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RESEARCH ARTICLE

Chitosan-induced growth enhancement, piperine production and relative expression of piperine synthase gene in long pepper (*Piper longum* L.)

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Abstract

A study was undertaken to investigate the effects of chitosan (CS) at varying concentrations (0 g/L, 1 g/L, 2 g/L, 3 g/L and 4 g/L) on *Piper longum*. CS, a naturally occurring polysaccharide, has garnered attention for its potential to enhance plant growth and yield. The experiment involved foliar applications of CS at 2, 4 and 6 months after planting (MAP), followed by observations 1 month post-application (3, 5 and 7 MAP). This allowed for a comprehensive assessment of the impact of CS on the growth, physiological, biochemical and yield parameters of *P. longum*. Notably, the findings highlighted that foliar spraying of CS at lower concentrations (1 g/L and 2 g/L) significantly stimulated the growth and yield attributes and expression of the piperine synthase gene in *P. longum*. These concentrations positively affected various parameters, including shoot length, physiological functions, biochemical processes and yield metrics. Conversely, higher concentrations of CS (3 g/L and 4 g/L) exhibited inhibitory effects, leading to compromised performance across the assessed parameters. Moreover, these concentrations produced poorer results than the control treatment, highlighting the detrimental effects of excessive CS application on *P. longum*. Overall, these findings emphasize the importance of optimizing CS concentrations for effective enhancement of growth and yield in *P. longum* cultivation, while also highlighting the potential risks associated with excessive CS application.

Keywords

chlorophyll content; photosynthetic rate; Piperaceae; piperine synthase gene; volatile oil; water use efficiency

Introduction

Piper longum Linn., commonly known as thippali or long pepper, is a member of the Piperaceae family and is renowned for its medicinal properties, particularly in treating respiratory disorders (1). Indigenous to the Indo-Malayan region, it is distributed widely across tropical and subtropical regions, including the Indian subcontinent, Sri Lanka, Malaysia, Singapore, Bhutan, Myanmar, Assam, Tamil Nadu and the Western Ghats from Konkan to Kerala (2). However, the demand for long pepper in the pharmaceutical industry has led to unsustainable harvesting practices, resulting in the depletion of natural populations in Kerala's forests (1). The growing demand for long pepper could be met through innovative and sustainable agro-techniques. Chitosan (CS), derived from chitin, is a naturally occurring polysaccharide found in the cell walls of fungi, crustaceans and insects

(3). Known for its biocompatibility, non-toxicity and biodegradability, CS has shown promising effects in promoting plant growth, enhancing secondary metabolite production and mitigating biotic and abiotic stresses (4).

Given the potential of metabolites in *P. longum* and the growing interest in enhancing their production, this study aimed to investigate the effects of CS on various aspects of *P. longum*, including plant growth promotion, piperine content, yield and the relative expression of the piperine synthase gene. The study aimed to identify the optimal concentration of CS for improving the growth and yield of *P. longum* while increasing its piperine content, a key bioactive compound in the plant. Additionally, the study examined the expression of the piperine synthase gene, which is involved in the biosynthesis of piperine, to elucidate the molecular mechanisms underlying the observed effects of CS. By examining the effects of CS on *P. longum* at both physiological and molecular levels, this study enhances our understanding of how natural compounds can be leveraged to improve the cultivation and production of medicinal plants. The findings of this research provide valuable insights for developing sustainable agricultural practices that can meet the increasing demand for medicinal herbs while conserving natural resources.

Materials and Methods

The field trial was conducted at College of Agriculture, Vellayani during 2021-2022. The location is situated at 8° 27' 59.0256'' North latitude and 76° 58' 3.27'' East longitude at an altitude of 26 m above mean sea level. The 2 months old rooted cuttings of *P. longum* were planted in polybags containing soil, sand and farm yard manure (1:1:1) and staking was given 3 MAP. CS at 4 different concentrations was applied as a foliar spray at 2, 4 and 6 MAP. The experiment design was a Completely Randomized Design (CRD) with 5 treatments and 4 replications.

Preparation of chitosan solution

CS (3800-20000 Da) sourced from shrimp shells, with a degree of deacetylation exceeding 75.0%, obtained from HiMedia, was utilized for the study. Foliar solutions of CS at varying concentrations of 0 g/L, 1 g/L, 2 g/L, 3 g/L and 4 g/L were prepared by dissolving in 1 % acetic acid solution. The spray volume applied to the plants varied depending on their growth stage, with each foliar application amounting to 30, 40 and 50 mL at 2, 4 and 6 MAP respectively.

Plant growth parameters

The plant growth parameters, including shoot length, number of primary branches and number of spike-bearing branches, were recorded 1 month after each foliar application, at 3, 5 and 7 MAP from 4 plants per replication. Additionally, the percentage increase in these parameters at 7 MAP compared to the control treatment was recorded. Observations regarding the days to spike emergence, flowering and spike maturity were also observed. The number of days from planting to the emergence of the first spike was recorded for selected 20 plants from all the treatments and the mean value was calculated to determine the days to spike emergence. Furthermore, the number of days from the emergence of the spike to the initiation of flowering on the spike was observed for 10 tagged spikes of selected plants and the mean value was calculated for recording days to flowering. Lastly, the number of days from spike emergence to spike maturity, indicated by a greenish-black color, was observed for 10 tagged spikes of selected plants and the mean value was recorded for days to spike maturity.

Physiological parameters

One month after each foliar spray, physiological parameters including stomatal conductance, photosynthetic rate and water-use-efficiency were assessed using a portable photosynthetic system (Model: CIRAS-3 Ver. 1.06, Amesbury, U.S.A). Additionally, the concentration of chlorophyll in the leaves was measured to evaluate the plant's health, photosynthetic capacity and chloroplast development, following the procedure (5). Stomatal distribution, indicated by stomatal density, was recorded using the epidermal imprinting technique (6).

Enzyme activity

In vitro enzyme activity assays were conducted to gain a deeper understanding of the biochemical effects of CS. For this, leaf samples were collected 1 month after each foliar application and 200 mg of sample were ground in phosphate buffer using a mortar and pestle. The resulting homogenate was then centrifuged for 15 min at 5000 rpm and 40 °C and the supernatant was collected. collected for determining peroxidase (7) and catalase (8) activities.

Analysis of Piperine, oleoresin and volatile oil content

The spikes, harvested at the fully mature unripe (greenish-black) stage, were collected, dried and powdered to determine piperine content. Spectrometric analysis measured the amount of piperine in the dried spikes. The samples were extracted in a volumetric flask using 100 mL of acetone. Subsequently, the absorbance of the solution was measured at 337 nm using a UV spectrophotometer (Biospectrometer-Eppendorf), with acetone serving as the blank. The piperine content of the samples was determined by referencing a piperine standard curve (9). Additionally, oleoresin and volatile oil content were analyzed from the dried spikes using a Soxhlet extractor and Clevenger apparatus respectively, with acetone as the solvent.

qRT - PCR analysis

The expression profiling of the piperine synthase gene was conducted using Quantitative Real-time PCR (qRT-PCR). RNA was extracted from the leaves using TRIzol reagent (Hi-Media) 24 h after the second CS (4MAP) application. Subsequently, 1000 ng of RNA was used to synthesize cDNA using the RT Easy kit (G-bioscience) following the manufacturer's instructions. The cDNA was then amplified using previously reported gene-specific primers: forward primer 5´-TTGGCGATATCGGAGCACTC-3´ and reverse primer 5´-CGATCCCGCCGCAAATAAAG-3´ for the piperine synthase gene and forward primer 5'-ACATCCGCTGGAAGGTGC-3' and reverse primer 5'-TCTGTATGGTAACATTGTGCTC-3' for the actin gene. Each sample and replication were used for qRT-PCR assay of the piperine synthase gene, with the actin gene as reference. The assay was conducted in a final volume of 20 µL, including 10 µL SYBR GREEN master mix (G-bioscience), 1 µL of template cDNA and 2 µL of primer (25 µM each) on a CFX96 Real-time PCR (Bio-Rad. U.S.A). The thermal cycling conditions comprised initial denaturation at 95°C for 3 min, followed by 35

cycles of denaturation at 95°C for 45 sec, annealing at 51.2°C (piperine synthase) and 47.5°C (actin) for 30 sec, extension at 72 ° C for 45 sec and final extension at 72°C for 3 min. The expression profile of the piperine synthase gene was compared with the reference actin gene using the comparative Ct method.

Yield parameters

Mature greenish-black spikes were harvested from the plants and counted up to 1 year and the sum of all the spikes were recorded as total number of spikes per plant per year. The length and girth of the spikes were measured using thread and a measuring tape and the mean value were recorded in centimetre (cm). Additionally, dark green mature spikes were picked and the weight of the freshly harvested spikes was measured at each picking up to one year using an electronic balance, the average was taken and expressed in gram (g). These spikes were then dried at 60°C for 96 h until consistent weights were obtained and the dry weight of the spikes was recorded. The entire plants were uprooted one year after planting and the roots were collected. The weight of the freshly collected roots was measured to determine the fresh weight and then the roots were dried at 60°C until consistent weights were obtained to obtain the dry weight of the roots. The percentage yield increase in shoot and root yield in terms of dry weight up to one year after planting compared to the control treatment was also recorded.

Statistical analysis

The collected data underwent statistical analysis using analysis of variance (ANOVA) through the "GRAPES" online software (10). This software provides a platform for comprehensive statistical analysis, facilitating the interpretation of results and drawing meaningful conclusions from the data.

Results

The results revealed significant variations in all plant growth parameters studied across all observation stages. Notably, the highest shoot length was observed in plants treated with CS 1 g/L concentration among all treatments performed. The shoot length was recorded as 59.57 cm, 124.62 cm and 150.25 cm at 3, 5 and 7 MAP respectively. The data recorded at 7 MAP reflected an increase of 19.75% over the control treatment (Fig. 1a; Supplementary Table 1).

A similar trend in data as in shoot length was observed for number of primary and spike-bearing branches. The highest number of primary branches was observed in plants treated with CS at a concentration of 1 g/L. The highest number of primary branches, 4.75, 5.75 and 7.75, was recorded at 3, 5 and 7 MAP respectively, at CS concentration of 1 g/L. Similarly, concerning the number of spike-bearing branches, the highest values were recorded in plants treated with CS at 1 g/L, with 3.75, 7.00 and 8.50 branches observed at the respective three stages of observation. In all stages of observation, higher concentrations of CS (3 and 4 g/L) exhibited lower performance than the control treatment in terms of number of primary and spike-bearing branches. At seven MAP, there was an increase of 34.78% and 21.42% in the number of primary and spike-bearing branches respectively, in the CS 1g/L treatment compared to the control (Supplementary Table 2).

Plants treated with CS concentration of 1 g/L showed early spike emergence (73.38 days), while a longer duration (85.87 days) of spike emergence was observed in plants treated with 4 g/L CS concentration. Similarly, early spike maturity, with a duration of 59.83 days, was observed in plants treated with a lower concentration of CS (1 g/L). Furthermore, CS at 2 g/L also showed early emergence, flowering and spike maturity compared to the control treatments. Conversely, higher concentrations of CS, 3 g/L and 4 g/L concentration, showed a delay in these parameters compared to the control treatment (Supplementary Table 3).

The photosynthetic system is crucial in regulating overall plant growth and productivity. Hence, it was investigated to elucidate the effect of CS treatments on stomatal conductance, photosynthetic rate and water-use-efficiency (WUE). The findings, shown in Supplementary Table 4, revealed that the highest values for these parameters were consistently obtained with CS concentration of 1 g/L. Similarly, stomatal distribution and conductance showed similar trends, with significant differences observed in plants treated with 1 g/L CS concentration, exhibiting the highest stomatal conductance of 496.50 mmoles/m2s (Fig. 1b). This trend was also reflected in the photosynthetic rate and WUE, with the highest values recorded at 1 g/L CS, reaching 8.87 µmol CO_2/m^2s and 6.83 mmol CO2 H2O/mol respectively (Fig. 1c-d). Given that these characteristics rely on gas diffusion from the atmosphere to the chloroplast, it was investigated whether they are influenced by stomatal distribution. The observations revealed that the application of the lower concentration (1 g/L) CS concentration resulted in a higher number of stomata (247.75/mm^2) (Fig. 1e). To elucidate further the positive or negative effects of CS, chlorophyll concentration was determined. The results showed an increasing trend in plants subjected to lower concentrations of CS (Supplementary Table 6). Specifically, plants treated with 1 g/L CS exhibited a higher chlorophyll content of 1.875 mg/g (Fig. 1f). These findings suggest that lower concentrations of CS positively influence chlorophyll content, stomatal distribution and photosynthetic parameters, ultimately enhancing plant growth and productivity.

The activity of antioxidant enzymes, catalase and peroxidase were determined for the investigation on the effect of CS on plant stress. It was observed that CS intensified the activity of these enzymes *in vitro*, particularly when plants were subjected to lower concentrations of CS (1 g/L) (Supplementary Table 7). Foliar spray of 1 g/L CS concentration resulted in enzyme activities of 19.86 activity/g min for peroxidase (POX) and 933.48 activity/g min for catalase (CAT). Conversely, a significant decline in enzyme activity was observed at higher CS concentrations (3 and 4 g/L). The results indicate that across all 5 treatments, lower concentrations of CS exhibited elevated enzyme activity compared to higher concentrations and to the control treatment (Fig. 2 a-b). These findings suggest that lower concentrations of CS might improve plant's antioxidant defense mechanisms, thereby mitigating oxidative stress and promoting overall plant health and resilience.

Piper longum L. is renowned for its potent extracts, namely oleoresin and volatile oil, which possess significant medicinal properties. Therefore, enhancing these

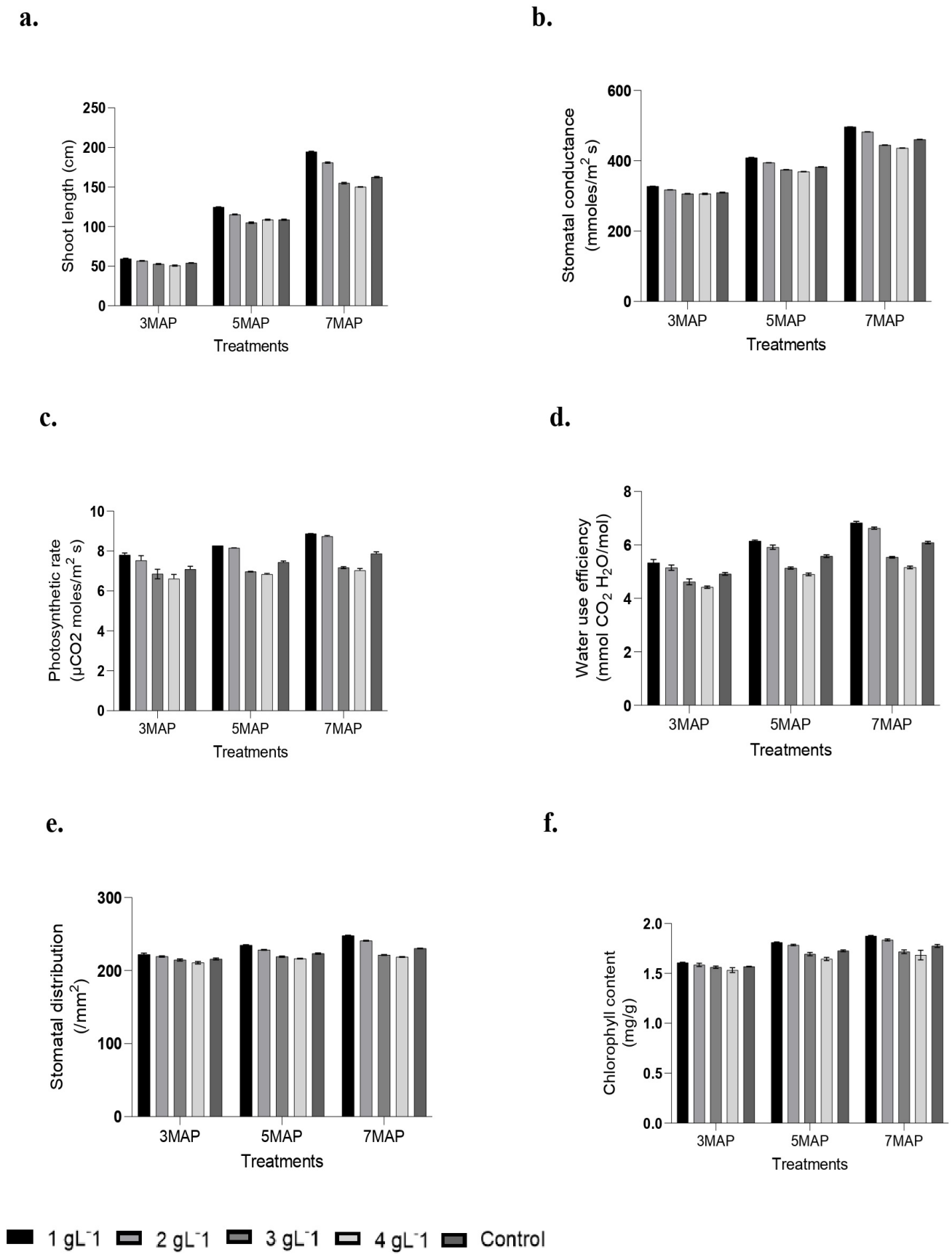

Fig. 1. **(a)** Shoot length of the plant after one month of each foliar treatment **(b)** Stomatal conductance of plant **(c)** Photosynthetic rate **(d)** water use efficiency **(e)** Stomatal distribution **(f)** chlorophyll content of plant one month after each foliar application of CS on 2, 4, 6 MAP. The plot is statistically significant according to the one-way ANOVA, $p< 0.05$. Represented values are the mean ± SE, n=4 r=3.

characteristics is crucial. For this, the quantity of oleoresin and volatile oil in both treated and control samples were analysed. The results indicate that CS at a concentration of 1 g/L showed the highest quantity among all the treatments observed (Fig. 2 c -d). Specifically, the highest quantities of oleoresin and volatile oil collected were 3.39 g/plant and 0.46 g/plant respectively. When comparing the yield, it was found that CS at 1 g/L resulted in a 25.86% increase in oleoresin yield and a 27.82% increase in volatile oil yield compared to the control treatment (Supplementary Table 8). These findings suggest that CS at 1 g/L positively influences the production of oleoresin and volatile oil in *P. longum* L., potentially enhancing its medicinal properties and utility.

Through spectrophotometric analysis, the acetone extract of spike samples revealed varying amounts of piperine content across different treatments. The highest piperine content, at 1.34%, was observed in plants treated with 1 g/L CS, while the least amount of piperine content, at 0.72%, was recorded in plants treated with a higher concentration of CS, 4 g/L. Among all the 5 treatments, lower concentrations of CS (1 and 2 g/L) exhibited higher yield of piperine, followed by the control plants (Fig. 3a; Supplementary Table 9) and similar observations were recorded in gene expression. The expression level of the gene piperine synthase, involved in piperine metabolism, was significantly affected by CS foliar treatment. Lower concentrations of CS induced upregulation of the piperine synthase gene compared to other treatments. Compared to non-CS-treated plants, gene expression increased by 1.8-fold and 1.3-fold in plants subjected to CS at 1 g/L and 2 g/L respectively (Fig. 3b; Supplementary Table 9). Conversely, there was a downregulation of the piperine synthase gene at higher concentrations of CS compared to the control treatment. These findings underscore the importance of optimizing CS concentration for maximizing piperine content and gene expression in *P. longum* L.

As most of the studied parameters were significantly influenced by CS application the overall yield was also assessed. It was observed that the highest number of spikes (181.50) was recorded in plants treated with CS at 1 g/L, while the lowest number of spikes (154.50) was observed in plants treated with CS at 4 g/L. Furthermore, the longest spike (3.25 cm) was observed in plants treated with CS at 1 g/L, with a similar trend observed for spike girth, where the highest girth (9.97 mm) was recorded at the same concentration. Plants treated with CS at 1 g/L and 2 g/L exhibited higher numbers and dimensions (length and girth) of spikes compared to the control treatment (Supplementary Table 10). Additionally, the highest spike yield (163.35 g/plant fresh, 29.40 g/plant dry) was recorded in plants treated with CS at 1 g/L and root yield was also higher in this treatment, with a fresh weight of 47.08 g/plant and a dry weight of 8.24 g/plant. Consistently, at all stages of observation, the foliar spray treatment with CS at 1 g/L exhibited the highest values for the above-mentioned yield parameters (Supplementary Table 11-12). Over the control treatment, a 21.73% and 31.84% increase was recorded in spike and root yield. Additionally, CS at 2 g/L also respectively resulted in higher yields compared to the control treatment. However, higher concentrations of CS at 3 g/L and 4 g/L were observed to be inhibitory, resulting in lower yields compared to the control treatment. These findings suggest that optimizing the

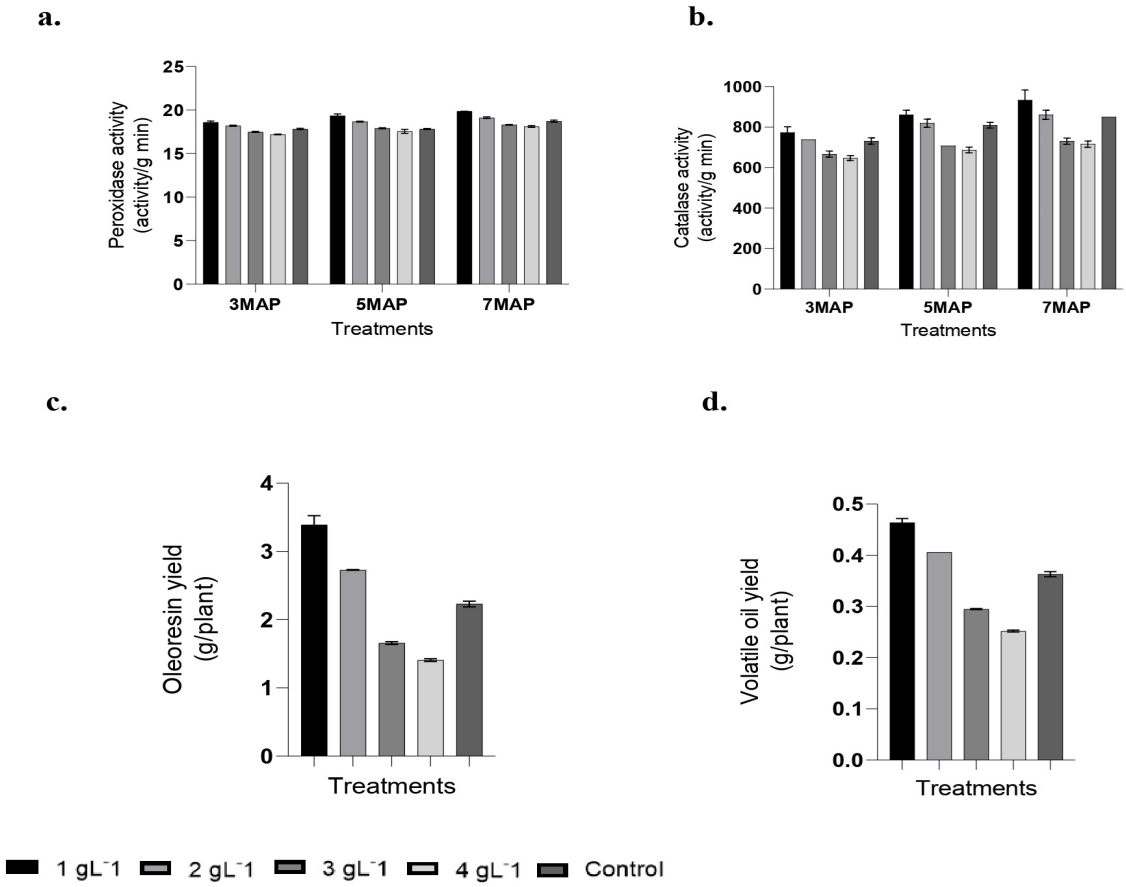

Fig. 2. (a-b) Activity of enzyme peroxidase **(a)** and catalase **(b)** after one month in the leaves of *P longum* subjected to foliar spray of CS at 2, 4 and 6 MAP. (**c-d)** the yield of oleoresin **(c)** and volatile oil **(d)** in dried spikes of *P longum* subjected to the same foliar spray. The plot is statistically significant according to the one-way ANOVA, $p < 0.05$. Represented values are the mean ± SE, n=4 r=3

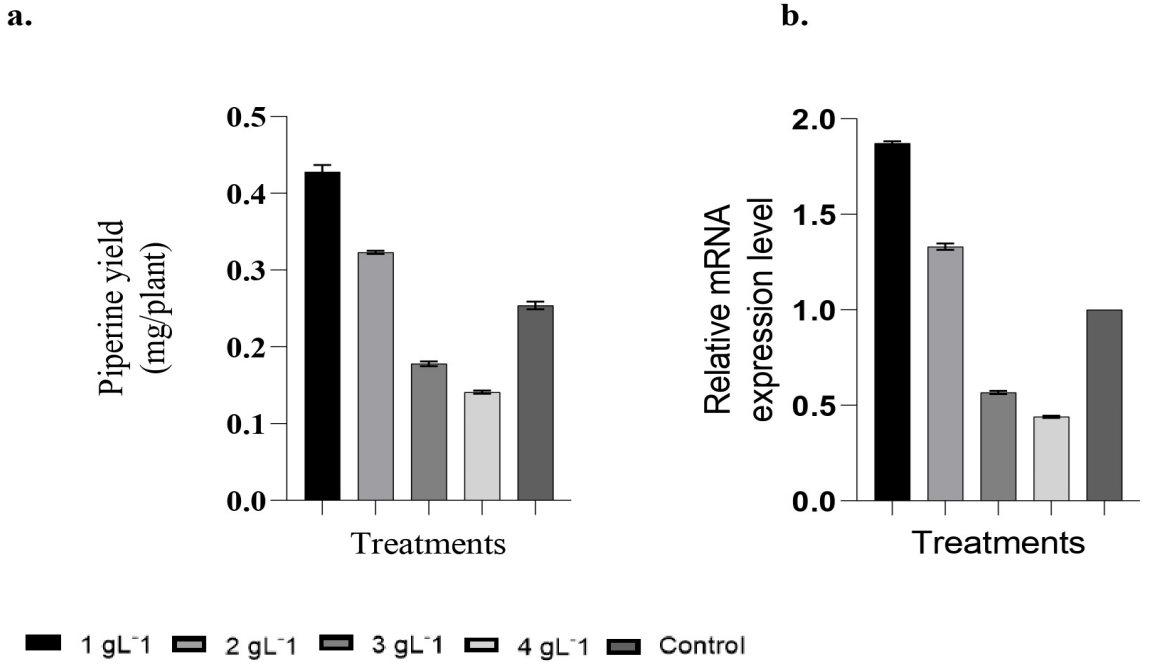

Fig. 3. (a) Piperine yield from the one-year collected dried spike *of P longum* subjected to foliar spray of CS at 2,4 and 6 MAP. **(b)** Relative expression of piperine synthase gene compared to reference gene actin using q-PCR. The plot is statistically significant according to the one-way ANOVA, p< 0.05. Represented values are the mean ± SE, n=4.

concentration of CS is crucial for maximizing yield parameters in *P. longum* L.

Discussion

The study demonstrated a significant improvement in plant growth and yield through foliar application of CS in *P. longum*. This enhancement mirrors similar findings across various crops, including watermelon (11), strawberry (12), tomato (13), cordyline (14), Chinese cabbage (15) and perilla (16). Lower concentrations of CS at 1 g/L and 2 g/L showed enhanced plant growth and yield compared to the control. Conversely, higher concentrations at 3 g/L and 4 g/L were found to be inhibitory. These results underscore the potential of CS as a promising tool for enhancing the productivity *piper longum*, emphasizing the importance of optimizing application concentrations to maximize its beneficial effects while avoiding potential inhibitory effects.

Interestingly, these findings differ from those reported (17), where higher concentrations of CS at 10 g/L led to substantial improvements in all plant growth indices compared to lower concentrations of 5 g/L and 7.5 g/L in black pepper (*Piper nigrum* L.). These inconsistencies highlight the importance of considering specific crop species and environmental conditions when determining the optimal concentration of CS for plant growth enhancement.

Chitosan activation of plant hormones including IAA (auxin), cytokinin and gibberellic acid encourages the proliferation of root cells, thereby increasing nutrient uptake (18). Additionally, when applied, it was observed that leaves absorbed CS and provides amino acids, promoting plant metabolic processes, growth and development (19). Furthermore, study suggested that CS is involved in signal transduction related to various plant growth phenomena (20). Moreover, the osmotic adjustment CS induces in stomatal cells leads to enhanced stomatal opening and CO_2 integration. This stimulation of plant physiological systems, including nutrition absorption, cell division, cell elongation, enzyme activation and protein synthesis, is directly associated with plant growth (21).

In the study, significant variations were observed among the treatments in terms of the days to emergence of spike, days to flowering and days from emergence to maturity of spike. Specifically, the concentration of CS at 1 g/L demonstrated early spike emergence, flowering and spike maturity compared to the higher concentrations and the control. Similar observations of improved flowering with CS application have been noted in various crops, including *Dendrobium* (22), *Gladiolus* (23) and potatoes (24). The early floral responses observed due to CS application could be attributed to enhanced nutrient availability, protein synthesis, cell proliferation, enzyme activity and plant vegetative growth (21, 22). This suggests that CS plays promote early flowering and reproductive development in plants.

The highest stomatal conductance (496.50 mmoles/ m2s), stomatal distribution (247.75/ mm^2), photosynthetic rate (8.87 µmol CO_2/m^2s) and WUE (6.83 mmolCO2 H2O/mol) were recorded in plants treated with foliar spray of CS concentration of 1g/L. Another study (25) support these findings who observed enhanced stomatal distribution in soybean following the foliar application of CS. Similarly, improved photosynthetic rate and stomatal conductance were reported in lemon grass (20) and *Calendula officinalis* (26) upon CS application. Due to CS application increased WUE has also been documented in plants such as *Helianthus annuus* (27) and *Calendula officinalis* (26).

Chitosan enhances the concentration of photosynthesis-related chlorophyll a, b and carotenoid pigments in leaves, leading to increased formation of organic molecules and sugars by enhancing the fixation of carbon

dioxide and water, thereby improving photosynthesis (28). CS oligomers within the cell can enhance enzyme activity by translocating into the nucleus and chloroplast, thereby improving antioxidative and photosynthetic activities (29). According to a study, CS-induced PSII stabilization, as evidenced by the upregulation of Fv/Fm, Fv'/Fm' and PSII genes, might have increased photosynthetic activity (20). The higher chlorophyll content and enzyme activity in photosynthetic cells may contribute to enhanced stomatal conductance, CO_2 absorption and increased photosynthetic rate in plant leaves (21). Both stomatal (stomatal distribution, stomatal conductance and WUE) and non-stomatal factors (chlorophyll content) modified by CS application significantly influence photosynthesis (30).

In this study, chlorophyll content was significantly enhanced by applying CS at 1 g/L, with the highest value (1.875 mg/g) observed at 7 MAP. These findings are consistent with those reported in chilli (31), *Dracaena surculose* (32), tomato (13) and lemon grass (20). The effects of CS on chlorophyll content have been attributed to its ability to block chlorophyllase, which is involved in the catabolic route for chlorophyll and stimulate the expression of genes involved in chlorophyll biosynthesis (33).

Antioxidant enzymes such as catalase (CAT) and peroxidase (POD) exhibited significant variation in activity with the application of CS at all 3 stages of observation (Fig. 2 a-b). The highest activities of CAT (933.48 activity/g min) and POD (19.86 activity/ g min) were recorded in plants treated with CS 1 g/L at 7 MAP. These results are consistent with the findings reported in orange (34), peach (35), turmeric (36), tomato (37) and lemongrass (20). The enhancement in antioxidant activity, including POD, superoxide dismutase (SOD) and CAT, upon exposure to CS, could be attributed to the upregulation of photosynthetic pigments and stomatal conductance, possibly mediated by the regulation of genes in the nucleus and chloroplast (38-40). CS treatment induces the generation of oligomers within the cell, which migrate to the nucleus and chloroplast to synthesize enzymes, thereby increasing antioxidative activity (29). By detoxifying hydrogen peroxide (H_2O_2) and superoxide radicals, CS treatment effectively protects plants from lipid peroxidation and oxidative damage (27).

Chitosan has demonstrated a capacity to enhance the production of secondary metabolites, as evidenced by the highest piperine content recorded at a concentration of CS 1 g/L. Similar enhancements in secondary metabolite production have been reported in various plant species. For example, CS application has led to increased production of flavonoids in *Hypericum perforatum* (41), phenolics in *Origanum vulgare* (42), curcumin in *Curcuma longa* (43), phenols and glycosides in *Stevia rebaudiana* (44), menthol in *Mentha piperita* (45), and xanthone in *Gentiana dinarica* (46). Additionally, essential oils in basil (47), *Thymus daenensis* (48), lemon grass (49), peppermint (50) and Java citronella (33) have been reported to improve upon CS application. The biosynthesis of piperine, a secondary metabolite in *P. longum*, follows the phenylpropanoid pathway. Phenylalanine ammonia-lyase (PAL) serves as the first enzyme in this pathway (51), where the aromatic portion of piperine likely originates. Thus, CS may stimulate the piperine production by activating genes associated with the phenylpropanoid pathway (52).

The expression analysis of the piperine synthase gene upon CS application provides valuable insights into the impact of CS on piperine production in *P. longum*. It was observed that foliar spray treatment with CS at concentrations of 1 g/L and 2 g/L significantly increased the expression of the piperine synthase gene compared to the control. This finding aligns with the notion proposed (53), suggesting that CS may influence the activity of genes and enzymes involved in secondary metabolite production. Several studies have reported CS's influence on gene expression associated with secondary metabolite biosynthesis. It was demonstrated that CS induces artemisinin biosynthesis by upregulating the ADS and DBR2 genes in *Artemisia annua* (54). Similarly, it was found that CS enhances jasmonic acid biosynthesis by upregulating the OPR gene in *Melissa officinalis* L. (55). A study suggested that CS activates the phenylpropanoid pathway, thereby affecting the production of various secondary metabolites (56). Piperine synthase is an enzyme responsible for catalyzing the conversion of piperidine and piperoyl-CoA into piperine, the final step in the synthesis pathway. The increased expression of the piperine synthase gene, particularly in the treatment with CS1 g/L, may explain the elevated piperine content observed in this treatment. This upregulation of gene expression could be attributed to activating of various signalling pathways, including secondary messengers, transcription factors and oxidative bursts triggered by CS and its oligosaccharides. By activating NADPH oxidase and inducing an oxidative burst leading to the production of H_2O_2, CS may ultimately stimulate enzyme activity such as PAL, thereby enhancing the phenylpropanoid pathway and secondary metabolite production (57). Overall, the treatment with CS at 1 g/L exhibited the highest expression of the piperine synthase gene among the tested concentrations, correlating with the highest piperine content observed in this treatment.

The significant enhancement in yield observed in the study upon foliar spray treatment with a lower concentration of CS (1g/L) is consistent with increased yield in response to CS application in a range of crops including soybean (58), sweet basil (53), sweet pepper (59), bell pepper (60), strawberry (61), potato (24), tomato (13) and artichoke (62). The improved yield observed with the exogenous application of CS can be attributed to its multifaceted effects on various physiological processes in plants. Firstly, CS has been shown to positively impact photosynthetic parameters, such as stomatal conductance, photosynthetic rate and chlorophyll content, thereby enhancing the efficiency of photosynthesis and carbon assimilation. This ultimately leads to increased biomass production and yield. Additionally, CS promotes nutrient uptake in plants, facilitating the absorption of essential nutrients from the soil. This improves nutrient availability for plant growth and development, thereby increasing yield.

Furthermore, CS has been found to stimulate cell division and elongation, leading to increased proliferation of root and shoot cells. This enhanced cell division promotes plant growth and development, ultimately translating into higher yield. Moreover, CS accelerates the transport of assimilates from the source to sink tissues within the plant. By facilitating the movement of sugars and other nutrients, CS

ensures that adequate resources are supplied to develop fruits, seeds or other reproductive structures, promoting their growth and development and ultimately increasing yield. Overall, the beneficial effects of CS on photosynthetic parameters, nutrient uptake, cell division and assimilate transport collectively contribute to the significant improvement in yield observed in plants treated with CS (21, 63).

The observed significant effect of CS foliar spray on root yield in the study aligns with findings reported in previous research. It was reported that different concentrations of CS led to enhanced root weight in bean plants (64). Similarly, another study suggested that CS enhances root cell development by inducing specific enzymes such as chitinases, pectinases and glucanases (14). CS's ability to induce ion uptake may also play a role in root development, acting as a signal for root initiation and ultimately resulting in higher root biomass. As roots are responsible for water and nutrient absorption, any enhancement in root development induced by CS treatment can substantially impact overall crop production. Therefore, the observed increase in root yield in response to CS foliar spray underscores the potential of CS as a beneficial tool for improving crop productivity, by directly influencing above-ground growth and enhancing below-ground root development and function.

In the study, it has been observed that increased concentrations of CS led to the inhibition of plant growth, physiological and biochemical parameters and subsequently yield. Foliar application of chitosan at higher concentrations increased the thickness of CS layer on the plants leaf surface. This might have inhibited the absorption of sunlight, which is indicated by low chlorophyll content at higher concentrations. Moreover, the stomatal conductance at higher concentrations was also observed to be low, which might have affected the entry of CO_2. This likely led to reduced photosynthesis, which in turn stunted plant growth, yield and production of secondary metabolites. Previous studies have also documented the inhibitory effects of high concentrations of CS on growth in wheat seedlings (65) and palm seedlings (66). Notably, elevated levels of CS (ranging from 2.5% to 4.5%) have been linked to inducing adverse effects on the foliage of *Brassica juncea* plants aged 2-3 weeks (67).

Conclusion

The study investigates the optimal concentration of CS to enhance plant growth, piperine content, yield and molecular-level responses in *P. longum*. The findings indicate that lower concentrations of CS, specifically 1 g/L and 2 g/L, resulted in improved growth and yield compared to higher concentrations and the control group. Moreover, foliar application of CS at 1 g/L demonstrated the maximum enhancement in piperine content compared to the control treatment. The findings were further supported by gene expression analysis using piperine synthase primers, which confirmed the increase in piperine content in spikes due to CS application at 1 g/L and 2 g/L. However, the higher concentrations of CS were inhibitory to plant growth and yield. CS, a non-toxic and eco-friendly biopolymer, holds promise for substantial enhancement in the growth and yield of commercially exploited medicinal plants like *Piper longum* L. However, the results of this investigation and existing literature, underscore the importance of optimizing the concentration of CS for exogenous application to promote plant growth in various plant species effectively. Further studies are warranted to elucidate the specific signal regulation and transduction components involved in the mechanism of action of CS. This will provide valuable insights into the precise mode of action of CS and facilitate its optimal utilization in *Piper longum* cultivation and potentially other crops as well.

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Authors' contributions

Conceptualization, methodology and supervision by DSN. Formal analysis and investigation by AK. Manuscript preparation, editing and review by AK, DSN, SKB, SA, MRV and VV. Resources supplied by DSN, SKB, SA and MRV. All authors read and approved the final manuscript.

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During the preparation of this work the author(s) used Grammarly in order to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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