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Effect of chitosan on *in vitro* growth and bioactive metabolite production in *Lavandula angustifolia* Mill.

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Abstract

Objective

Low production of bioactive metabolites remains one of the major limitations in commercial production of *Lavandula angustifolia.* Chitosan has been proposed as an effective elicitor for enhancing secondary metabolite biosynthesis under *in vitro* conditions. This study aimed to evaluate the effect of chitosan, a biological derivative of chitin, on the growth and active compound content of lavender plants cultured *in vitro*.

Materials and methods

A completely randomized design with four chitosan concentrations (0, 50, 100, and 200 mg L^{-1}) was used. Moreover, ten biological replicates per treatment were used for *in vitro* cultured *Lavandula angustifolia* plantlets. Vegetative growth parameters, including plant height, branch number, leaf number, fresh weight, and dry weight were recorded, after four weeks of culture. GC–MS was used for analysing volatile metabolites and HPLC was applied for quantifying phenolic compounds.

Results

The results of the current study showed that plant growth and metabolite accumulation are significantly influenced by chitosan ($p < 0.05$). Using chitosan (100 mg L^{-1}) produced the highest biomass, except for number of branches that the greatest number of branches was recorded at 50 mg L^{-1}. This treatment, in comparison with the control increased plant height (90%), fresh weight (199%), and dry weight (142%). At this concentration, the highest levels for quercetin, rosmarinic acid, luteolin, apigenin, caffeic acid, and ferulic acid were 95.6 ppm, 90.5 ppm, 88.7 ppm, 83.9 ppm, 81.4 ppm, and 70.6 ppm, respectively. In contrast, 50 mg L^{-1} chitosan induced the accumulation of 2-nitroethyl propionate, whereas 200 mg L^{-1} reduced both growth and phenolic accumulation, indicating inhibitory effects at higher concentrations.

Conclusion

The current research showed that chitosan can successfully increase the growth and secondary metabolite production of *Lavandula angustifolia* cultured *in vitro* in a concentration-dependent manner. Overall, 100 mg L^{-1} was the optimum concentration because it produced the highest biomass and phenolic accumulation, although the maximum branch number was observed at 50 mg L^{-1}. Our results showed that supplementing *in vitro* culture media with 100 mg L^{-1} chitosan can be used as a practical strategy for improving the production of valuable bioactive compounds in lavender. For validation of the underlying mechanisms and support commercial application, it would be better to perform further molecular and greenhouse studies.

Key words: active compounds, chitosan, *in vitro*, lavender

Paper Type: Research Paper.

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Introduction

Lavender biologically classified as *Lavandula angustifolia* Mill is belongs to the Lamiaceae family (Lamiaceae), which comprises approximately 39 species (Sayafi et al., 2025). These aromatic plants were characterized by its grayish-green leaves and purple flowers, which contain a complex mixture of fragrant and biologically active volatile compounds (Prusinowska et al., 2014). Lavender originated in the Mediterranean region (Demasi et al., 2018; de Melo Alves Silva et al., 2023) now widely cultivated worldwide (Husseini et al., 2016; Demasi et al., 2018).

Recently, extraction methods and techniques using natural compounds have been widely studied and considered as a useful and effective strategy for increasing the production of secondary metabolites in medicinal plants cultivated *in vitro*. Among them, chitosan has been widely investigated. Chitosan is a biodegradable diacetylated derivative of chitin. Chitin has a high ability to stimulate plant defense responses and activate biosynthetic pathways related to phenolic compounds, flavonoids and other bioactive metabolites. The results of various studies have shown that chitosan supplementation increases the accumulation of rosmarinic acid and phenolic compounds in medicinal plants such as lemon balm (Melissa officinalis) and basil (Ocimum basilicum) (Kim et al., 2005; Fooladi Vanda et al., 2019). This supplement also improves biomass production at optimal concentrations. Chitosan can increase the biosynthesis of secondary metabolites in laboratory cultures of several medicinal plants (Sun et al., 2023). This supplement does this by activating defense-related signaling pathways, including jasmonic acid-mediated responses and phenylpropanoid metabolism. However, factors such as plant species, culture conditions, and elicitor concentration affect the response of medicinal plants to chitosan. In addition, excessive levels potentially inhibit growth. However, information on the effects of chitosan on biomass accumulation and targeted production of bioactive compounds in laboratory cultures of lavender (*Lavandula angustifolia*) is very limited. Lavender is considered an important natural source of secondary metabolites, especially for pharmaceutical and therapeutic applications (Chianese et al., 2023). However, its cultivation in Iraq remains challenging due to the harsh environmental conditions, including extreme temperature and heat stress. These stresses negatively impact plant growth and productivity and may result in crop failure. Under such condition, plant tissue culture techniques provide a promising solution to support lavender propagation and restoration, helping to overcome the limitations imposed by desertification, elevated temperature and degraded soils associated with global warming (Viana et al., 2022). *In vitro* culture systems enable the efficient production of lavender independent of geographical constraints, thereby increasing plant availability and uniformity (Neumann et al., 2020). Compared with mother plants, cell cultures offer the advantage of continuous, year-round production of secondary metabolites under well-controlled environmental conditions, ensuring consistent quality, purity and yield (Alrawi et al., 2022). Furthermore, metabolite accumulation can be enhanced by supplementing nutrient media with biotic or chemical elicitors (Abed et al., 2020), a widely used strategy in plant biotechnology. Among natural biotic elicitor, chitosan had been shown to effectively stimulate secondary metabolites production in a wide range of medicinal plant species (Fooladi Vanda et al., 2019). Therefore, this study aimed to investigate the effect of different chitosan concentrations on *in vitro* growth, phenolic accumulation, volatile metabolites, and biomass production of *Lavandula angustifolia*, and to identify the optimum elicitor concentration.

Materials and methods

Seed source and germination: Lavender Seeds were obtained from Wadi seeds, a specialized production seed enterprise located in the southern region of Iraq. Upon acquisition,

the seeds were transported and also stored under controlled laboratory conditions at the Plant Tissue Culture Laboratory, Center of Desert Studies, University of Anbar, until further use. Germination was conducted at a constant temperature of 25 ± 1°C under aseptic conditions. For the germination process, a basal culture medium consisting of half-strength Murashige and Skoog (½ MS) salts, supplemented with 0.5 mg L^{-1} gibberellic acid (GA_3) and 7 g L^{-1} agar, was prepared. Moreover, all components were dissolved in distilled water, in addition to the pH was adjusted to 5.7 using 1 N NaOH or HCl before sterilization. The medium was gently heated to achieve full dissolution, dispensed into glass culture tubes (10 mL per tube), sealed with sterile caps and sterilized in an autoclave at 121°C, 1.04 kg cm^{-2} for 20 minutes. Moreover, after sterilization, the tubes were positioned in a slanted orientation to provide a larger surface area for seed placement (Miclea et al., 2018). Surface sterilization of the seeds was carried out using 3% sodium hypochlorite (NaOCl) for 15 minutes, followed by three rinses with sterile distilled water (3 minutes each rinse), without the addition of ethanol or Tween-20. Notably, under a laminar airflow hood, sterilized seeds were aseptically placed on the surface of the prepared germination medium (100 tubes in total). The cultures were later transferred to a controlled growth chamber maintained at 25 °C, a 16-hour photoperiod, in addition to a light intensity of 40–60 μmol m^{-2} s^{-1}. Germination commenced within two weeks. After four weeks (March 1, 2025), the germination percentage was recorded. Selection of seedlings for subsequent *in vitro* propagation was performed based on uniform morphological characteristics, including similar plant height, leaf development, and overall vigor. For minimizing experimental variation, seedlings with abnormal growth, contamination, or developmental differences were not used for further study. (Sekhi et al., 2025).

Establishment and preparation of multiplication medium: For the shoot multiplication phase, a culture medium was prepared using full-strength MS salts (4.74 g L^{-1}) supplemented with two plant growth regulators: 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyl adenine (BA) at concentrations of 0.5 mg L^{-1} in addition to 2 mg L^{-1}, respectively. Because initial optimization experiments and previous reports had shown that low concentrations of 2,4-D, when combined with higher levels of cytokinin, could increase cell division. They also improved the regeneration capacity of nodal explants without inducing excessive callus formation, we used this combination in this study. Therefore, low concentrations of 2,4-D (0.5 mg/L) were used to stimulate primary meristem activity. While BA (2 mg/L) was used to promote lateral branch proliferation. With this selection, regeneration of the dominant branch and callus formation were negligible. The composition of the multiplication medium was done based on the protocol reported by Sekhi et al., (2025), with no modifications. They have shown that it supported efficient shoot multiplication of *Lavandula angustifolia* under *in vitro* conditions. Each treatment was replicated ten times to ensure statistical reliability. The pH of the medium was adjusted to 5.7, in addition to 7 g.L^{-1} agar was added as a solidifying agent. Then completed dissolution, the medium was autoclaved under standard conditions (121°C for 20 minutes) and allowed to cool. Vegetative shoot segments approximately 1 cm in length were excised from the previously germinated seedlings and cultured on the prepared medium under aseptic conditions. Cultures were maintained in the growth chamber under the same environmental conditions as before (25

°C, 16-h photoperiod, 40–60 µmol m^{-2} s^{-1}). Within four weeks, multiple shoots developed, providing sufficient material for the subsequent elicitation experiment (Sekhi et al., 2025).

Preparation of bioactive compound elicitation medium: Following the completion of the multiplication stage, the obtained plantlets were transferred to MS media supplemented with varying concentrations of chitosan 0, 50, 100, and 200 mg L^{-1} to investigate the elicitor's influence on growth and secondary metabolite accumulation. These chitosan concentrations were selected based on previous studies (Mohammed et al., 2020; Fooladi Vanda et al., 2019; Elateeq et al., 2021). These concentrations included control (0 mg/L), low level of stimulus (50 mg/L), medium concentration (previously reported to maximize biomass and secondary metabolite production, i.e. 100 mg/L), and relatively high concentration (to assess potential inhibitory effects associated with excessive stimulus, i.e. 200 mg/L). Each treatment included ten replicates, and cultures were maintained under identical environmental conditions (25 °C, 16-hour photoperiod, 40–60 µmol m^{-2} s^{-1} light intensity) (Mohammed et al., 2020). The time used to evaluate the *in vitro* growth of lavender (*Lavandula angustifolia*) seedlings after chitosan treatment was four weeks. A ruler was used to measure the plant height (cm). This was measured from the base of the stem to the tip of the branch. All visible lateral branches produced in each seedling were counted as the number of branches. All fully developed leaves on each seedling were considered as the number of leaves. Fresh weight (g) measurements were made immediately after removing the seedlings from the culture medium. To remove excess moisture, the roots were gently dried with sterile filter paper. Dry weight (g) was determined after drying the samples in a compressed air oven at 70°C until constant weight was reached (usually 48 hours). An analytical balance was then used to weigh the samples. Ten seedlings were evaluated for each treatment. The average of these ten values was then used for statistical analysis.

Extraction and analysis of bioactive compounds: Bioactive compounds were extracted and analysed using GC–MS for volatile metabolites and HPLC for phenolic compounds as described below.

Extraction and analysis of volatile compounds by GC-MS: The extraction and qualitative analysis of bioactive compounds (Volatile and semi-volatile compounds) were carried out following the method described by Mohammed et al. (2020), with slight modifications to optimize compound recovery. Fresh green tissues of Lavender plantlets from each chitosan treatment were harvested, and 100 mg of fresh tissue per replicate was used for extraction. Samples were finely homogenized a in ceramic mortar and pestle with 1 mL of analytical-grade of methanol (99%) to ensure complete cellular disruption and efficient solubilization metabolites. The resulting homogenate was quantitatively transferred to a 10 mL volumetric flask, and the was volume adjusted to 10 mL with methanol (99%) to maintain uniform solvent concentration across all samples. The mixture was then filtered through Whatman No. 1 filter paper to remove insoluble and obtain a clear filtrate suitable for chromatographic analysis. The methanolic extracts were analyzed immediately by Gas Chromatography–Mass Spectrometry (GC–MS) to identify volatile and also semi-volatile secondary metabolites. The system GC–MS was operated under standard analytical conditions and each extract was injected in triplicate to ensure reproducibility and also

measurement precision. Instrumentation: Agilent 7890B GC coupled to a 5977A MSD. Column: HP-5 (30m 0.25mm, 0.25 μm). Injection: split/Splitless mode, film). injection volume 1μL. Carrier gas: helium at 1.0 min^{-1}. Oven program: initial 50° C (2min), ramp C 3° min^{-1} to C, 250° hold 5 min. Ionization: electron impact (EI) at 70 eV. Mass range: m/z 40-500. To identify compounds, we compared their mass spectra and inhibition indices with spectra in the National Institute of Standards and Technology (NIST) mass spectral library. Positively identified compounds were those with a similarity index (coefficient of agreement) of ≥80% (or ≥90%, according to laboratory identification criteria). Where valid reference standards were available, the identity of compounds was further confirmed by comparison of inhibition times and mass spectra with the standards. Consequently, it is enabling reliable characterization of the chemical profiles and detection of dominant bioactive constituents in both treated and control plantlets (Mohammed et al., 2020). Where, it was reported the retention time (RT) and relative percentage area for each identified peak.

Extraction and analysis of phenolic compounds by HPLC: For the determination of phenolic compounds (non-volatile compounds), extraction was performed using dried powdered plant material. A total of 3 g powdered of tissue from each treatment was extracted with 12 mL chloroform under continuous stirring for 8 hours at room temperature to ensure maximum extraction efficiency. The mixture was than sonicated in an ultrasonic bath for 15 to minutes to enhance solvent penetration and solubilization of phenolics. After sonication, 100 mL of butanol was added to the extract, which was subsequently transferred to a separating funnel for phase partitioning. The butanol layer, containing the majority of phenolic compounds, was collected and concentrated to dryness using a rotary under evaporator reduced to obtain pressure a crude phenolic extract. This extraction procedure was repeated three times to accumulate sufficient quantities of for extract analytical replication. Each extract was analyzed with High-performance liquid chromatography (HPLC), (Sykum-German) system; Separation was performed on C-18 column (250 × 4.6 mm) at 30°C. Acetonitrile and water in 75:25 (v/v) were set as the mobile phase. The elution mode was in an isocratic program with a flow rate of 1.4 mL·min-1; injection volume (20μl); UV detection was at (210 nm). Quantitative method was performed by external calibration. Compound identification and quantification were conducted by the comparing chromatographic retention times and UV–visible spectra with those of authentic phenolic standards. All standards were obtained from Sigma-Aldrich (USA) (Abed et al., 2020).

Evaluation of growth parameters and statistical analysis: The *in vitro* growth performance of *Lavandula angustifolia* seedlings was evaluated four weeks after chitosan treatment. Plant height (cm) was measured. It was done from the base of the stem to the tip of the branch. The number of fully developed branches and leaves for each seedling was recorded. Fresh weight (g) was determined immediately after removing the seedlings from the culture medium. It gently dried to remove excess moisture. While, dry weight (g) was measured after drying the samples in a compressed air oven at 70°C until constant weight was reached. A completely randomized design (CRD) with four chitosan treatments (0, 50, 100 and 200 mg/L) was used in this study. Each treatment consisted of ten biological replicates (one seedling per culture dish represented one biological replicate). Samples were taken from each biological replicate to

perform GC-MS and HPLC analyses. To ensure the analytical precision of the analysis, three replicates (technical replicates) were performed for each extract. The mean value of the replicates was used for statistical analysis. Shapiro-Wilk and Levene tests were used to assess the assumptions of normality and homogeneity of variance. One-way analysis of variance (ANOVA) was performed using GenStat software (version 12, VSN International Ltd., UK). Since the data met the assumptions of parametric analysis ($P > 0.05$), Comparison of treatment means was performed using the least significant difference (LSD) test at the 5% probability level ($P < 0.05$). OriginPro 2025b was used to plot the concentration of phenolic compounds. Pearson correlation coefficients were used to evaluate the relationships between growth traits, GC-MS compounds, and phenolic metabolites. Correlation matrices were displayed as heat maps using PAST version 4.12b. In these maps, the size of the circle and the intensity of the color indicated the strength and direction of the correlations, respectively.

Results

Effect of different concentrations of chitosan on the growth of lavender plantlet: Statistical analysis showed in Table 1 demonstrated difference among the tested chitosan concentration (0, 50, 100 and 200) mg L^{-1}, with the longest average branch length observed, the highest stimulatory effect on the number of green branches and leaves was observed at 50 mg with concentration at 10.45 mm, considerably surpassing the control average of 5.12 mm. The increased branch development correlated with improvements in leaf pigment concentration. however, plants treated with this concentration a notable increased compared with control group (0 mg L^{-1}) and higher concentrations (100 and 200) mg L^{-1}. The addition of the bio stimulant chitosan was shown to enhance the production of biomass and secondary metabolites in lavender plants. Does of chitosan seemed to improve the vegetative growth of the plantlets, and both fresh and dry weights of plantlets increased with chitosan concentration up to 100 mg L^{-1}. Maximum fresh and dry weights of plantlets (0.398 g and 0.063 g) were observed at 100 mg chitosan L^{-1}, in comparison to control (0.133 g and 0.026 g). That same concentration (100 mg L^{-1}) also resulted in the highest plantlet height (2.375 cm) while the shortest height was recorded for the control (1.250 cm) (Figure1). Increased fresh and dry weights observed may in part be explained by the increase in branch length. Although the highest number of branches was recorded at 50 mg L^{-1} chitosan, 100 mg L^{-1} produced significantly greater plant height, fresh weight, and dry weight, indicating that this concentration was more effective for overall biomass production.

Effect of chitosan on the phytochemicals in lavender plantlet: By using GC-MS, the selected phytochemical compounds in lavender (*Lavandula angustifolia*.) were analyzed for their relative abundance (%). This was done in lavender treated with different concentrations of chitosan (C0- C200) with the results presented in Table 2. In this table, C0, C50, C100, and C200 denote the control and increasingly chitosan treated samples, respectively. The lavender phytochemicals analyzed were esters, anhydrides, alcohols, and some heterocyclic compounds and their candidacy was made in spiro[2.3]hexan-4-one. In the results, spiro[2.3]hexan-4-one was found only in the C200 treatment (8.61%) with 4,6-Dimethoxy-5-nitropyrimidine which was found in C0 (2.41%), C50 (2.51%) and C200 (7.71%) but absent in C100.

Figure 1. Effects of different chitosan concentrations (C0= MS medium without chitosan; C50= MS+ 50 mg L^{-1} chitosan; C100= MS+100 mg L^{-1} chitosan; C200=MS+200 mg L^{-1}chitosan) on the growth traits of *Lavandula angustifolia* Plantlet grown *in vitro*

Table 1. Effect of Different Concentrations of Chitosan on the Growth Performance of Lavender Plantlet

Chitosan concentration mg L^{-1}	Plantlet height (cm)	Number of branches	Number of leaves of the Plantlet	Fresh weight of the Plantlet (g)	Dry weight of the Plantlet (g)
C0(0)	1.250^{d}	2^{b}	10^{b}	0.133^{b}	0.026^{c}
C50(50)	1.625^{b}	5^{a}	13^{a}	0.292^{a}	0.040^{b}
C100(100)	2.375^{a}	4^{a}	12^{a}	0.398^{a}	0.063^{a}
C200(200)	1.500^{cd}	2^{b}	10^{b}	0.24^{ab}	0.030^{bc}
LSD 5%	**0.471**	**1.11**	**2.06**	**0.126**	**0.011**

Moreover, C0 (2.70%) and C50 (2.91%) had Dimethyl pimelate which increased to C200 (10.36%). 2,2′-(1,4-piperazinediyl)bis[N-(4-methoxyphenyl)succinimide] attained its peak relative abundance at C3 (17.62%). 2-Nitroethyl propionate was only found in C50 (2.43%). The principal compound, Bis(2-ethylhexyl) adipate, had high to variable levels in all treatments showing the highest in C50 (89.41%). 3-Butyn-1-ol was present in C0 (6.19%) and C100 (7.51%) but was absent in all other treatments. cis-Aconitic anhydride exhibited a decreasing trend starting in C0 (21.97%) and finishing in C200 (7.92%).

Effect of chitosan on phenolics in lavender plantlet: Statistically significant differences ($P< 0.05$) identified with the analysis of variance in the chitosan treatments (C0, C50, C100, C200) with the phenolic compounds (Figure 2A-F). Costin treated Groups (C50, C100, C200) it

showed that higher level of bioactive phenolic compounds compared with the control (C0). The concentrations of rosmarinic acid, ferulic acid, caffeic acid, quercetin, apigenin, and luteolin increased with higher chitosan dosage in a dose-dependent manner.

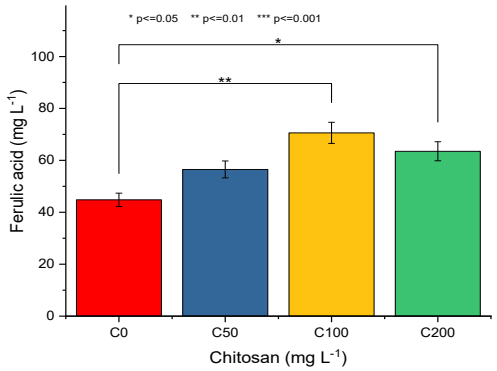
Table 2. Some phytochemicals profiles identified in chitosan-treated lavender using GCMS

RT	Identity	Chitosan			
		C0	C50	C100	C200
6.627	spiro[2.3]hexan-4-one	n.d	n.d	8.61	n.d
9.180 -10.721	4,6-Dimethoxy-5-nitropyrimidine	2.41	2.51	n.d	7.71
17.766- 17.974	Dimethyl pimelate	2.70	2.91	n.d	10.36
19.904- 20.302	2,2′-(1,4-piperazinediyl)bis[N-(4-methoxyphenyl)succinimide]	3.44	2.74	n.d	17.62
20.207	2-Nitroethyl propionate	n.d	2.43	n.d	n.d
23.089 - 23.894	Bis (2-ethylhexyl) adipate	63.29	89.41	71.14	56.39
24.950	3-Butyn-1-ol	6.19	n.d	7.51	n.d
25.201- 28.438	cis-Aconitic anhydride	21.97	n.d	12.74	7.92

n.d: non-detected

Statistically, the peak concentrations were reached at treatment C100, while C200 showed significant reduction in phenolic content (figure 2A-F). In basil (*Ocimum basilicum*), moderate chitosan concentrations led to a marked increase in phenolic content and antioxidant activity, while higher doses inhibited growth (Kim et al., 2005). Similarly, chitosan at a concentration of 100 mg/L increased rosmarinic acid accumulation in *Melissa officinalis* bud cultures by activating jasmonally mediated signaling pathways. The response pattern observed in lavender mimics increased metabolite accumulation at moderate chitosan concentrations followed by inhibition at higher levels. These results confirm the accuracy of the response pattern observed within the Lamiaceae family (Fooladi Vanda et al., 2019). Analyzing the changes in phenolic compounds with treatment C100, HPLC analysis indicated that quercetin (95.6 ppm, figure 2A), rosmarinic acid (90.5 ppm, figure 2B), luteolin (88.7 ppm, figure 2C), apigenin (83.9 ppm, figure 2D), caffeic acid (81.4 ppm, figure 2E) and ferulic acid (70.6 ppm, figure 2F) were the most abundant phenolic compounds in descending concentration order.

Correlation analysis: Pearson correlation analysis revealed distinct associations among vegetative growth traits and phenolic metabolites (Tab. 3). Plant length (PL) showed a very high positive correlation with fresh weight (FW; r=0.95) and dry weight (DW; r= 0.99), confirming that shoot elongation directly reflects biomass accumulation. Notably, PL was strongly correlated with all quantified phenolic compounds, including rosemarinic acid (r=0.81), ferulic acid (r=0.85), caffeic acid (r=0.82), quercetin (r=0.80), luteolin (r=0.82), and also apigenin (r=0.79). These relationships indicate that improved vegetative vigor was linked to closely secondary enhanced metabolite biosynthesis. Fresh and dry biomass each demonstrated similarly strong positive correlations with phenolic contents (r =0.85- 0.91), further supporting the concept of metabolic intensification accompanying increased plant growth. The number of branches and leaves showed weak or inconsistent correlations with phenols (r = 0.81-0.46), suggesting that these traits contribute less directly to phenolic productivity than total or biomass shoot length.



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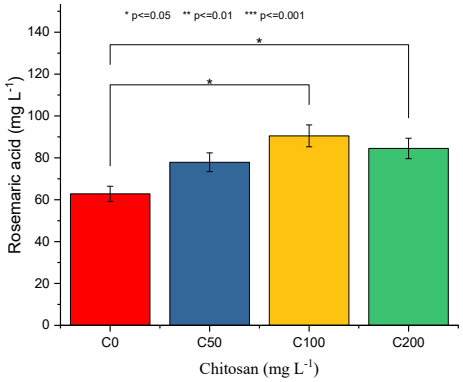

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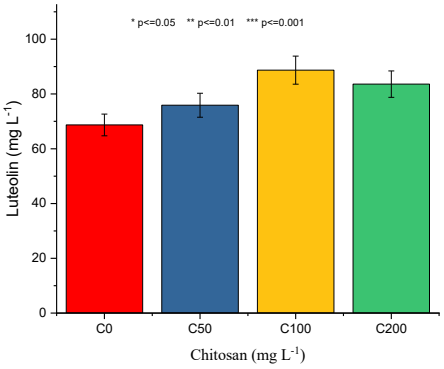

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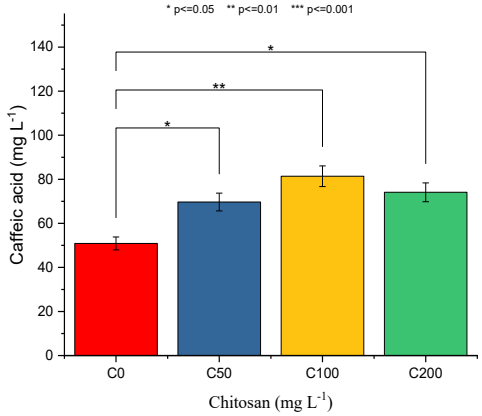

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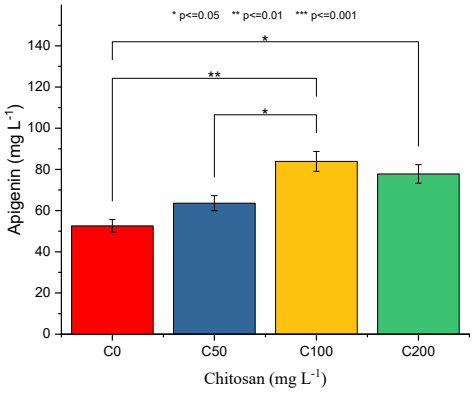

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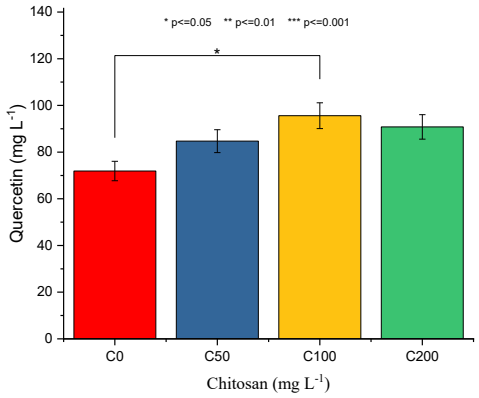

F

Figure 2. Effects of Chitosan treatments on selected phenolic compounds in *Lavandula angustifolia* Plantlet grown *in vitro*: (A) ferulic acid, (B) rosmarinic acid, (C) caffeic acid, (D) luteolin, (E) quercetin and (F) apigenin, the x-axis represents the phenolic compounds analyzed, while the y-axis indicates the applied concentrations of chitosan

Correlations among phenolic metabolites: Highly strong and statistically significant positive correlation were illustrated among the phenolic compounds themselves. Rosmarinic acid was almost perfectly correlated with quercetin (r =0.9998), ferulic acid (r = 0.994), caffeic acid (r =0.995), luteolin (r =0.980), and apigenin (r = 0.975). Likewise, ferulic acid was correlated strongly with caffeic acid (r =0.984) in addition to quercetin (r = 0.994). These near-unity correlations strongly indicate a shared biosynthetic regulation consistent with the coordinated activity of phenylpropanoid and flavonoid pathways, resulting in a parallel accumulation of these compounds rather than independent metabolite regulation.

Chitosan treatments and growth–metabolite relationships: Clear divergence was observed among the chitosan treatment effects (CH1-CH8). Treatments CH1 showed Strong positive correlations with PL (r = 0.95), FW (r = 0.80), DW (r = 0.93), and phenolic accumulation (r ≈ 0.64 - 0.72), identifying this treatment as the most effective elicitor combination for both growth promotion and secondary metabolite induction. In contrast, CH2-CH4 exhibited significant negative correlations with all major growth parameters (PL; R= -0.43 to -0.56); DW; r =-0.55to -0.67). and no meaningful positive association with phenolics (most r ≈ 0 or negative). These findings indicate that certain chitosan concentrations or formulations likely induced physiological stress, suppressing biomass accumulation and decoupling secondary metabolism from vegetative performance. CH5 and CH6 were positively associated with branching and leaf number (r = 0.78–0.94) but showed weak or negative correlations with phenolic compounds, implying preferential stimulation of morphogenesis rather than carbon allocation to secondary metabolism. CH7 demonstrated mixed responses, displaying positive links to CH1 yet negative correlations to biomass and phenolic traits, reflecting variable elicitor sensitivity. CH8 displayed moderate negative correlations with growth and metabolites, suggesting partial inhibition at higher concentrations or under less favorable elicitation regimes.

Pearson correlation heatmap: Pearson correlation heatmap showing relationships among vegetative growth traits (PL, branches, leaves, FW, DW), chitosan treatments (CH1-CH8), and phenolic compounds (rosmarinic acid, ferulic, caffeic acid; quercetin, luteolin, apigenin) in lavender plantlets. Blue circles indicate positive correlations, red circles indicate negative correlations, with color intensity and circle size proportional to correlation strength (-1 to +1). Values inside cells represent correlation coefficients (r). Pearson correlation analysis (Figure 3) demonstrated strong positive relationships between plant length (PL), fresh weight (FW), and dry weight (DW) (r = 0.94–0.99), confirming that shoot elongation was closely associated with biomass accumulation. These traits also exhibited strong positive correlations with all quantified phenolic compounds, including rosmarinic acid (r = 0.81), ferulic acid (r = 0.85), caffeic acid (r = 0.82), quercetin (r = 0.80), luteolin (r = 0.82), and apigenin (r = 0.79), indicating coordinated enhancement of growth and secondary metabolism. Phenolic metabolites were almost perfectly intercorrelated, with correlation coefficients exceeding r = 0.97, reflecting tight metabolic coupling through the phenylpropanoid–flavonoid biosynthetic network. Among chitosan treatments, CH1 displayed strong positive correlations with both growth parameters (r = 0.80–

0.95) and phenolic compounds (r = 0.63–0.72), identifying this treatment as the most effective elicitor.

Table 3. Pearson coefficients of correlation among nineteen parameters of *Lavandula angustifolia*

	PL*	Branch	leaves	FW	DW	CH1	CH2	CH3	CH4	CH5	CH6	CH7	CH8	Ros	Fer	Caf	Que	Lut	Api
PL		0.46	0.46	0.05	0.01	0.05	0.44	0.49	0.57	0.91	0.76	0.58	0.83	0.19	0.15	0.18	0.20	0.18	0.21
Branch	0.55		1	0.31	0.37	0.67	0.44	0.44	0.40	0.22	0.06	0.88	0.33	0.63	0.67	0.54	0.64	0.77	0.82
leaves	0.55	1		0.31	0.37	0.67	0.44	0.44	0.40	0.22	0.06	0.88	0.33	0.63	0.67	0.54	0.64	0.77	0.82
FW	0.99	0.69	0.69		0.06	0.20	0.58	0.63	0.68	0.84	0.60	0.87	0.53	0.11	0.11	0.09	0.12	0.15	0.19
DW	0.99	0.63	0.63	0.94		0.07	0.33	0.38	0.45	0.99	0.64	0.55	0.82	0.26	0.23	0.25	0.27	0.26	0.31
CH1	0.95	0.33	0.33	0.80	0.94		0.35	0.40	0.50	0.67	0.95	0.32	0.85	0.35	0.29	0.37	0.36	0.28	0.32
CH2	-0.56	-0.57	-0.57	-0.42	-0.67	-0.65		0.00	0.02	0.87	0.47	0.27	0.77	0.99	0.96	0.98	0.97	1.00	0.94
CH3	-0.51	-0.56	-0.56	-0.37	-0.62	-0.60	1.00		0.01	0.84	0.46	0.29	0.77	0.93	0.98	0.97	0.91	0.94	0.87
CH4	-0.43	-0.60	-0.60	-0.32	-0.55	-0.50	0.98	0.99		0.73	0.38	0.37	0.82	0.86	0.90	0.91	0.84	0.85	0.78
CH5	-0.09	0.78	0.78	0.16	0.01	-0.33	-0.13	-0.16	-0.27		0.09	0.43	0.23	0.94	0.86	0.97	0.93	0.75	0.72
CH6	0.25	0.94	0.94	0.40	0.36	0.05	-0.53	-0.55	-0.62	0.91		0.80	0.38	0.95	1.00	0.86	0.97	0.89	0.84
CH7	0.42	-0.11	-0.12	0.13	0.45	0.68	-0.73	-0.71	-0.64	-0.57	-0.20		0.22	0.91	0.99	0.88	0.90	0.94	0.98
CH8	-0.18	-0.67	-0.67	-0.47	-0.18	0.15	-0.23	-0.24	-0.18	-0.77	-0.62	0.78		0.51	0.60	0.44	0.51	0.69	0.67
Ros	0.81	0.37	0.37	0.89	0.74	0.65	0.01	0.07	0.14	-0.06	0.05	-0.09	-0.49		0.01	0.00	0.00	0.02	0.03
Fer	0.85	0.33	0.33	0.89	0.77	0.71	-0.04	0.02	0.10	-0.14	-0.00	0.01	-0.40	0.99		0.02	0.01	0.01	0.01
Caf	0.82	0.46	0.46	0.92	0.75	0.63	-0.02	0.03	0.09	0.05	0.14	-0.12	-0.56	1.00	0.98		0.01	0.04	0.05
Que	0.80	0.36	0.36	0.88	0.73	0.64	0.03	0.09	0.16	-0.07	0.03	-0.10	-0.49	1.00	0.99	0.99		0.02	0.02
Lut	0.83	0.23	0.23	0.85	0.74	0.72	0.00	0.06	0.15	-0.25	-0.11	0.06	-0.31	0.99	0.99	0.96	0.98		0.00
Api	0.79	0.18	0.18	0.81	0.69	0.68	0.06	0.13	0.22	-0.28	-0.16	0.02	-0.31	0.97	0.99	0.95	0.98	1.00	

*PL= Plantlet length, branches= number of branches, FW= fresh weight, Dwdry weight, CH1= spiro[2.3]hexan-4-one, CH2= 4,6-Dimethoxy-5-nitropyrimidine, CH3= Dimethyl pimelate, CH4= 2,2′-(1,4-piperazinediyl)bis[N-(4-methoxyphenyl)succinimide], CH5= 2-Nitroethyl propionate, CH6= Bis[2-ethylhexyl) adipate, CH7= 3-Butyn-1-ol, CH8= cis-Aconitic anhydride, Ros rosemarinic acid, Fer= ferulic acid, Caf= caffeic acid, Que= quercetin, Lut= luteolin, Api= apigenin

Conversely, CH2–CH4 showed pronounced negative correlations with plant growth ($r \approx -0.42$ to -0.67) and negligible or negative associations with secondary metabolites, suggesting that excessive chitosan application imposed physiological stress conditions that suppressed biomass production and disrupted metabolic induction. Treatments CH5 and CH6 were positively associated with vegetative branching but weakly or negatively related to phenolic contents, indicating preferential allocation towards morphogenesis rather than secondary metabolite biosynthesis.

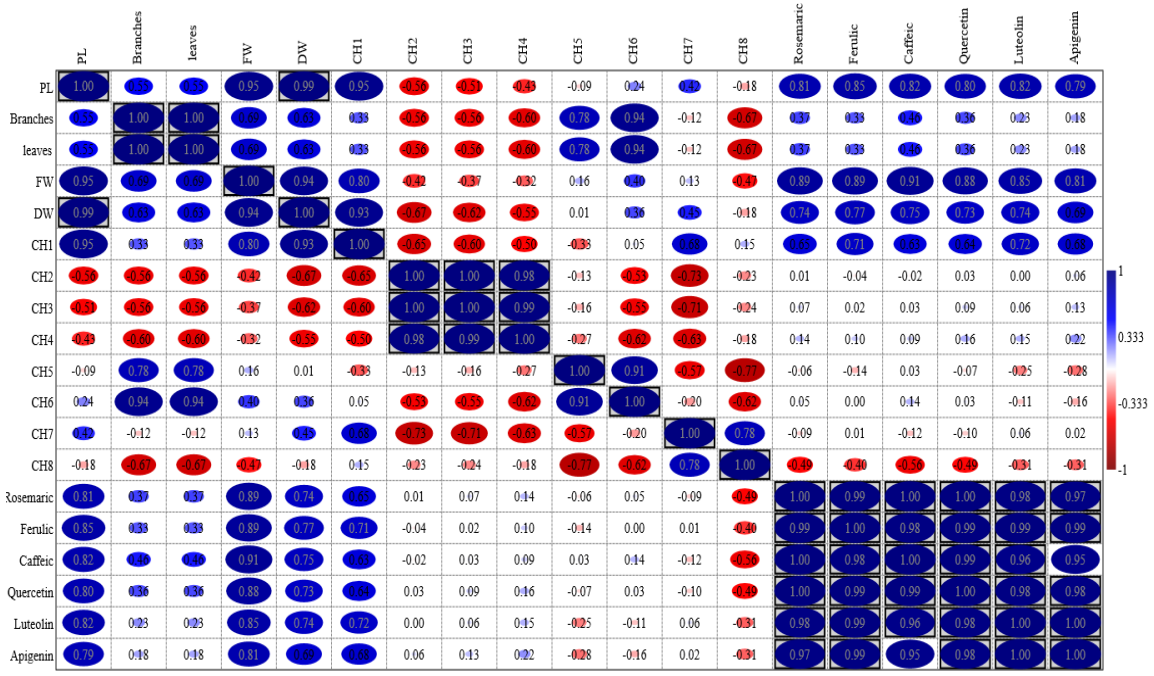

Figure 3. Pearson correlation heatmap of nineteen parameters of *Lavandula angustifolia.* (PL= Plantlet length, branches= number of branches, FW= fresh weight, Dwdry weight, CH1= spiro[2.3]hexan-4-one, CH2= 4,6-Dimethoxy-5-nitropyrimidine, CH3= Dimethyl pimelate, CH4= 2,2′-(1,4-piperazinediyl)bis[N-(4-methoxyphenyl)succinimide], CH5= 2-Nitroethyl propionate, CH6= Bis[2-ethylhexyl) adipate, CH7= 3-Butyn-1-ol, CH8= cis-Aconitic anhydride), Red and blue color as in scale

Discussion

Considering the recorded growth parameters, increasing chitosan concentrations up to 100 mg L^{-1} significantly enhanced the growth of lavender plantlets, whereas a further increase to 200 mg L^{-1} resulted in growth inhibition. These findings are consistent with previous studies on different plant species (Nourozi et al., 2014). For instance, in *Agastache foeniculum,* chitosan concentration of 100 mg L^{-1} stimulated biomass production, while fresh weight was decreased at 200 mg L^{-1}. Similarly, Elateeq et al. (2021) reported that 100 mg L^{-1} chitosan increased fresh and dry weight of *Ginkgo biloba* callus tissues, although a decline in fresh weight occurred at 200 mg L^{-1}. In this study, chitosan was applied as a biostimulant to enhance both vegetative growth and secondary metabolite accumulation in lavender tissue cultures. The observed improvements align with previous reports describing chitosan as an effective growth promoter (Pirbalouti et al., 2017; Atteya et al., 2023). Most growth parameters, including plant height, fresh weight, and dry weight, increased with chitosan concentration up to 100 mg L^{-1}, whereas the maximum branch number was observed at 50 mg L^{-1}. This growth stimulation is attributed to chitosan's role as a natural biopolymer that enhances nutrient uptake, particularly nitrogen and phosphorus, thereby supporting metabolic activities necessary for biomass accumulation. Increased nutrient assimilation promotes higher metabolic rates, which in turn elevate fresh weight, via water and organic matter accumulation, and dry weight, through the buildup of protein and carbohydrates.

Plant height increased markedly with rising chitosan concentration, peaking at 100 mg L^{-1}. This enhanced elongation is due to chitosan- mediated activation of auxin biosynthesis or signalling pathways, which promote cell wall loosening and longitudinal cell expansion. However, this vertical growth was accompanied by a reduction in leaf number and vegetative branches, suggesting a possible trade-off between shoot elongation and lateral organ development. Importantly, the chitosan stimulatory effect is concentration-dependent. At 200 mg L^{-1}, most of the growth traits were significantly decreased, indicating that excessive chitosan may exert an inhibitory effect. Chitosan shifts from a biostimulant to a stress-inducing agent. Excessive elicitation can disrupt membrane integrity, impair nutrient uptake, and cause ionic imbalance in plant tissues. Furthermore, sustained activation of defense responses leads to elevated ROS levels, resulting in oxidative stress and diversion of energy away from primary metabolism and growth processes. Under such conditions, carbon and energy resources are preferentially allocated toward stress mitigation rather than biomass accumulation. This metabolic burden explains the observed reduction in plant height, fresh weight, dry weight, and phenolic content at 200 mg L^{-1}. Similar inhibitory effects at high chitosan concentrations have been reported in *Agastache foeniculum*, *Ginkgo biloba*, and basil species, confirming that supra-optimal elicitation imposes physiological constraints on *in vitro* plant growth. Beyond growth enhancement, chitosan induced notable changes in lavender phytochemistry. As a polyglucosamine derivative of chitin, chitosan is well-known for priming plant defense mechanisms by activating secondary metabolic pathways (Fooladi Vanda et al., 2019; Li et al., 2020; Roman-Doval et al., 2023; Sun et al.2023). This activation is typically mediated through jasmonate and salicylate-dependent signalling routes, which in chitosan collectively increase the synthesis of defense-related secondary metabolites. Metabolomic profiling in this study revealed the exclusive appearance of spiro[2.3]hexan-4-one at the highest chitosan concentration (C200), along with substantial increases in Dimethyl pimelate and 2,2′-(1,4-piperazinediyl)bis[N-(4-methoxyphenyl)succinimide]. These results suggest an upregulation of pathways associated with fatty acid-derived and nitrogenous secondary metabolites under strong elicitation pressure (Zhang et al., 2020). Consistently high levels of di(2-ethylhexyl) adipate across treatments indicate the possibility of external contamination resulting from storage of the extract in plastic tubes (reaction of methanol solvent with plastic). Moreover, the observed decline in cis-Aconitic anhydride with increasing chitosan concentration suggests a possible metabolic shift away from the tricarboxylic acid (TCA) cycle toward secondary metabolite synthesis. Such reallocation of carbon flux is a common strategy in stress-responsive plants and aligns with the enhanced production of protective compounds under elicitation (Obata and Fernie, 2012). The results confirm the biostimulatory capacity of chitosan to enhance both diversity and abundance of phyto-volatiles in the medicinal and aromatic plants such as lavender. This enhancement could increase the therapeutic, industrial, and commercial value of lavender essential oil and extracts (Moghaddam and Mehdizadeh, 2017; Alhassan et al., 2025). Overall, the correlation patterns support three key biological insights: Vegetative vigor is tightly linked to phenolic biosynthesis. Traits reflecting total biomass (PL, FW, DW) are better predictors of metabolite accumulation than architectural traits (branch or leaf number). Phenolic compounds

are co-regulated. Near-perfect positive correlations strongly support the involvement of a coordinated biosynthetic pathway rather than metabolite-specific regulation. Chitosan elicitation is dose- and formulation-dependent. The addition of chitosan at progressively higher concentrations to nutrient media stimulated the accumulation of phenolic compounds in lavender tissues, with the effect peaking at a concentration of 100 mg/L. This accumulation is believed to represent a defensive response to scavenge free radicals (Delgado-Rivera et al., 2026; Sharma et al., 2019). Previous studies have indicated that chitosan activates jasmonic acid, which plays a pivotal role in the biosynthesis of enzymes associated with plant defense mechanisms, such as catalase and peroxidase, and in the production of phytoalexins, including key enzymes like polyphenol oxidase, chalcon synthase, and phenylalanine ammonium lyase (PAL), which is crucial for activating the phenylpropanoid pathway (Fooladi Vanda et al., 2019; Sharma et al., 2019). The study also confirmed a clear positive correlation between PAL enzyme activity and total phenol accumulation, with the two indicators following a similar pattern. This molecular regulation of phenolic content is primarily attributed to the level of PAL gene expression, which directs metabolic flow toward phenol production via the phenylpropanoid pathway (Fooladi Vanda et al., 2019). The key enzymes in this pathway [PAL, Cinnamate 4-Hydroxylase (C4H), and Coumarate: CoA Ligase (4CL)] work sequentially to convert the amino acid phenylalanine to the active compound p-coumaroyl-CoA. This p-coumaroyl-CoA represents a central branching point from which carbon atoms are distributed into multiple biosynthetic pathways responsible for the production of a wide range of secondary phenolic compounds (Sharma et al., 2019). Low or optimized treatments (e.g., CH1) synchronized growth promotion with metabolite induction, whereas other treatments suppressed biomass or favored structural growth without biochemical enhancement. The correlation matrix revealed that optimized chitosan treatment enhances both plant growth and phenolic biosynthesis simultaneously, while suboptimal treatments disrupt this relationship. Phenolic metabolite accumulation in lavender appears most strongly driven by overall biomass production and shared biosynthetic pathway activation rather than vegetative morphology alone. Future investigations incorporating transcriptomic, proteomic, and enzymatic analyses are needed to elucidate the regulatory mechanisms controlling chitosan-induced metabolic pathways and to deepen the understanding of secondary metabolite biosynthesis in lavender.

Conclusion: This study showed that the growth and secondary metabolism of lavender plantlets grown *in vitro* were significantly impacted by chitosan supplementation. At 100 mg L^{-1} chitosan, growth traits such as plant height, fresh weight, and dry weight were maximally enhanced, suggesting that this concentration is ideal for vegetative development. Inhibitory effects were noted at higher concentrations [200 mg L^{-1}), indicating a limited effective range. Furthermore, chitosan treatment altered metabolite composition in a dose-dependent manner, according to phytochemical and phenolic analyses. Key phenolics [quercetin, rosmarinic acid, luteolin, apigenin, caffeic acid, and ferulic acid) were most abundant in the 100 mg L^{-1} treatment, whereas unique compounds like spiro[2.3]hexan-4-one only showed up at 200 mg L^{-1}. According to these results, chitosan supplementation at moderate levels promotes advantageous metabolic

reprogramming, while high levels may upset the primary metabolic balance. Although its effects were highly concentration-dependent, chitosan generally functioned as an efficient biostimulant for lavender *in vitro*. To elucidate the regulatory mechanisms behind these alterations, in addition to validating their applicability in large-scale cultivation systems, more transcriptomic and biochemical research is advised.

Despite the encouraging results, this study has a number of limitations that should be noted. First, the study was conducted under controlled vitro culture conditions, which may not accurately reflect the complex physiological and environmental interactions experienced by lavender under field conditions. Therefore, the applicability of these results to large-scale cultivation or natural systems remains uncertain. Second, the study evaluated a relatively narrow range of chitosan concentrations (0 to 200 mg L^{-1}). The inclusion of intermediate concentrations or higher could provide a more detailed understanding of the dose-response relationship and help identify precise thresholds between stimulatory and inhibitory effects. Third, although insights into secondary metabolisms were obtained, phytochemical analysis was limited to a selected group of phenolic compounds. A broader metabolomic approach, coupled with transcriptomic profiling, would be necessary to comprehensively clarify the regulatory networks and biosynthetic pathways influenced by chitosan treatments. Furthermore, while the physiological mechanisms connecting chitosan application to enhance growth and metabolites accumulation were inferred, they were not directly validated at the molecular level. Future studies employing gene expression analysis, enzyme activity assays, and hormonal profiling would be essential to confirm the underlying signaling pathways and metabolic controls. Finally, this study focused solely on short-term growth responses of lavender plantlets. Long-term assessments are required to determine the sustained impact of chitosan on developmental parameters such as flowering, essential oil yield, plant morphology, and tolerance to abiotic stress under greenhouse and field conditions. Addressing these limitations in future research will enable a more comprehensive understanding of the role of chitosan as a bio-stimulant and support its effective application in lavender cultivation.

List of abbreviations

BA: benzyladenine, CRD: completely randomized design, Cn: concentration level, GA3: gibberellic acid, LSD: least significant difference, MS: Murashige and Skoog, NIST: National Institute of Standards & Technology, ppm part per million, P: probability, spp: more species, TCA: tricarboxylic acid.

Authors contribution

The authors confirm contribution to the paper as follows: study conception and design: MAM, AFA; data collection: MSA and BHS: analysis and interpretation of results: AFA, MA. Author. MA. Author: draft manuscript: MAM. AOM. All authors reviewed the results and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Finding

The authors declare that this work didn't receive any private or official support.

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical considerations

Not applicable. This study focused on **wheat** and did not involve human subjects or animal experiments that require ethical approval.

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تأثیر کیتوزان بر رشد درون‌شیشه‌ای (*in vitro*) و تولید متابولیت‌های زیست‌فعال در *Lavandula angustifolia* Mill.

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چکیده

هدف: تولید پایین متابولیت‌های زیست‌فعال همچنان یکی از محدودیت‌های اصلی در تولید تجاری اسطوخودوس (*Lavandula angustifolia*) محسوب می‌شود. کیتوزان به‌عنوان یک محرک مؤثر برای افزایش زیست‌ساخت متابولیت‌های ثانویه در شرایط *in vitro* پیشنهاد شده است. این مطالعه با هدف ارزیابی اثر کیتوزان، که یک مشتق زیستی از کیتین است، بر رشد و میزان ترکیبات فعال گیاهان اسطوخودوس کشت‌شده در شرایط *in vitro* انجام شد.

مواد و روش‌ها: یک طرح کاملاً تصادفی با چهار غلظت کیتوزان شامل ۰، ۵۰، ۱۰۰ و ۲۰۰ میلی‌گرم در لیتر مورد استفاده قرار گرفت. همچنین، برای هر تیمار، ده تکرار زیستی از گیاهچه‌های اسطوخودوس کشت‌شده در شرایط *in vitro* استفاده شد. پارامترهای رشد رویشی شامل ارتفاع گیاه، تعداد شاخه‌ها، تعداد برگ‌ها، وزن تر و وزن خشک پس از چهار هفته کشت ثبت شدند. برای تجزیه وتحلیل متابولیت‌های فرّار از روش GC–MS و برای اندازه‌گیری ترکیبات فنولی از روش HPLC استفاده شد.

نتایج: نتایج مطالعه حاضر نشان داد که رشد گیاه و تجمع متابولیت‌ها به‌طور معنی‌داری تحت تأثیر کیتوزان قرار گرفتند ($p < 0.05$). استفاده از کیتوزان با غلظت ۱۰۰ میلی‌گرم در لیتر بیشترین میزان زیست‌توده را تولید کرد، به‌جز تعداد شاخه‌ها که بیشترین تعداد شاخه در غلظت ۵۰ میلی‌گرم در لیتر مشاهده شد. این تیمار در مقایسه با شاهد، موجب افزایش ارتفاع گیاه (۹۰ درصد)، وزن تر (۱۹۹ درصد) و وزن خشک (۱۴۲ درصد) شد. در این غلظت، بیشترین مقادیر ترکیبات کوئرستین، اسید رزمارینیک، لوتئولین، آپیژنین، اسید کافئیک و اسید فرولیک به‌ترتیب برابر با ۹۵/۶ ppm، ۹۰/۵ ppm، ۸۸/۷ ppm، ۸۳/۹ ppm، ۸۱/۴ ppm و ۷۰/۶ ppm بود. در مقابل، کیتوزان با غلظت ۵۰ میلی‌گرم در لیتر موجب تجمع ترکیب 2-nitroethyl propionate شد، در حالی‌که غلظت ۲۰۰ میلی‌گرم در لیتر باعث کاهش رشد و کاهش تجمع ترکیبات فنولی گردید که نشان‌دهنده اثرات بازدارنده در غلظت‌های بالاتر است.

نتیجه‌گیری: پژوهش حاضر نشان داد که کیتوزان می‌تواند رشد و تولید متابولیت‌های ثانویه در اسطوخودوس کشت‌شده در شرایط *in vitro* را به‌صورت وابسته به غلظت افزایش دهد. به‌طور کلی، غلظت ۱۰۰ میلی‌گرم در لیتر به‌عنوان غلظت بهینه شناخته شد، زیرا بیشترین میزان زیست‌توده و تجمع ترکیبات فنولی را ایجاد کرد؛ اگرچه بیشترین تعداد شاخه در غلظت ۵۰ میلی‌گرم در لیتر مشاهده شد. نتایج ما نشان داد که افزودن ۱۰۰ میلی‌گرم در لیتر کیتوزان به محیط‌های کشت *in vitro* می‌تواند به‌عنوان یک راهبرد عملی برای بهبود تولید ترکیبات زیست‌فعال ارزشمند در اسطوخودوس مورد استفاده قرار گیرد. برای اعتبارسنجی مکانیسم‌های زیربنایی و حمایت از کاربرد تجاری، انجام مطالعات مولکولی و گلخانه‌ای بیشتر پیشنهاد می‌شود.

کلمات کلیدی: اسطوخودوس، ترکیبات فعال، کیتوزان، *in vitro*

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