW at 160 ppm (Fig. 3.). These findings confirm the generation of oxidative stress under ChNP treatment.

Proline Accumulation and Total Protein Content

Proline accumulation is a key indicator of plant responses to abiotic stress. Proline content was measured to evaluate the stress status of treated plants, as it accurately indicates physiological changes due to stress (Fig. 4.). The content of this amino acid increased across all treatments, suggesting its role as an osmoprotectant under ChNP-induced stress. In the control plants, proline content was 0.47 mg g^{-1} FW, which rose to 0.57 mg g^{-1} FW at 160 ppm (1.2-folds). Notably, the increase was gradual and consistent across doses, indicating a proportional response to stress levels.

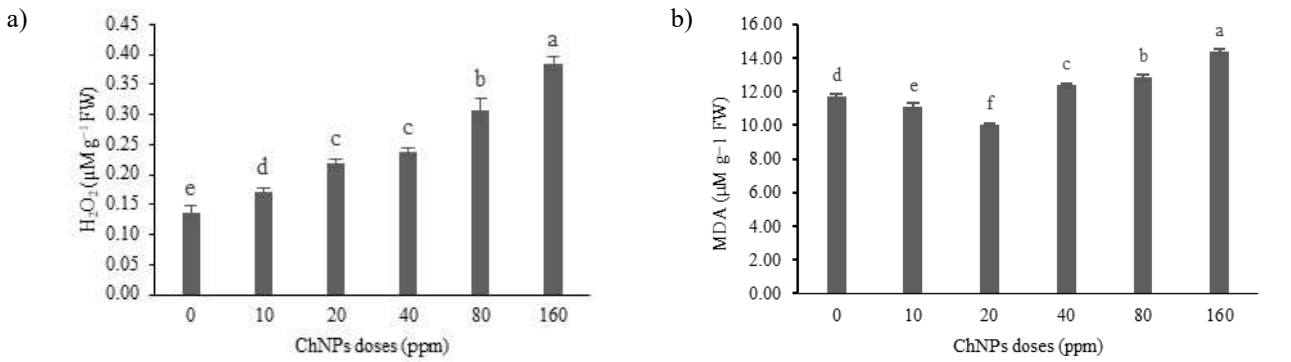

Fig. 3. The effect of different concentrations of ChNPs on oxidative injury indices, including a) H_2O_2 and b) malondialdehyde (MDA) in the leaves of *Salvia nemorosa* after two weeks of exposure. Values are given as means ± standard deviation (S.D.) from three biological replicates. Columns with different letters indicate significant differences at $P \leq 0.05$ (Duncan's test).

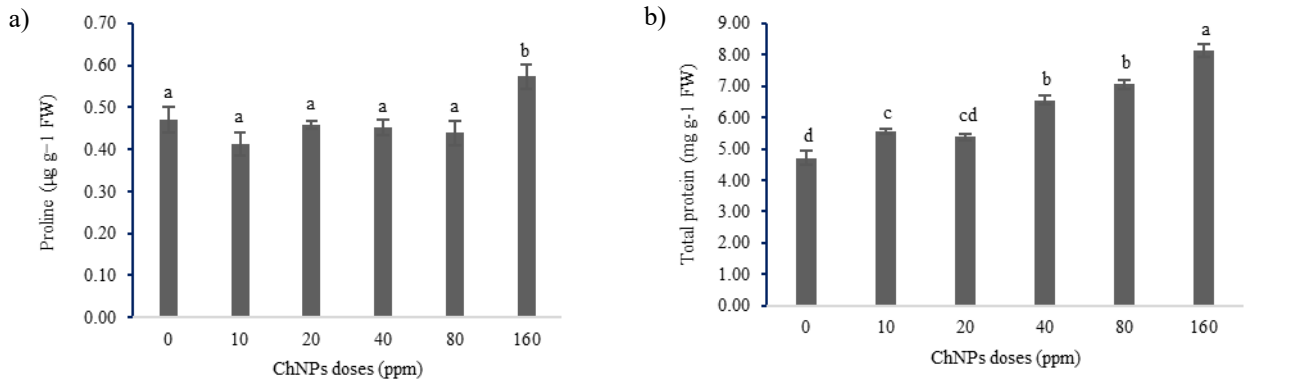

Fig. 4. The effect of different concentrations of ChNPs on Oxidative injury indices, including a) proline and b) total protein content in the leaves of *Salvia nemorosa* after two weeks of exposure. Values are given as means ± standard deviation (S.D.) from three biological replicates. Columns with different letters indicate significant differences at $P \leq 0.05$ (Duncan's test).

The total protein content also rose progressively with rising ChNP concentrations. Control plants had a protein content of 4.72 mg g^{-1} FW, which increased to 6.54 mg g^{-1} FW at 40 ppm and peaked at 160 ppm (8.14 mg g^{-1} FW). The observed increase suggests the activation of stress-responsive protein synthesis, including antioxidant and defense-related proteins, to counteract the stress induced by ChNPs.

Antioxidant Enzyme Activities

The activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) showed a marked increase with rising concentrations of ChNP, indicating an upregulated antioxidant defense system. The oxidative stress responses of *S. nemorosa* to ChNP exposure were assessed through the activities of these key antioxidant enzymes (Table 1). These enzymes are crucial in neutralizing reactive oxygen species (ROS) generated by nanoparticles within the cellular environment, thereby mitigating oxidative damage (Faizan et al., 2024). SOD activity displayed a dose-dependent increase across the ChNP treatments. The lowest activity was observed at 20 ppm ChNPs (2.46 U mg^{-1} protein), significantly lower than the control (2.96 U mg^{-1} protein; $P \leq 0.05$). However, a marked enhancement was recorded at higher doses, peaking at 160 ppm with a maximum activity of 4.77 U mg^{-1} protein. This trend suggests an elevated ROS scavenging requirement at higher nanoparticle concentrations, reflecting an intensified oxidative stress response.

Table 1. ChNPs affect the biochemical parameters of *Salvia nemorosa* after two weeks of exposure. Values are given as means ± standard deviation (S.D.). Rows with different letters indicate significant differences at $P \leq 0.05$ (Duncan's test)—abbreviation: CAT, catalase; POD, peroxidase; SOD, superoxide dismutase.

		ChNPs doses (ppm)					
		0	10	20	40	80	160
Antioxidant enzymes activity	SOD activity (U mg^{-1} protein)	2.96±0.10^{d}	3.11±0.07^{d}	2.46± 0.13^{e}	3.74±0.09^{c}	4.14±0.08^{b}	4.77±0.18^{a}
	POD activity (µkat. mg^{-1} protein)	0.93±0.04^{c}	0.77±0.11^{d}	0.56±0.15^{e}	1.19±0.08^{b}	1.63±0.14^{b}	2.07±0.18^{a}
	CAT activity (µkat. mg^{-1} protein)	72.02±0.80^{c}	39.63±1.07^{d}	31.19±0.31^{e}	90.69±1.38^{b}	92.11±0.49^{b}	112.45±0.58^{a}
Phenolic compounds	Total phenol (mg GAE g^{-1} DW)	10.02±0.50^{f}	50.43±0.65^{b}	61.07±1.19^{a}	28.40±0.54^{d}	29.92±0.34^{c}	15.93±0.41^{e}
	Total flavonoids (mg QE g^{-1} DW)	3.95±0.34^{d}	7.80±0.21^{a}	5.12±0.11^{b}	4.63±0.15^{c}	4.63±0.10^{c}	2.91±0.08^{e}
	Total anthocyanin (nmol g^{-1} FW)	24.06±0.23^{c}	24.22±0.35^{b}	24.57±0.67^{a}	35.39±0.41^{d}	30.44±0.27^{d}	28.56±0.28^{d}

CAT activity followed a biphasic trend, initially declining at lower ChNP doses before significantly increasing at higher concentrations. The minimum activity was recorded at 20 ppm ChNPs (31.19 U mg^{-1} protein), while the highest activity (112.45 U mg^{-1} protein) was observed at 160 ppm. Notably, at 80 ppm and 160 ppm ChNPs, CAT activity was significantly elevated compared to the control (72.02 U mg^{-1} protein), highlighting its role in detoxifying ROS under high oxidative stress conditions. POD activity demonstrated a similar dose-dependent pattern, with lower activity at 10 and 20 ppm ChNPs (0.77 and 0.56 U mg^{-1} protein, respectively). In contrast, the highest activity was recorded at 160 ppm ChNPs (2.07 U mg^{-1} protein), representing a significant increase over the control (0.93 U mg^{-1} protein) (Table 1). This result suggests robust POD activation as a defense mechanism against ROS accumulation at elevated ChNP levels.

Secondary Metabolites

Based on Table 1, the total phenol (TPC), total flavonoid (TFC), and total anthocyanin (TAC) contents in *S. nemorosa* leaves were significantly influenced by varying concentrations of ChNPS. The TPC showed a dynamic response to ChNP exposure, with significant variations across treatments ($P \leq 0.05$). The highest TPC was observed at 20 ppm ChNPs (61.07 mg g^{-1} DW), representing a 6-fold increase compared to the control (10.02 mg g^{-1} DW). At 10 ppm ChNPs, TPC was also significantly elevated (50.43 mg g^{-1} DW). However, higher doses of ChNPs (40–160 ppm) led to a decline in TPC, with the lowest value recorded at 160 ppm (15.93 mg g^{-1} DW) (Table 1). This data suggests a dose-dependent modulation of phenolic biosynthesis, with an optimal response at moderate ChNP concentrations.

The TFC peaked at 10 ppm ChNPs (7.80 mg g^{-1} DW), reflecting a significant increase compared to the control (3.95 mg g^{-1} DW). At 20 ppm, TFC decreased slightly (5.12 mg g^{-1} DW) but remained higher than control levels. Beyond 20 ppm, TFC declined further, reaching the lowest value at 160 ppm (2.91 mg g^{-1} DW). These results indicate that low to moderate doses of ChNP positively regulate flavonoid accumulation, whereas higher concentrations may suppress flavonoid biosynthesis. Anthocyanin accumulation exhibited a distinct dose-dependent trend. The highest TAC was recorded at 40 ppm ChNPs (35.29 mg g^{-1} DW), representing a significant increase compared to the control (24.06 mg g^{-1} DW). Moderate enhancement was observed at 80 ppm (30.44 mg g^{-1} DW) and 160 ppm (28.56 mg g^{-1} DW) (Table 1). Conversely, lower doses of ChNPs (10 and 20 ppm) did not significantly affect TAC, with values comparable to the control. This pattern suggests that moderate ChNP does stimulate anthocyanin biosynthesis, possibly as part of a stress-adaptive response.

Enzymes in the Phenylpropanoid Pathway

The activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAT), key enzymes in the phenylpropanoid biosynthetic pathway, were significantly affected by different

doses of ChNPs (Fig. 5). PAL activity showed a dose-dependent response to ChNP treatment. The highest PAL activity was recorded at 20 ppm ChNPs, which exhibited a 4.8-fold increase compared to the control. PAL activity reached 5.98 U mg^{-1} protein at this dose, significantly higher than the control value of 1.25 U mg^{-1} protein ($P \leq 0.05$). At 10 ppm, PAL activity increased significantly, reaching 4.56 U mg^{-1} protein, a 264% enhancement relative to the control. However, at higher doses, PAL activity declined progressively. At 40, 80, and 160 ppm, the activities were 3.12, 2.67, and 1.84 U mg^{-1} protein, representing reductions of 48%, 55%, and 69%, respectively, compared to the peak activity at 20 ppm.

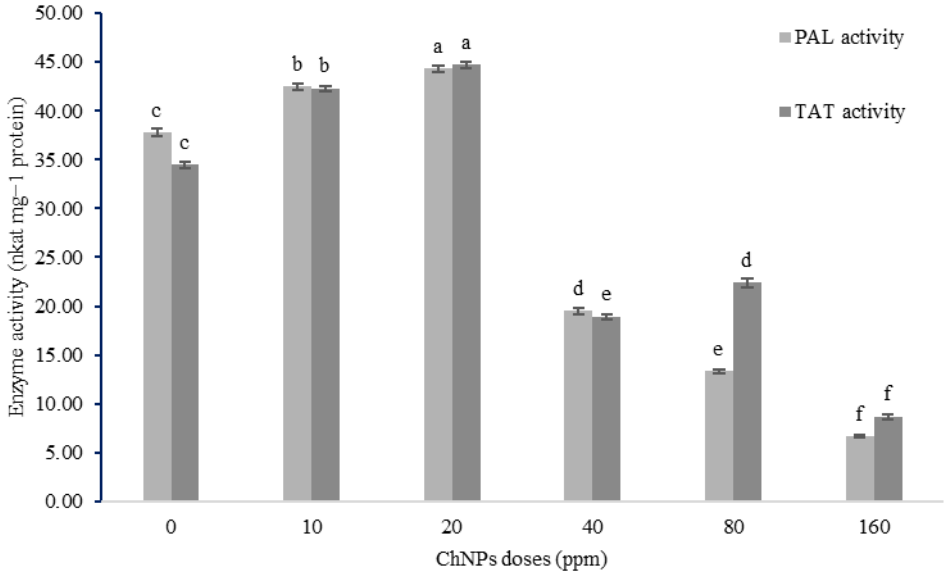

Fig. 5. The effect of different concentrations of ChNPs on the activity of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAT) in the leaves of *Salvia nemorosa* after two weeks of exposure. Values are given as means ± standard deviation (S.D.) from three biological replicates. Columns with different letters indicate significant differences at $P \leq 0.05$ (Duncan's test).

TAT activity displayed a similar trend, with notable increases at low to moderate ChNP doses. The maximum TAT activity was observed at 40 ppm, reaching 3.84 U mg^{-1} protein, a 2.8-fold increase compared to the control value of 1.37 U mg^{-1} protein ($P \leq 0.05$). At 10 and 20 ppm, TAT activities were 2.96 U mg^{-1} protein and 3.42 U mg^{-1} protein, representing 116% and 150% increases, respectively, over the control. However, at 80 and 160 ppm, TAT activity decreased to 2.31 U mg^{-1} protein and 1.72 U mg^{-1} protein, corresponding to 40% and 55% reductions from the peak activity at 40 ppm.

The results indicate that ChNP exposure modulates the PAL and TAT activities dose-dependently. Low to moderate ChNP doses (10–40 ppm) significantly enhanced the enzymatic activities, leading to increased biosynthesis of phenolic compounds, flavonoids, and anthocyanins. The most substantial enhancement in PAL activity (4.8-fold) occurred at 20 ppm, while TAT activity peaked (2.8-fold) at 40 ppm. Higher ChNP concentrations (80–160 ppm) led to a notable decline in both enzyme activities, likely due to stress-induced inhibition. These findings reveal the optimal doses of ChNPs for stimulating phenylpropanoid biosynthesis through PAL and TAT activation, providing insights into the regulatory effects of nanoparticles on plant secondary metabolism.

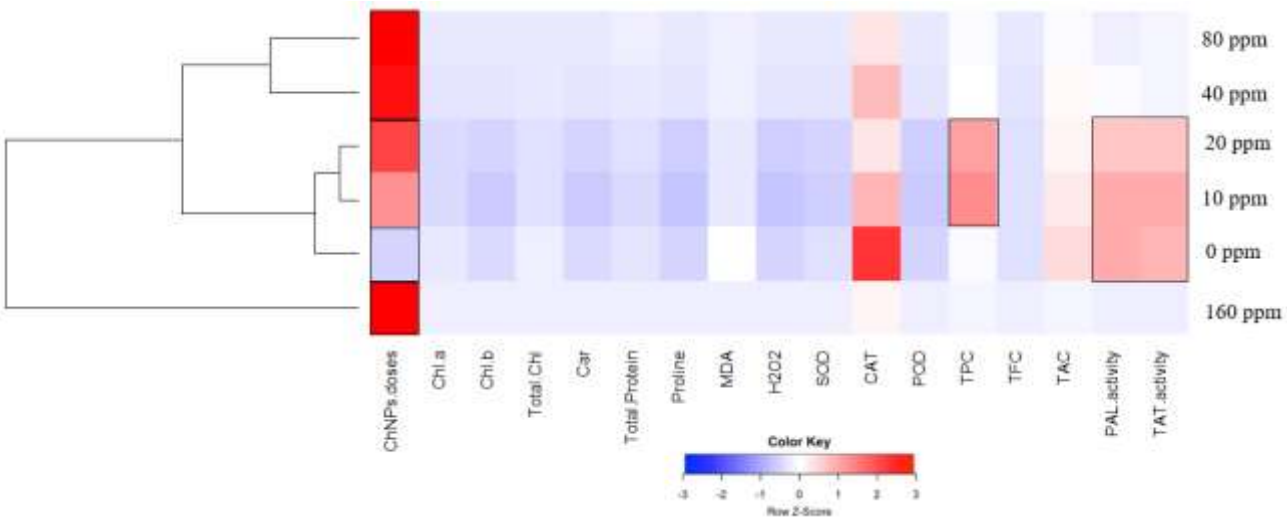

Fig. 6. Heatmap analysis and Pearson's correlation coefficient (ρ) calculated for the ChNP-treated and untreated samples. Heatmap compares the changes in all studied traits. The normalization procedure consisted of mean row-centering with color scales—*a*bbreviation: ChNPs doses, chitosan nanoparticles doses; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; PAL activity, phenylalanine ammonia-lyase activity; TAT activity, tyrosine aminotransferase activity.

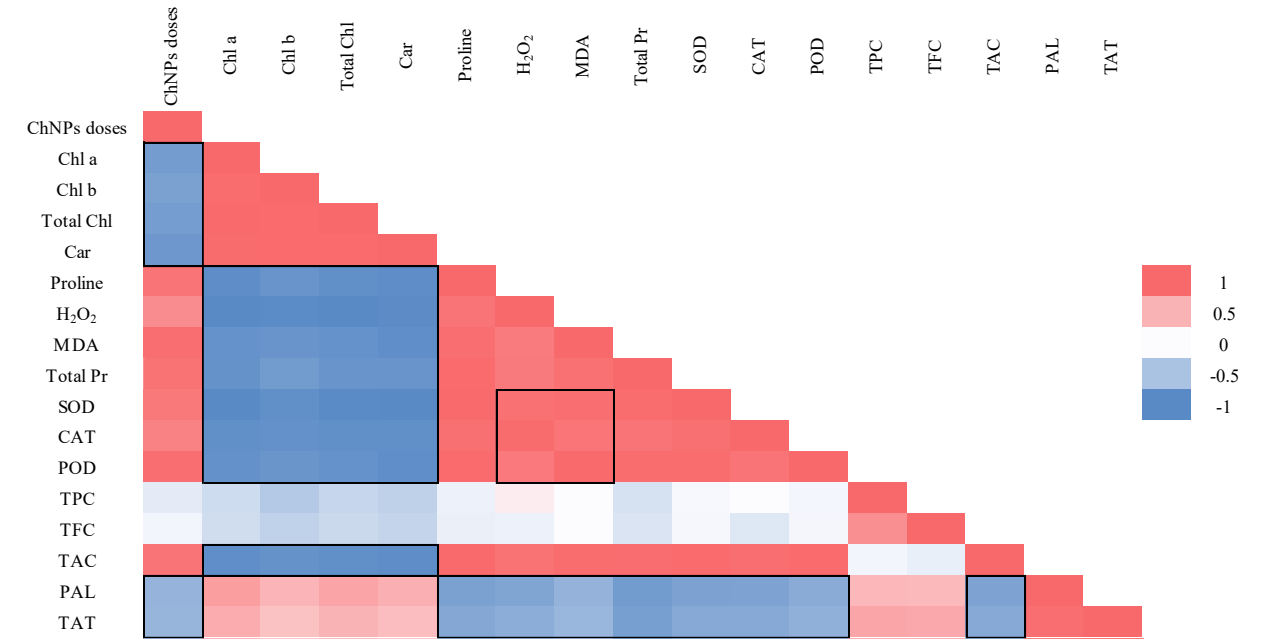

Fig. 7. Heatmap analysis showing the correlations between all studied parameters. Correlations coefficients were calculated based on Pearson's correlation. Red shows the positive effect, whereas blue indicates the negative effect. The color indication refers to the correlation coefficients—*a*bbreviation: ChNPs, chitosan nanoparticles; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; PAL activity, phenylalanine ammonia-lyase activity; TAT activity, tyrosine aminotransferase activity.

A heatmap was created to illustrate the physiological and biochemical responses of *S. nemorosa* to various ChNP concentrations (Fig. 6). This visual classification highlights four principal clusters, effectively distinguishing the plants based on their exposure levels and corresponding reactions. The first cluster represents the control group, comprising plants unexposed to ChNPs, indicating baseline physiological conditions. The second cluster includes those with low ChNP doses of 10 and 20 ppm, showing moderate physiological changes. Plants responding to moderate ChNP exposure levels of 40 and 80 ppm form the third cluster, where notable alterations in stress-related parameters emerge. The fourth and most distinct cluster contains plants exposed to the highest ChNP dose of 160 ppm, where substantial

physiological and biochemical disruptions were observed, indicating severe stress conditions. This clustering underscores the dose-dependent impact of ChNPs on *S. nemorosa*, emphasizing the need to carefully regulate nanoparticle applications to prevent adverse effects on plant health.

The analysis presented in Fig. 7 highlights a distinct relationship between ChNP concentrations and various plant defense mechanisms. As ChNP concentrations increase, enzymatic and non-enzymatic antioxidants exhibit the most potent positive correlations, indicating a pronounced dose-dependent response. Conversely, the levels of photosynthetic pigments such as chlorophyll display the most negative correlations with ChNP concentrations, suggesting a detriment to the photosynthetic system under nanoparticle stress. In addition, phenolic compounds, except for total anthocyanin content (TAC), generally decrease with higher ChNP exposure, reflecting a negative correlation. This data also shows a negative relationship between photosynthetic pigments and both categories of antioxidants and TAC. Notably, non-enzymatic antioxidants like hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and proline have a positive correlation with enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). Among these enzymes, strong positive correlations exist: SOD with CAT ($r_{0.01} = 0.958$) and POD ($r_{0.01} = 0.980$), and CAT with POD ($r_{0.01} = 0.934$). This complex interplay underscores the adaptive shifts in plant biochemistry under ChNP-induced stress.

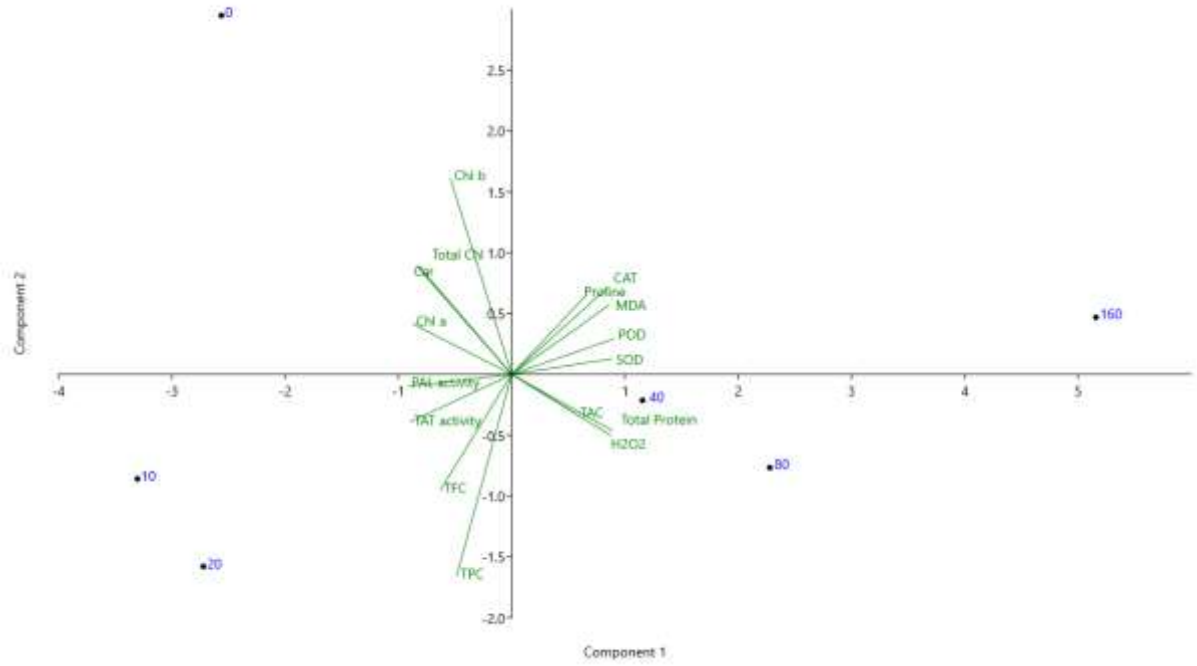

Fig. 8. PCA biplot of the analyzed responses of *S. nemorosa,* two weeks after exposure to different doses of ChNPs—*a*bbreviation: TPC, total phenol content; TFC, total flavonoid content; TAC, total anthocyanin content; Car, carotenoid; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; PAL activity, phenylalanine ammonia-lyase activity; TAT activity, tyrosine aminotransferase activity.

The Principal Component Analysis (PCA) of the effects of ChNPs on *S. nemorosa* highlights distinct treatment groupings based on the physiological and biochemical responses of the plants to varying nanoparticle doses (Fig. 8). The control group occupies the far-left region of the PCA plot, characterized by optimal photosynthetic conditions, high levels of chlorophyll and carotenoids, and minimal oxidative stress. This group represents the baseline, where no nanoparticle exposure has interrupted natural plant growth or metabolism.

Low concentrations of ChNPs (10 and 20 ppm) induce moderate oxidative stress, accompanied by a slight increase in H_2O_2 and MDA levels. These doses trigger a mild upregulation of antioxidant enzymes such as SOD, CAT, and POD, which is sufficient to mitigate cellular stress and maintain photosynthetic pigment levels. These responses indicate that the plants adapt effectively to these lower doses, with minimal disruption to photosynthesis.

At moderate doses (40 and 80 ppm), the oxidative stress becomes more pronounced, with substantial increases in H_2O_2 and MDA levels, suggesting higher levels of cellular damage. Antioxidant defenses are significantly upregulated to counteract this oxidative damage, characterized by heightened activity of SOD, CAT, and POD enzymes. However, photosynthetic pigments such as Chl *a*, -*b*, and carotenoids begin to decline, indicating that photosynthetic machinery is becoming compromised. These doses mark the transition from tolerable stress to more detrimental effects, as physiological and biochemical responses suggest the plants deal with a higher burden of nanoparticle-induced stress. The highest dose (160 ppm) induces severe oxidative stress, with the plants exhibiting extreme increases in oxidative stress markers and maximal activation of antioxidant defenses. Despite these efforts, the damage overwhelms the plants' capacity to adapt. Chlorophyll and carotenoid levels decrease drastically, signaling a breakdown in photosynthesis and overall metabolic function. Additionally, secondary metabolites such as proline and total proteins increase, reflecting the plants' desperate attempts to survive under extreme conditions.

This dose-dependent analysis underscores the increasing impacts of ChNPs, from moderate adaptive stress at low doses to significant oxidative damage and metabolic impairment at high doses. The 40-ppm dose, closer to the responses observed at 80 ppm, is a critical threshold where stress symptoms become markedly detrimental yet not as severe as those at the highest concentrations. These findings are essential for understanding the tolerable limits of ChNP applications and optimizing nanoparticle use in agriculture to augment plant resilience while minimizing adverse effects.

Discussion

The present study examined the dose-dependent effects of ChNPs on the physiological and biochemical parameters of *S. nemorosa*. The focus was on assessing how various concentrations of ChNPs influence photosynthetic pigments, oxidative stress markers, antioxidant enzyme activities, and secondary metabolites. This research is significant because it elucidates the dual role of ChNPs as beneficial stress mitigators at optimal doses and stress inducers at higher concentrations. Such insights are crucial for optimizing ChNP application in agriculture to enhance plant resilience while minimizing potential phytotoxic effects.

Photosynthetic Pigments

The observed dose-dependent reduction in Chl *a*, -*b*, and total chlorophyll content with increasing ChNP concentrations is consistent with the stress-induced disruption of photosynthetic machinery. Chlorophyll biosynthesis is a highly regulated process involving multiple enzymatic steps, including the conversion of protochlorophyllide to chlorophyllide by the enzyme protochlorophyllide oxidoreductase (POR) (Tanaka & Tanaka, 2007). High concentrations of ChNPs likely interfere with these enzymatic processes by directly inhibiting enzyme activity or inducing oxidative stress that damages the enzymes involved. The results indicated that rising levels of H_2O_2 and MDA correlate with decreased total chlorophyll content ($r_{0.01} = -0.951$ and $r_{0.01} = -0.880$ for H_2O_2 and MDA, respectively), likely due to oxidative damage to chloroplast membranes and thylakoid structures.

ChNPs influenced the levels of Chl *a*, -*b*, and total chlorophyll in a dose-dependent manner. Low concentrations (10 ppm) had negligible effects on Chl *a* level but slightly reduced Chl *b* and total chlorophyll, suggesting that minimal nanoparticle-induced stress did not disrupt the photosynthetic apparatus significantly. However, higher concentrations caused a pronounced

decline in chlorophyll content, likely due to oxidative damage and impaired chlorophyll biosynthesis. Chlorophyll biosynthesis is a highly regulated process involving multiple enzymatic steps, including the conversion of protochlorophyllide to chlorophyllide by the enzyme protochlorophyllide oxidoreductase (POR) (Tanaka & Tanaka, 2007). High concentrations of ChNP likely interfere with these enzymatic processes, either by directly inhibiting enzyme activity or inducing oxidative stress that damages the enzymes involved. Contrary to our findings, ChNPs have been shown to increase chlorophyll content and reduce ROS production under saline conditions (Balusamy et al., 2022).

The decrease in carotenoid content follows a similar trend, as carotenoids are integral components of the photosynthetic apparatus and are susceptible to oxidative degradation. Carotenoids play a dual role in photosynthesis; they act as accessory pigments for light harvesting and as antioxidants that protect chlorophyll from photo-oxidative damage (Hashimoto et al., 2016). Higher ChNP concentrations may overwhelm the antioxidant capacity of carotenoids, as indicated by their decreased levels in response to excessive ROS under stress conditions. This finding aligns with studies on other plant species, where nanoparticle exposure led to a decline in chlorophyll and carotenoid content due to oxidative stress (Azimi et al., 2021; Patel et al., 2020).

Oxidative Stress and Antioxidant Defense

Based on the results, ChNPs influenced oxidative stress markers and antioxidant enzyme activities in a dose-dependent manner, indicating a complex interplay between stress induction and the plant's defense mechanisms. In the current study, the levels of H_2O_2 and MDA increased progressively with higher ChNP doses, peaking at 160 ppm. This reflects elevated reactive oxygen species (ROS) production, which disrupts cellular homeostasis and causes lipid peroxidation. ROS generation is a well-documented response to nanoparticle exposure, as ChNPs can penetrate plant cells and interact with cellular components, inducing oxidative stress (Faizan et al., 2024). For instance, in *Phaseolus vulgaris*, high concentrations of ChNP resulted in elevated H_2O_2 levels and MDA content, corroborating our findings (Alenazi et al., 2024). Interestingly, moderate ChNP doses (10–40 ppm) induced less oxidative stress, with lower H_2O_2 and MDA levels than higher doses. This suggests that low-to-moderate concentrations of ChNPs are less disruptive to cellular redox balance, where ChNPs at 20 ppm enhance stress tolerance while minimizing oxidative damage.

The antioxidant defense enzymes, including SOD, CAT, and POD, play a vital role in mitigating ROS-induced damage. In this study, these enzymes exhibited a dose-dependent increase in activity, particularly at higher ChNP concentrations (80–160 ppm). SOD, which catalyzes the dismutation of superoxide radicals to H_2O_2, showed a significant increase at 80 ppm and peaked at 160 ppm. Similar trends were observed in barley (Behboudi et al., 2018) and wheat (Behboudi et al., 2019) under drought stress, where ChNPs enhanced SOD activity as a primary defense mechanism against ROS (Behboudi et al., 2018). Elevated SOD activity suggests that plants exposed to higher ChNP doses actively counteract excessive superoxide radicals.

CAT, responsible for decomposing H_2O_2 into water and oxygen, also showed the highest activity at 160 ppm. This aligns with studies on *Triticum aestivum*, where ChNPs stimulated CAT activity to detoxify ROS and prevent oxidative damage (Behboudi et al., 2019). However, at lower doses (10–20 ppm), CAT activity remained stable, indicating that H_2O_2 levels were within manageable limits for the plant's defense system. Likewise, POD, which catalyzes the reduction of H_2O_2 using phenolic compounds as substrates, demonstrated a similar dose-dependent trend. Its peak activity at 160 ppm suggests a strong activation of the enzymatic scavenging pathway under severe oxidative stress. Comparable findings were reported in *Solanum lycopersicum* under Cd stress, where ChNPs enhanced POD activity, particularly under high-stress conditions (Faizan et al., 2021).

Secondary Metabolites and Phenolic Biosynthesis

Pathway

Nano-chitosan significantly affected the biosynthesis of secondary metabolites, as evidenced by changes in TPC, TFC, TAC and the activities of key enzymes in the phenolic biosynthesis pathway, such as PAL and TAT. Low doses (e.g., 10–20 ppm) enhanced the accumulation of these compounds, improving stress resilience, while excessive doses had inhibitory effects. ChNPs appear to stimulate SOD activity as a primary defense mechanism against ROS and help plants ameliorate oxidative stress (Ji et al., 2022; Sadak et al., 2022).

Phenolic compounds are non-enzymatic antioxidants that combat the harmful effects of ROS and free radicals (Saleem et al., 2022). Flavonoids and anthocyanins, derivatives of phenolic compounds, also neutralize ROS, showcasing potent antioxidant properties. Phenolic compounds are non-enzymatic antioxidants that combat the harmful effects of ROS and free radicals. Flavonoids and anthocyanins, derivatives of phenolic compounds, also neutralize ROS, showcasing potent antioxidant properties (Saleem et al., 2022). Enhanced TPC, TFC, and TAC under low and moderate ChNP doses can be attributed to the activation of ROS signaling, which stimulates phenolic biosynthesis. Likewise, PAL and TAT, critical enzymes in the phenylpropanoid pathway, showed peak activities at 20 ppm, indicating increased metabolic flux toward phenolic compound biosynthesis. Similar findings in *Vitis vinifera* confirm the elicitor role of ChNPs in enhancing antioxidant enzyme activities and phenolic compound accumulation, boosting plant tolerance to abiotic stress (Panahirad et al., 2023).

The elicitor properties of ChNPs may stem from their interaction with plant cells, triggering signaling pathways that enhance secondary metabolite biosynthesis. However, excessive ChNPs appeared to disrupt cellular homeostasis and reduce metabolic activity. These results emphasize the biostimulant potential of ChNPs for sustainable agriculture, highlighting their capacity to enhance antioxidant defenses and stress tolerance when applied at optimal concentrations.

Dose-Dependent Effects of ChNPs

The dual role of ChNPs observed in this study underscores the importance of dose optimization. At low to moderate doses (10–40 ppm), ChNPs enhance stress tolerance by stimulating antioxidant defense systems and secondary metabolite production. However, excessive ROS generation overwhelms the defense mechanisms at high doses (80–160 pm), leading to cellular damage and metabolic disruption.

Conclusion

This study reveals the dual role of ChNPs in either promoting or impairing the growth and metabolism of *S. nemorosa*, depending on their concentration. on. At lower doses, ChNPs promote antioxidant defense, improve secondary metabolite production, and support plant resilience, while higher concentrations induce oxidative stress, reducing photosynthetic pigments and metabolites. This research emphasizes the importance of optimizing ChNP concentrations for enhancing plant growth and secondary metabolite accumulation, providing practical applications for medicinal plant cultivation. The findings also underscore the necessity of careful management when applying nanoparticles in agriculture to balance beneficial and detrimental effects on plant health and productivity.

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