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Nano-Chitosan as a Biotic Elicitor for Enhancing Secondary Metabolite Production in Basil Callus Cultures (Ocimum Basilicum L.)

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ABSTRACT: Ocimum basilicum L. is one of the important medicinal and aromatic plants, widely known for its high content of phenolic compounds and flavonoids with significant antioxidant properties. The present study was conducted to investigate the effect of nano-chitosan on biomass accumulation, phytochemical production and antioxidant activity in basil callus cultures. Callus cultures were induced using leaf explants cultured on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L 6-benzylaminopurine (BAP). The nano-chitosan was applied at 0, 5, 10, 25, 50, 75 and 100 mg L-1. After 6 weeks elicitation, biomass accumulation, total phenolic content, total flavonoid content, antioxidant activity and major phenolic compounds were assessed.

Nano-chitosan significantly boosted callus growth and phytochemicals. The maximum biomass, phenolic content and flavonoid content was observed at 50 mg/L nano-chitosan. Antioxidant activities of elicited cultures were determined by DPPH, ABTS and FRAP and showed significant increase. HPLC analysis showed an increase in the accumulation of rosmarinic acid, chicoric acid, caffeic acid, quercetin, kaempferol and apigenin after treatment with nano-chitosan. Correlation analysis revealed a positive correlation among phenolic content, flavonoid content and antioxidant activity.These results suggest that under carefully regulated in vitro conditions, nano-chitosan can be utilized as an efficient elicitor to enhance phytochemical output in basil callus cultures.The results suggest the possible use of nano-chitosan as a sustained elicitation approach for commercial phytochemical synthesis under controlled in vitro settings and offer important information for its application in medical plant biotechnology.

INTRODUCTION

Aromatic and medicinal plants are key sources of a range of bioactive compounds used in food, cosmetics, pharmaceuticals, and dietary supplements. These plants are characterized by their ability to produce a wide variety of secondary metabolites with antioxidant, antibacterial, anti-inflammatory, and even anticancer properties. These compounds include phenolic acids, flavonoids, alkaloids, and terpenoids (Nadeem et al., 2022).

Thanks to its rich chemical composition and distinctive medicinal properties, basil (Ocimum basilicum L.), belonging to the mint family (Lamiaceae), is among the most economically valuable medicinal plants. This is due to its content of numerous bioactive compounds such as , rosmarinic acid, and caffeic acid derivatives, all of which play a pivotal role in its health and biological effects.(Kwee & Niemeyer, 2011;Kiferle et al. 2011).

Despite basil's significant therapeutic potential, conventional cultivation often results in variations in phytochemical levels due to differences in agricultural and climatic conditions. Plant tissue culture offers a suitable solution to address this challenge. It provides a controlled environment that promotes the more stable synthesis of beneficial secondary metabolites. Callus cultures

are among the most commonly used methods in laboratory studies to stimulate the production of these compounds because of their rapid growth and ability to produce a wide variety of metabolites.(Espinosa-Leal et al., 2018; Chandran et al., 2020; Kolewe et al., 2008).

One effective method for promoting the accumulation of secondary metabolites in plant tissue cultures is the use of stimulation techniques. Stimuli contribute to increased synthesis of bioactive compounds such as phenols, flavonoids, and other essential metabolites by activating biological pathways responsible for the plant's defense response pathways (Ramakrishna & Ravishankar, 2011).Chitosan is one of the most extensively studied natural polymers as an effective biostimulant characterized by its biodegradability and compatibility with biological systems. Chitosan has demonstrated its ability to promote the formation of secondary metabolites, enhance antioxidant activity, and stimulate plant growth (Al-Hadrami et al., 2010).Furthermore, the recent innovation of nanochitosan, with its properties such as increased effective surface area and improved interaction with plant cells, has significantly enhanced its catalytic performance. Nanotechnology has become an effective means of improving chitosan's efficacy in promoting gene expression, plant defense mechanisms, and the accumulation rates of phytochemicals (Wang et al., 2024; Anjum et al., 2021).Documentary evidence supports the fact that nano-catalysts effectively enhance antioxidant responses and increase the production of bioactive compounds (Cao et al., 2025; Wang et al., 2024).Despite the successes achieved with nano-chitosan in many medicinal plants, research on its effects on basil callus cultures remains limited. Therefore, the current study aimed to analyze the effects of different nano-chitosan concentrations on the biomass growth of basil callus cultures, as well as to evaluate its impact on the synthesis of phenolic and flavonoid compounds, antioxidant activity, and the levels of essential phenolic compounds in this plant.

2. MATERIALS AND METHODS

2.1 Plant Material and Culture Establishment

We bought Ocimum basilicum L. seeds from a commercial seller. Following a complete 15-minute wash under running water, the seeds were surface sterilized for 1 minute using 70% ethanol and then for 15 minutes using a 2% sodium hypochlorite solution that contained Tween-20. After being rinsed five times with sterile distilled water, the sterilized seeds were grown on hormone-free MS medium (Murashige & Skoog, 1962).

Cultures were maintained at 25 ± 2°C using a 16-hour light/8-hour dark photoperiod. Four-week-old seedlings were used to create leaf explants.

2.2 Preparation of Nano-Chitosan

Nano-chitosan particles were prepared using a modified ionic gelation method based on the procedure described by Calvo et al. (1997). Chitosan was dissolved in 1% acetic acid and then mixed with sodium tripolyphosphate (TPP) while continuously stirring. The resulting nanoparticles were collected by centrifugation, washed with distilled water, and freeze-dried before to use.

2.3 Characterization of Nano-Chitosan

Nano-chitosan particles were characterized using Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) to determine their functional groups, morphology, and particle size.

2.4 Callus Induction

Leaf explants were grown in MS medium supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L BAP. The medium contained 8 g/L agar and 30 g/L sucrose. The pH was adjusted to 5.8 before autoclaving. A total of sixty explants were grown and evaluated after four weeks.

2.5 Nano-Chitosan Treatments

Fresh MS medium containing 0, 5, 10, 25, 50, 75, and 100 mg/L nano-chitosan was added to actively developing calli. There were five replicates of each treatment, each containing three callus fragments.

2.6 Biomass Determination

After six weeks of elicitation, fresh and dry biomass were measured.

2.7 Total Phenolic Content

The Folin-Ciocalteu technique was used to calculate the total phenolic content (Singleton et al., 1999).

2.8 Total Flavonoid Content

The aluminum chloride colorimetric test was used to quantify the total flavonoid content.

2.9 Antioxidant Activity

DPPH (Brand-Williams et al., 1995), ABTS (Re et al., 1999), and FRAP (Benzie & Strain, 1996) assays were used to measure antioxidant activity.

2.10 HPLC Analysis

Reverse-phase HPLC was used to quantify major phenolic constituents, such as rosmarinic acid, chicoric acid, caffeic acid, ferulic acid, quercetin, kaempferol, and apigenin.A reverse-phase C18 column (250 × 4.6 mm, 5 µm particle size) was used for the HPLC study. Using a gradient elution program, solvent A (0.1% formic acid in water) and solvent B (acetonitrile) made up the mobile phase. Compounds were detected at 280 nm while the flow rate was kept at 1.0 mL/min. Retention periods of verified standards and comparison with published chromatographic profiles served as the basis for identification.

2.11 Statistical Analysis

The data was expressed as the mean ± standard deviation (SD) of five separate replicates. The Shapiro-Wilk test was used to verify that the data distribution was normal prior to statistical analysis. Treatment differences were examined using the Least Significant Difference (LSD) test at $P \leq 0.05$ and one-way analysis of variance (ANOVA). Pearson correlation analysis was used to evaluate the relationships between phytochemical and antioxidant indicators. Statistical analyses were conducted using SPSS software version 26.

3. Results and Discussion

3.1 Callus Induction and Characterization of Nano-Chitosan

Leaf explants grown on MS medium supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L BAP successfully generated callus tissues within 10–14 days of incubation. The induced calli were primarily friable to semi-compact and exhibited a greenish-yellow coloration. These characteristics indicated actively growing callus tissues suitable for subsequent secondary metabolite production studies.

Table 1. Ocimum basilicum Explants' Callus Induction Efficiency

Parameter	Value (%)
Callus induction	86.4
Survival rate	84.7
Browning rate	8.3
Contamination rate	5.6

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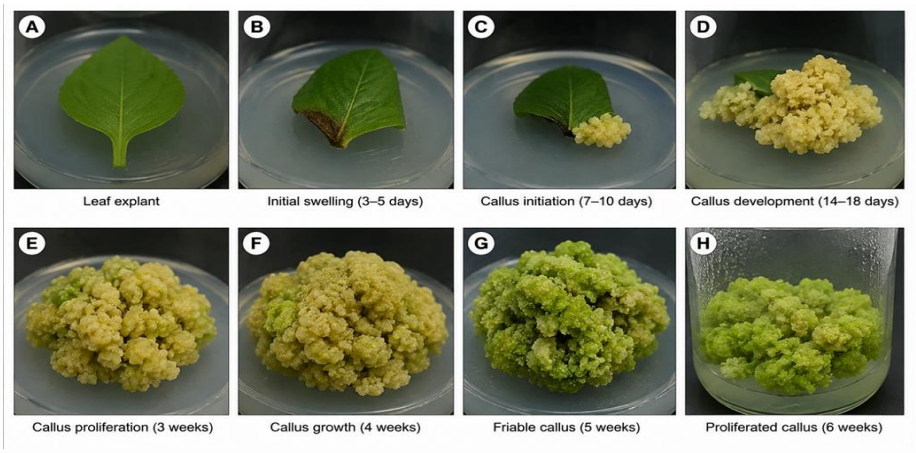

Figure 1. Ocimum basilicum leaf explants grown on MS media supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L BAP showed varying phases of callus induction and proliferation.

The relatively high frequency of callus induction observed in the present study suggests that the selected combination of auxin and cytokinin effectively promoted cellular dedifferentiation and callus proliferation. Callus induction and growth in Ocimum basilicum have been reported to depend strongly on the type and concentration of plant growth regulators used in the culture medium. The low percentages of contamination and browning observed in the present study further indicate the effectiveness of the surface-sterilization procedure and the established culture conditions.

Scanning Electron Microscopy (SEM)

The majority of the nano-chitosan particles were spherical, with sizes ranging from roughly 50 to 85 nm, according to SEM examination. With very little aggregation, the majority of particles seemed to be evenly scattered.

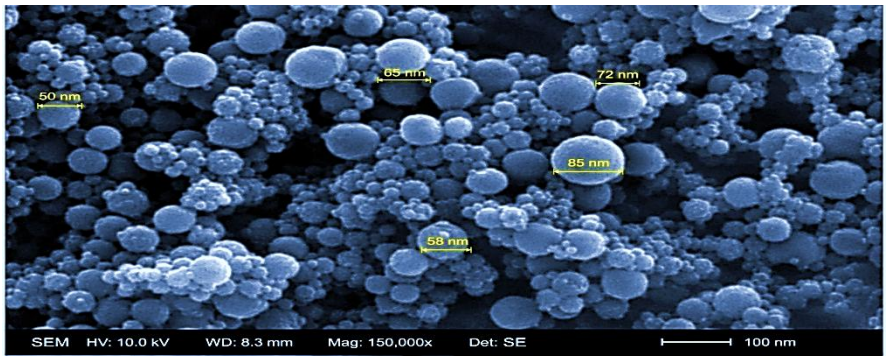

Figure 2.A scanning electron micrograph of spherically shaped nano-chitosan particles.

The effective production of nano-chitosan particles with primarily spherical morphology and particle sizes ranging from 50 to 85 nm (average ≈ 66 nm) was validated by SEM examination. A small amount of aggregation was seen, which is normal for chitosan nanoparticles because of interactions between molecules. The vast surface area provided by the nanoscale dimensions may promote secondary metabolite production and elicitor activity in basil callus cultures.

Fourier Transform Infrared Spectroscopy (FTIR)

The hydroxyl, amino, amide, and carbonyl functional groups were represented by distinctive absorption bands in the FTIR spectra at roughly 3428, 2924, 1651, 1542, 1384, and 1075 cm^{-1}.

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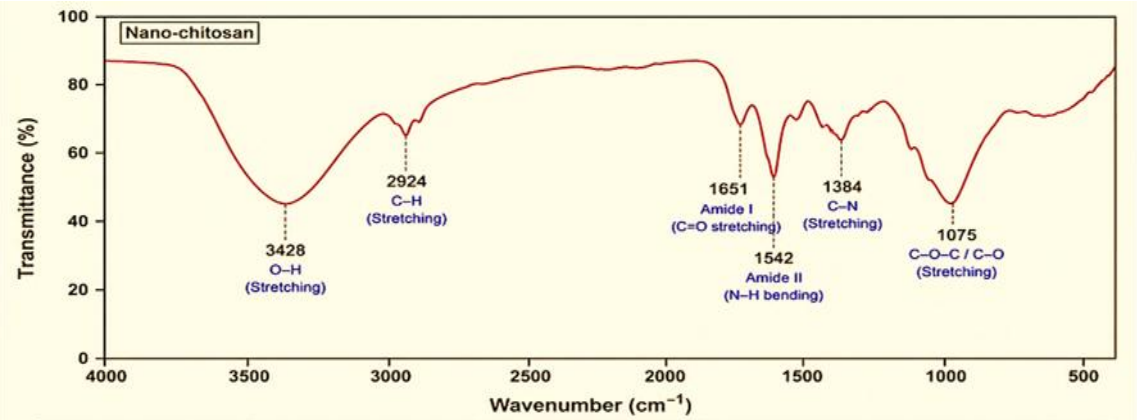

Wavenumber (cm^{-1})	Functional group	Vibrational mode	Assignment
3428	O–H	Stretching	Hydroxyl group (–OH)
2924	C–H	Stretching	Aliphatic C–H
1651	Amide I (C=O)	Stretching	Amide carbonyl (C=O stretching)
1542	Amide II (N–H)	Bending	N–H bending and C–N stretching
1384	C–N	Stretching	C–N stretching (amine)
1075	C–O–C / C–O	Stretching	C–O–C glycosidic linkage / C–O stretching

Figure 3. FTIR spectrum of nano-chitosan particles.

The successful formation of nano-chitosan particles was confirmed by SEM analysis, which revealed spherical to irregular nanoparticles with sizes ranging from 50 to 85 nm. FTIR analysis confirmed the presence of characteristic chitosan functional groups after nanoparticle formation. Similar morphological characteristics and FTIR profiles have been reported for chitosan nanoparticles prepared using ionic gelation methods. The nanoscale dimensions of the particles may enhance their interaction with plant cells and thereby improve their elicitation efficiency.

3.2 Effect of Nano-Chitosan on Biomass Accumulation

In basil callus cultures, nano-chitosan had a major impact on biomass accumulation. Up to 50 mg/L of elicitor, fresh and dry biomass grew gradually before somewhat declining at higher concentrations.

Table 2. Effect of Nano-Chitosan on Biomass Accumulation

Treatment	Dry Weight (g/L)	Fresh Weight (g/L)
Control	12.6 ± 1.1 d	194.3 ± 9.1 d
NC5	15.8 ± 1.3 cd	226.5 ± 10.4 cd
NC10	18.7 ± 1.5 c	261.8 ± 11.6 c
NC25	22.8 ± 1.7 b	309.7 ± 12.5 b
NC50	26.4 ± 1.8 a	348.6 ± 13.2 a
NC75	24.9 ± 1.6 ab	332.1 ± 12.8 ab
NC100	21.5 ± 1.4 bc	291.5 ± 11.7 bc

The values show the mean ± SD (n = 5). Significant differences at $P < 0.05$ are indicated by different letters.

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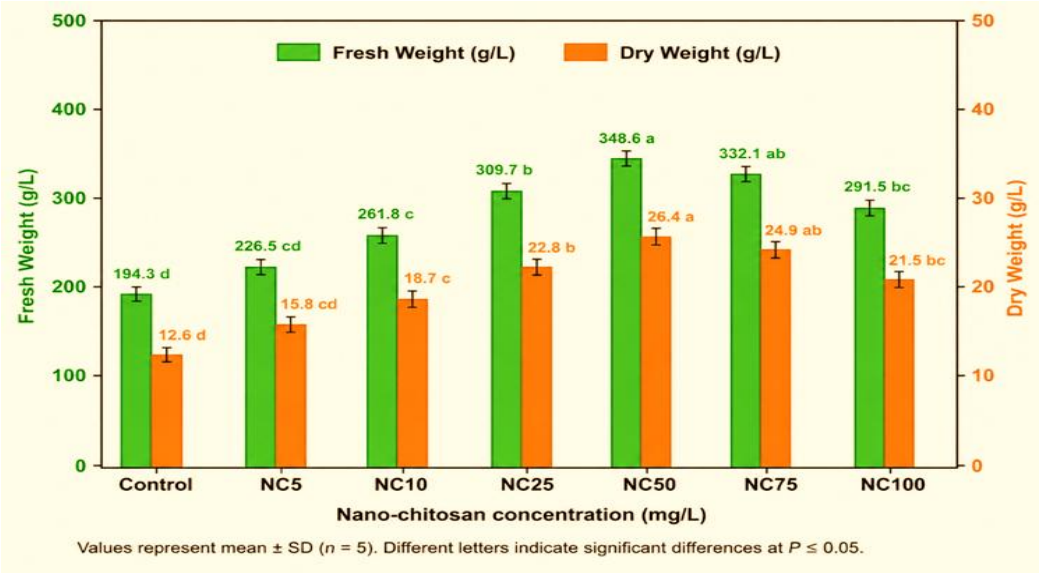

Figure 4. The buildup of fresh and dry biomass in basil callus cultures is affected by the concentration of nano-chitosan.

Compared with the untreated control, nano-chitosan markedly increased biomass accumulation. The 50 mg/L treatment produced the highest fresh and dry weights, indicating that this concentration was optimal for growth promotion under the conditions of the present study. The slight decrease in biomass observed at 75 and 100 mg/L may indicate a concentration-dependent physiological response, in which higher elicitor levels impose additional stress and may redirect cellular resources toward defense and secondary metabolism. Nevertheless, biomass values at all tested nano-chitosan concentrations remained higher than those of the control, indicating an overall positive effect of nano-chitosan on callus growth. Similar concentration-dependent effects of nano-chitosan on plant growth and biomass accumulation have been reported, with moderate concentrations promoting growth while higher concentrations may exert inhibitory effects (Chaudhary et al., 2021; Asgari-Targhi et al., 2018).

3.3 Effect of Nano-Chitosan on Total Phenolic and Flavonoid Contents

In basil callus cultures, nano-chitosan elicitation markedly increased the accumulation of total phenolic and flavonoid chemicals.

Table 3. Effect of Nano-Chitosan on Total Phenolic and Flavonoid Contents

Treatment	TFC (mg QE/g DW)	TPC (mg GAE/g DW)
Control	8.9 ± 0.7 e	15.8 ± 1.3 e
NC5	11.1 ± 0.9 d	19.4 ± 1.6 de
NC10	13.8 ± 1.1 cd	23.6 ± 1.9 cd
NC25	17.4 ± 1.4 bc	28.4 ± 2.2 b
NC50	21.7 ± 1.6 a	33.8 ± 2.5 a
NC75	19.8 ± 1.5 ab	30.7 ± 2.3 ab
NC100	16.5 ± 1.3 c	26.9 ± 2.1 bc

The values show the mean ± SD (n = 5). Significant differences at $P < 0.05$ are indicated by different letters.

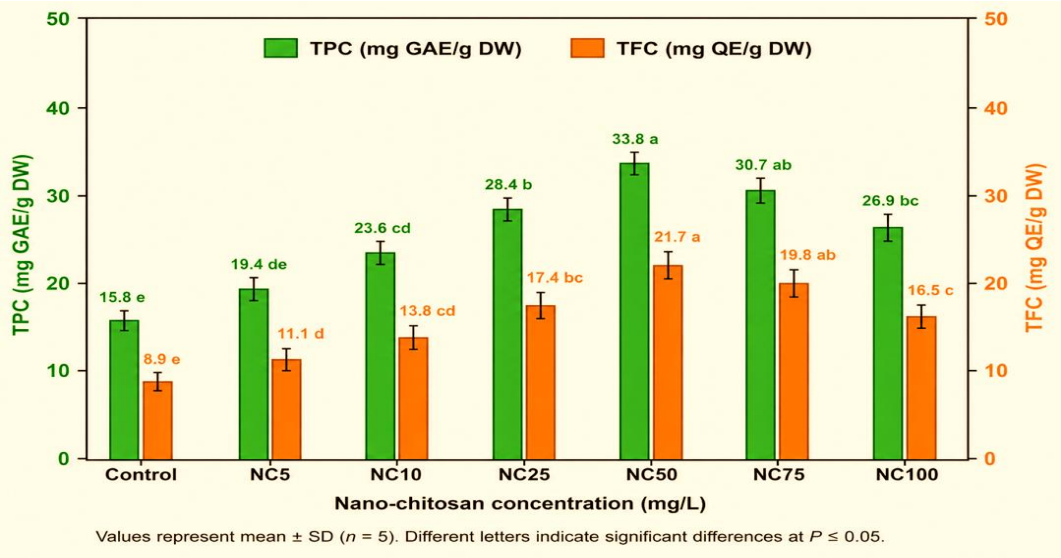

Figure 5. Total phenolic content (TPC) and total flavonoid content (TFC) in basil callus cultures are affected by the concentration of nano-chitosan.

Following nano-chitosan treatment, a considerable increase in total phenolic and flavonoid contents was observed. Compared with the untreated control, TPC and TFC approximately doubled at the optimum concentration of 50 mg/L. This enhancement may be associated with elicitor-induced activation of plant defense signaling and phenylpropanoid metabolism, which can promote the biosynthesis and accumulation of phenolic and flavonoid compounds. The decline observed at higher nano-chitosan concentrations may reflect an excessive elicitation response that imposes physiological stress and alters cellular metabolic balance. Nevertheless, metabolite levels remained higher than those of the untreated control, indicating the effectiveness of nano-chitosan as an elicitor for secondary metabolite production. Similar elicitor-dependent increases in phenolic and flavonoid accumulation have been reported in medicinal plant cultures, where elicitation activated defense-related pathways and enhanced secondary metabolite biosynthesis (Kianersi et al., 2022; Jain et al., 2024).

3.4 Effect of Nano-Chitosan on Antioxidant Activity

The antioxidant activity of basil callus extracts was greatly increased by nano-chitosan elicitation. Ferric reducing antioxidant power (FRAP), ABTS radical scavenging capacity, and DPPH radical scavenging activity all showed comparable response patterns.

Table 4. Antioxidant Activity of Basil Callus Extracts

Treatment	DPPH (%)	FRAP (µM TEAC)	ABTS (µM TEAC)
Control	55.9 ± 3.1 d	278 ± 15 e	312 ± 17 e
NC5	64.8 ± 3.8 cd	341 ± 18 d	381 ± 20 d
NC10	72.5 ± 4.2 c	397 ± 21 c	446 ± 23 c
NC25	82.7 ± 4.5 b	486 ± 24 b	554 ± 28 b
NC50	90.3 ± 5.0 a	603 ± 27 a	671 ± 31 a
NC75	91.1 ± 5.2 a	615 ± 28 a	688 ± 33 a
NC100	81.6 ± 4.6 b	509 ± 23 b	575 ± 26 b

The values show the mean ± SD (n = 5). Significant differences at $P < 0.05$ are indicated by different letters.

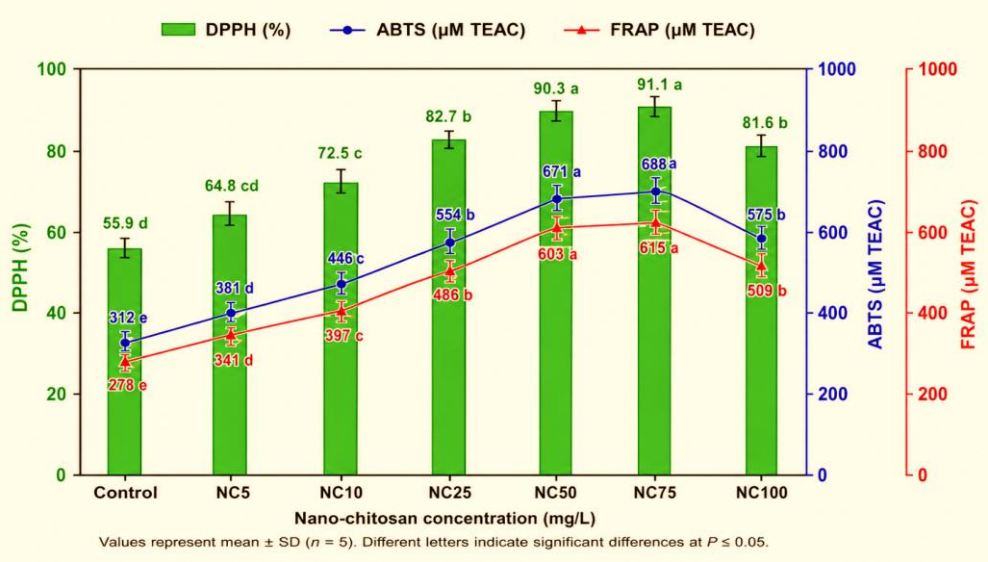

Figure 6. The values show the mean ± SD (n = 5). Significant differences at P < 0.05 are indicated by different letters.

Compared with the untreated control, nano-chitosan treatment markedly enhanced antioxidant activity. The highest antioxidant activities were observed at NC50 and NC75, which also showed increased total phenolic and flavonoid contents. This pattern suggests a possible association between the accumulation of phenolic and flavonoid compounds and the enhanced antioxidant capacity of the elicited cultures. Phenolic compounds and flavonoids are important contributors to antioxidant activity because of their ability to scavenge free radicals and participate in redox reactions. Accordingly, the higher levels of these secondary metabolites observed in nano-chitosan-treated cultures were consistent with the increased DPPH, ABTS, and FRAP values. The agreement among the three antioxidant assays indicates an overall enhancement of free-radical-scavenging and reducing capacity following nano-chitosan elicitation.

3.5 HPLC Analysis of Major Phenolic Compounds

HPLC analysis was used to further examine the impact of nano-chitosan on the accumulation of key phenolic chemicals. The majority of the examined compounds showed notable increases after being treated with nano-chitosan.

Table 5. HPLC Quantification of Major Phenolic Compounds in Basil Callus Cultures

Compound	Control (mg/g DW)	NC50 (mg/g DW)
Rosmarinic acid	8.24 ± 0.48	20.71 ± 1.19
Chicoric acid	1.12 ± 0.08	3.94 ± 0.26
Caffeic acid	0.68 ± 0.05	2.79 ± 0.18
Ferulic acid	0.49 ± 0.04	1.88 ± 0.14
Quercetin	0.43 ± 0.03	1.69 ± 0.11
Kaempferol	0.36 ± 0.03	1.37 ± 0.10
Apigenin	0.19 ± 0.02	0.91 ± 0.07

Values represent mean ± SD (n = 5).

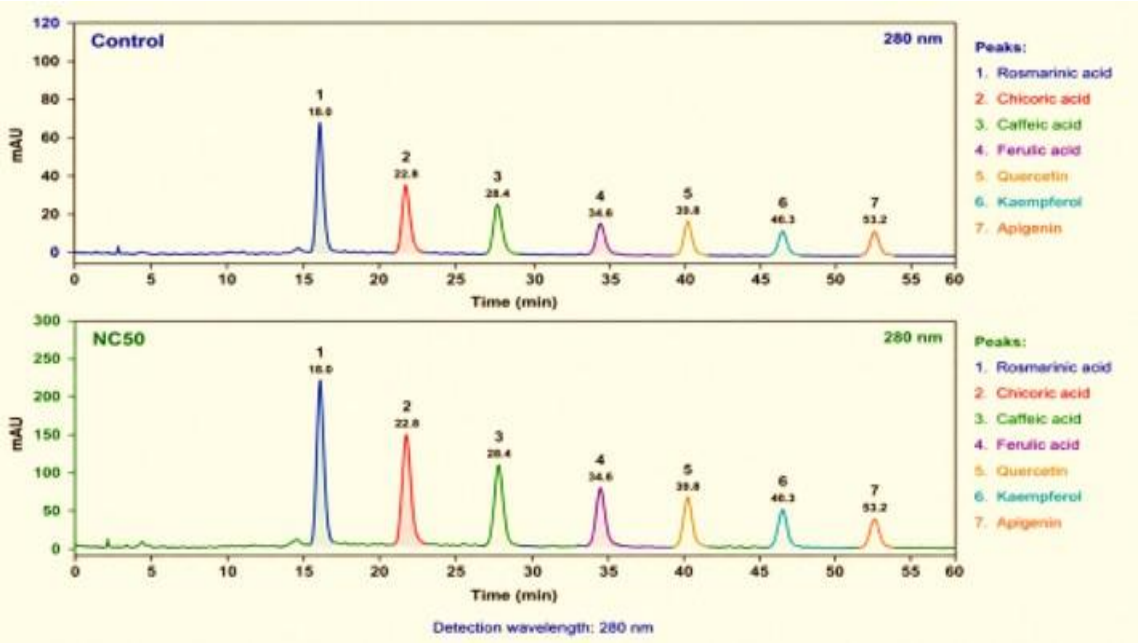

Figure 7.HPLC chromatograms of Ocimum basilicum callus cultures treated with NC50 and control.

Rosmarinic acid was the most abundant phenolic compound detected in both nano-chitosan-treated and untreated callus cultures. Treatment with nano-chitosan significantly increased the concentration of rosmarinic acid, reaching approximately 2.5-fold that of the untreated control at the optimal treatment level. This finding is consistent with previous studies showing that chitosan elicitation can enhance rosmarinic acid accumulation in Ocimum basilicum and stimulate phenylalanine ammonia-lyase (PAL) activity, an important enzyme in the phenylpropanoid pathway (Chibu et al., 2006; Kiferle et al., 2011). Significant increases were also observed in other phenolic compounds, including chicoric acid, caffeic acid, ferulic acid, quercetin, kaempferol, and apigenin. These compounds are important secondary metabolites that contribute to the biological and antioxidant properties of basil. Overall, the HPLC results demonstrated that nano-chitosan elicitation enhanced the accumulation of phenolic compounds and other bioactive phytochemicals in basil callus cultures. The pronounced increase in rosmarinic acid further suggests that nano-chitosan may stimulate phenylpropanoid metabolism in O. basilicum callus cultures.

3.6 Correlation Analysis

The associations between phenolic content, flavonoid content, and antioxidant activity were assessed using Pearson correlation analysis.

Table 6. Pearson Correlation Coefficients Among Measured Parameters

Parameter	TPC	TFC	DPPH	ABTS	FRAP
TPC	1.000	0.89	0.85	0.94	0.92
TFC	0.89	1.000	0.79	0.84	0.82
DPPH	0.85	0.79	1.000	0.88	0.86
ABTS	0.94	0.84	0.88	1.000	0.93
FRAP	0.92	0.82	0.86	0.93	1.000

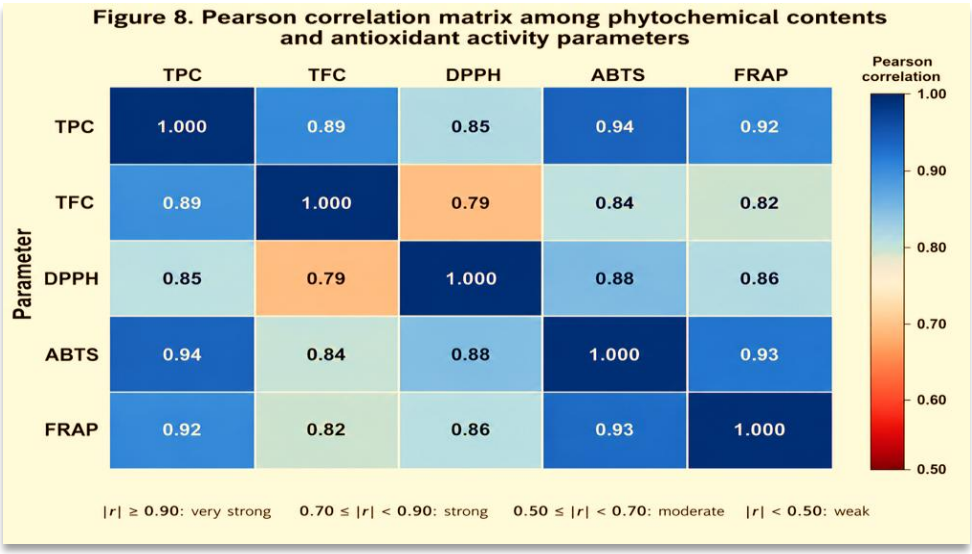

Figure 8.A Pearson correlation matrix shows the relationships between total phenolic content, total flavonoid content, and antioxidant activity traits.

Strong positive correlations were observed among total phenolic content, total flavonoid content, and antioxidant activity. The strongest correlation was observed between total phenolic content and ABTS activity (r = 0.94), followed by a strong correlation between total phenolic content and FRAP activity (r = 0.92). These results suggest that the accumulation of phenolic compounds contributed substantially to the enhanced antioxidant capacity of basil callus extracts. Similar associations between increased phenolic accumulation and enhanced antioxidant activity have been reported in medicinal plant cultures and elicitation studies. The positive correlations among the measured biochemical parameters further support the conclusion that nano-chitosan elicitation improved the biochemical quality of basil callus cultures and was associated with enhanced secondary metabolism, particularly phenylpropanoid-related metabolism.

4. CONCLUSION

The present study demonstrates that nano-chitosan is an effective biostimulant for enhancing biomass accumulation and phytochemical production in callus cultures of Ocimum basilicum L. Compared with untreated cultures, nano-chitosan treatments increased fresh and dry biomass, total phenolic content, total flavonoid content, and antioxidant activity, with the most pronounced effects observed at the optimal treatment concentration. Significant positive associations between phenolic and flavonoid contents and antioxidant activity further indicate that the enhanced accumulation of these secondary metabolites contributed to the improved biochemical properties of the treated cultures. The marked increase in rosmarinic acid and other phenolic compounds also suggests that nano-chitosan elicitation may stimulate phenylpropanoid-related metabolism in basil callus cultures. Overall, nano-chitosan represents a promising, environmentally friendly, and sustainable elicitor for enhancing the production of bioactive phytochemicals in O. basilicum callus cultures, with potential applications in plant biotechnology and the production of valuable secondary metabolites.

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