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Chitosan foliar spray enhances photosynthetic performance in drought-stressed *Piper longum* plants

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Abstract

This study, conducted at the Department of Plantation, Spices, Medicinal, and Aromatic Crops, College of Agriculture, Vellayani, aimed to assess the impact of foliar application of chitosan on photosynthetic performance in drought-stressed long pepper (*Piper longum* L.). Two concentrations of chitosan (0.5 and 1.0 g L^{-1}) were applied to drought stress (irrigated at 60% and 75 % field capacities) exposed plants and photosynthetic parameters were observed. Chitosan-treated plants, particularly at 1 g L^{-1} concentration, exhibited significantly higher relative water content, stomatal density, photosynthetic efficiency, transpiration rate, stomatal conductance, and water-use efficiency. Chitosan-treated plants also showed improved chlorophyll fluorescence parameters, chlorophyll content, and biomass yield compared to untreated controls. These findings suggest the potential of chitosan as a bio-elicitor against abiotic stresses in long pepper, warranting further research in this area.

Key words: Chitosan, drought, photosynthetic parameters, gas exchange parameters, infrared gas analyser, drought stress

Introduction

Globally, drought stress has emerged as a vital limiting factor for the productivity of crops. Worldwide drought has increased dramatically in recent years because of the increasing impacts of climate change. Water scarcity outbreaks are attributed to reduced precipitation or its complete absence, leading to diminished soil moisture levels and decreased water availability in the aboveground plant organs, including leaves and stems (Ristvey *et al.,* 2019).

Plants express an extensive series of reactions toward drought conditions that are generally shown by a range of modifications in their morphological, physiological, and biochemical parameters (Iqbal *et al.,* 2022). Photosynthesis is regarded as the most essential process on the Earth for sustaining life, as it is the sole method of capturing and converting light energy into chemical energy, which is then used by living organisms (Zargar *et al.,* 2017). It is also a crucial focus of research in the context of abiotic stress, as it plays a direct role in the vital processes of plants (Shen *et al.,* 2021). Light reactions that take place in chloroplast are highly water-dependent. Hence, drought would substantially affect photosynthetic processes (Qiao *et al.,* 2024).

During drought, the water potential of the soil decreases, which in turn directly impacts the plant's water potential (Sharma *et al.,* 2020). The initial response of plants to drought stress is to reduce transpiration by closing their stomata. Stomata optimize carbon gain while minimizing water loss, adjusting their aperture based on environmental conditions (Nakad, 2024). While stomatal closure helps to conserve water, it reduces the absorption of CO_2, restricts the transport of non-structural carbon and leads to a decline in photosynthetic activity (Talbi *et al.,* 2020).

Drought conditions cause a notable decline in net photosynthetic rate (Pn) and stomatal conductance (Gs). In winter wheat, both moderate and severe drought stress led to a substantial reduction in these parameters, which consequently reduced biomass and yield (Zhao *et al.,* 2020). Drought stress adversely affects chlorophyll fluorescence parameters, signalling a decline in photosynthetic efficiency (Abid *et al.,* 2017).

Long pepper (*Piper longum* L.) is a perennial herbaceous shrub or vine native to the Indo-Malaya region. It is used as spice and as medicine in the Indian traditional medical system Ayurveda. It contains many bioactive phytochemicals like alkaloids (piperine and piperlongumine), flavonoids, esters, and steroids. Its essential oils exhibit antimicrobial, anti-inflammatory, antioxidant, anticancer, neuro-pharmacological, and cardioprotective properties (Carsono *et al.*, 2022). The crop holds immense economic significance in the food and pharmaceutical industries.

While *P. longum* shows certain mechanisms for drought resistance, prolonged drought can still lead to significant stress, affecting its growth and medicinal properties. According to Krishnamurthy and Saji (2006), *P. longum* is a drought susceptible plant as black pepper (*Piper nigrum*). Being a susceptible crop, drought might affect its yield and quality. Chitosan foliar spray has demonstrated its ability to boost photosynthetic performance in plants under drought stress by enhancing physiological and biochemical responses. Dowom *et al.* (2022) reported enhanced chlorophyll content and photosynthetic efficiency in drought-stressed *Salvia abrotanoides* plants on the application of chitosan nanoparticles. This biopolymer has potential to be used in crop production due to their ability to scavenge reactive oxygen species (ROS) and enhance stress tolerance (Hidangmayum *et al.,* 2019).

This study aims to evaluate the effects of chitosan foliar spray on key photosynthetic parameters in *P. longum*, under drought-stressed conditions. By doing so, we hope to shed light on the potential of chitosan as a drought stress mitigator in long pepper cultivation.

Materials and methods

Plant materials and experimental site: The study was conducted in 2023-2024 at the Department of Plantation, Spices, Medicinal and Aromatic Crops, College of Agriculture Vellayani, Kerala Agricultural Unversity. Two-month-old rooted cuttings of long pepper plants, variety Viswam, released by Kerala Agricultural University, were used for the experiment. The potting mixture was composed of soil, farm yard manure (FYM), and sand in 1:1:1 ratio and was supplemented with 100 g of FYM per pot at monthly intervals. The plants were staked one month after transplanting and maintained in rain-out shelter conditions until 4 MAT, after which drought stress was imposed giving controlled irrigation. The stress was maintained for two weeks after which normal irrigation was resumed. The plants were grown under mild shade conditions with 10 h sunlight per day under 1200 μ mol m^{-2} h^{-1} at 30°C ± 2°C during day time and 25°C ± 1°C during night time. Observations were taken 10 days after imposing restricted irrigation.

Design of experiment and treatment details: The experiment was laid out in a completely randomized design (CRD) with nine treatments and three replications. Plants were given normal irrigation until 4 MAT. Chitosan foliar spray was given at two levels (0.5 g L^{-1} and 1.0 g L^{-1}) at transplanting, 2 MAT, 4 MAT and 6 MAT. The foliar solution was prepared by dissolving the specified quantity of chitosan in acetic acid solution (10 mL of acetic acid made up to 1 L distilled water). The drought stress was imposed at 4 MAT by the gravimetric method. The stress was also imposed at two levels (75% FC and 60% FC) along with the unstressed control plants (100% FC). The treatment details are given in Table 1.

Table 1. Treatment details

Treatment	Conditions
T1	100% FC + unsprayed control
T2	100% FC + 0.5 g L^{-1} chitosan
T3	100% FC + 1.0 g L^{-1} chitosan
T4	75% FC + unsprayed control
T5	75% FC + 0.5 g L^{-1} chitosan
T6	75% FC + 1.0 g L^{-1} chitosan
T7	60% FC + unsprayed control
T8	60% FC + 0.5 g L^{-1} chitosan
T9	60% FC + 1.0 g L^{-1} chitosan

Measurement of gas exchange parameters: The stomatal distribution (stomatal density) was determined using the epidermal imprinting technique as described by Hörmann *et al.* (2018) using an inverted microscope under 40X magnification. The number of stomata per unit area was counted and recorded in mm^{-2}. A portable photosynthesis system (LI-6400, LI-COR Inc, Lincoln, USA) was used to measure the photosynthetic gas exchange parameters of the sampled leaves between 08:30 and 11:30 hours. The ambient temperature was maintained at 27 ± 3°C. During the measurements, the CO_2 concentration, relative humidity, and air temperature in the leaf chamber were 365 ± 5 mmol m^{-2}, 60 ± 4.0%, and 26 ± 1.5°C, respectively. A range of 6 photosynthetically active radiation (PAR) levels (0, 400, 600, 800, 1000, and 1200 μmol/m²/h) at 30°C was provided by a cold LED light source. The automatic system recorded transpiration rate (E), net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and water-use efficiency (WUE).

WUE was calculated using the formula, WUE=Pn/E.

Measurement of chlorophyll fluorescence parameters: A portable photosynthesis system (LI-6400, LI-COR Inc, Lincoln, USA) was employed to measure chlorophyll fluorescence parameters, including the minimum chlorophyll fluorescence yield in the dark-adapted state (F_0), maximum fluorescence yield in the dark-adapted state (F_m), steady-state fluorescence yield (F_s), minimum fluorescence in the light-adapted state (F_0’), and maximum fluorescence yield in the light-adapted state (F_m’). Each measurement was repeated three times. Under 800 μmol m^{-2} s^{-1} light, the leaves of treated plants reached a steady state, after which F_s was recorded. Then, using saturated pulsed light (12,000 μmol m^{-2} s^{-1}), F_m’ was measured. Following the closure of the actinic light, far-red light was immediately activated, and F_0’ was measured after 2 s. The leaves were then dark-adapted for 30 min using a clip, and F_0 and F_m were subsequently measured.

Additional chlorophyll fluorescence parameters were calculated using the following formulas (Liang *et al.*, 2017)

Maximum quantum yield of photosystem II (PSII) (F_v/F_m) = (F_m - F_0)/F_m

Effective quantum yield of PSII (ΦPSII) = (F_m’ - F_s)/F_m’

Physiological parameters: Total chlorophyll content in leaves was determined following the method described by Sun *et al.* (2021). The relative water content of leaves was analyzed as prescribed by Noun *et al.* (2022).

Spike yield: The spike yield, both fresh and dry was determined up to one year after planting. The mature unripe greenish-black spikes were collected, weighed and dried at 60°C until constant weight was obtained.

Statistical analysis: The observations will be subjected to statistical analysis adopting standard procedures (ANOVA) by using KAU Grapes Software.

Results

Relative water content (RWC): The effect of chitosan on the relative water content (RWC) in drought-stressed *Piper longum* plants (Table 2) revealed significant differences among treatments. RWC decreased under drought, with the lowest value (61.25%) observed in plants irrigated at 60% field capacity (FC) without chitosan treatment. In contrast, higher chitosan concentrations significantly improved RWC. The highest RWC (95.39%) during drought occurred in plants irrigated at 100% FC and treated with 1 g L^{-1} chitosan foliar spray, followed by plants irrigated at 100% FC with 0.5 g L^{-1} chitosan and those at 75% FC with 1 g L^{-1} chitosan. Even plants irrigated at 60% FC showed higher RWC with chitosan treatment compared to untreated controls.

Gas exchange parameters: The effect of chitosan on stomatal distribution in *Piper longum* under drought stress demonstrates significant improvement in all gas exchange parameters compared to respective unsprayed treatments (Fig. 1-6). Under severe drought (60% FC), stomatal density was 239.79 stomata mm^{-2}, while the application of chitosan 1 g L^{-1} increased this to 259.47

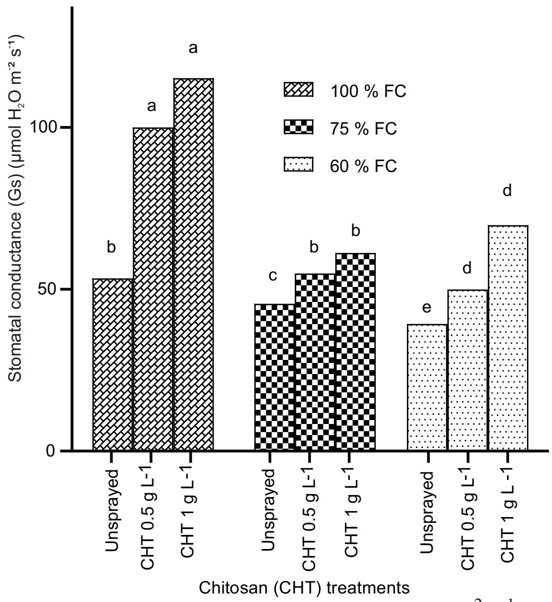

Fig.3. Stomatal conductance (mmoles H_2O m^{-2} s^{-1}) in drought stressed *P. longum* plants

stomata mm^{-2}, representing a 40.70 % increase. In moderate drought (75% FC), the unsprayed plants showed a stomatal density of 199.98 stomata mm^{-2}, which increased to 305.45 stomata mm^{-2} with 1 g L^{-1}chitosan, marking a 52.74 % increase. Under well-watered conditions (100% FC), the stomatal density was 239.79 stomata mm^{-2}, which when given a foliar spray with chitosan showed a significant improvement. With chitosan 1 g L^{-1}, the stomatal density increased to 337.49 mm^{-2} (45.69 % increase) and with chitosan 0.5 g L^{-1}, it increased to 269.15 mm^{-2} (12.24 % increase). The higher concentration of chitosan 1g L^{-1} has given significant improvement of 40-52 % increase compared to chitosan 0.5 g L^{-1}, which gave a rise of 12 to 35 % over the respective unsprayed treatments (Fig. 1). Imposition of drought led to reduction in Pn by 13.44 % for mild drought (75 % FC) and 86.14% for severe drought (60 % FC) over normal condition (100 % FC). The plants irrigated at 100 % FC and exposed to chitosan 1 g L^{-1}, recorded the highest Pn of 11.54 µmol CO_2 m^{-2} s^{-1}, 66.76 % increase over the respective unsprayed treatment. This was observed to be on par with that exposed to chitosan 0.5 g L^{-1}, with a Pn of 11.11 µmol CO_2 m^{-2} s^{-1} (60.54 % increase). The lowest photosynthetic rates were observed at 60 % FC, with Pn values of 0.96 µmol CO_2 m^{-2} s^{-1}. At 60 % FC, with the foliar spray of chitosan of 0.5 and 1 g L^{-1}, Pn was increased to 4.48 and 4.50 µmol CO_2 m^{-2} s^{-1}, respectively (Fig. 2). There was a decline of 15.01 % in stomatal conductance at 75 % FC and 26.53 % at 60 % FC. The highest stomatal conductance (115.27 mmol H_2O m^{-2} s^{-1}) was recorded in the treatment, 100 % FC with chitosan 1g L^{-1} with an increase of 115.22 % over the respective unsprayed treatment. This was followed by the treatment of 100 % FC with chitosan 0.5 g L^{-1}, with the stomatal conductance of 100.02 mmol H_2O m^{-2} s^{-1} (86.74 % increase). The lowest stomatal conductance was observed in 60 % FC not exposed to chitosan foliar spray 39.35 mmol H_2O m^{-2} s^{-1} (Fig. 3). Drought had a significant declining effect on transpiration rate (E) with decrease of 25.94 % (75 % FC) and 69.76 % (60 % FC) over normal condition (100 % FC) (Fig. 4). The highest E (1.94 mmol H_2O m^{-2} s^{-1}) during drought was observed in 100 % FC with chitosan 1 g L^{-1} with 48.89 % increase over the respective unsprayed treatment. The lowest E (0.39 mmol H_2O m^{-2} s^{-1}) among the treatments was observed at 60 % FC. With the application of chitosan 0.5 and 1 g L^{-1}, the E at 60 % FC improved to 0.95 and 1.30 mmol H_2O m^{-2} s^{-1}, respectively, corresponding to 128.20 % and 233.33 % increase. The impact of drought on WUE was evident (Fig. 5), with a significant decrease across unsprayed treatments, with 21.71 % and 54.11 % decline at 75 and 60 % FC over normal irrigation (100 % FC). Among the treatments, the highest WUE (6.90 µmol CO_2 mmol^{-1} H_2O) was recorded at 100 % FC with chitosan 0.5 g L^{-1} which represented a 29.77 % increase over the respective unsprayed control. This was followed by 100 % FC with chitosan 1 g L^{-1} with WUE of 5.95 µmol CO_2 mmol^{-1} H_2O, corresponding to a 12 % increase. The lowest WUE (2.44 µmol CO_2 mmol^{-1} H_2O) was observed at 60 % FC not exposed to chitosan application. With the foliar application of chitosan 0.5 and 1 g L^{-1}, WUE at 60 % FC enhanced to 3.48 and 4.70 µmol CO_2 mmol^{-1} H_2O, respectively. Drought imposition led to an increase in Ci by 20.05 % at 75 % FC and 23.97% at 60 % FC, compared to the normal condition (100 % FC). The internal CO_2 concentrations were the lowest (227.77 µmol CO_2 mol^{-1}) at 100 % FC with chitosan 0.5 g L^{-1}. This was observed to be on par with 75 % FC with chitosan 0.5 g L^{-1}. However, at a higher concentration of chitosan, Ci significantly increased over the lower concentration but was still less than the respective unsprayed treatment. The highest internal CO_2 concentration, 359.23 µmol CO_2 mol^{-1} was observed at 60 % FC, without chitosan foliar spray. This was on par with 75 % FC with no chitosan foliar spray with Ci of 347.70 µmol CO_2 mol^{-1} (Fig. 6).

Chlorophyll fluorescence: The effect of chitosan on the chlorophyll fluorescence parameters in *Piper longum* plants during drought revealed significant differences among the treatments (Fig. 7-8). Drought conditions led to a slight reduction in Fv/Fm, 0.73 at 75 % FC (corresponding to a 3.2 %

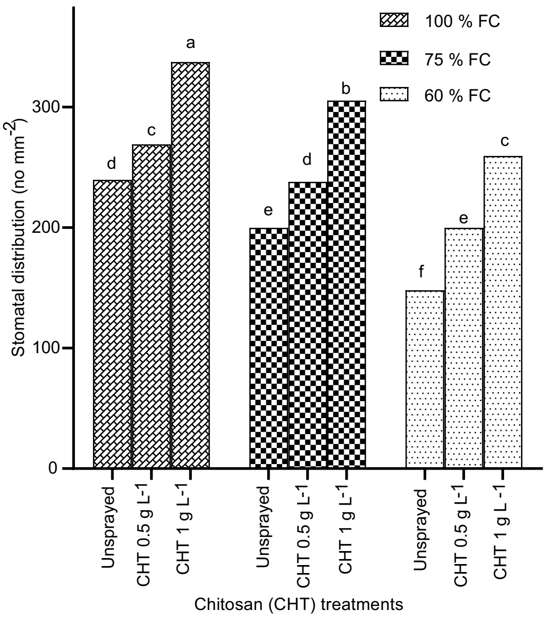

Fig.1. Stomatal distribution (no mm^{-2}) in drought stressed *P. longum* plants

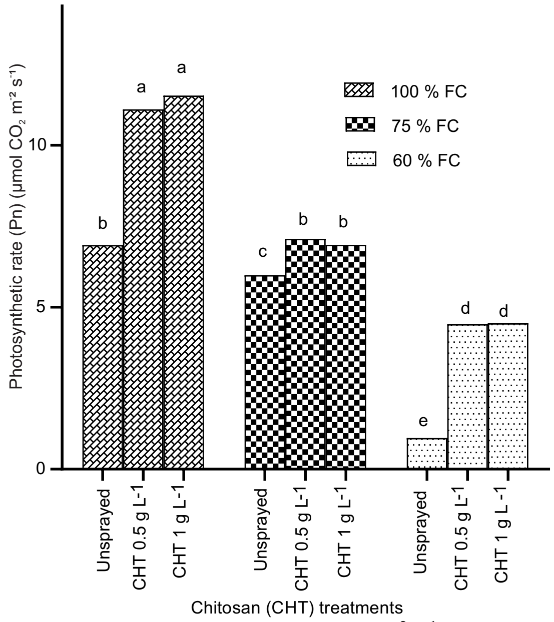

Fig.2. Photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) in drought stressed *P. longum* plants

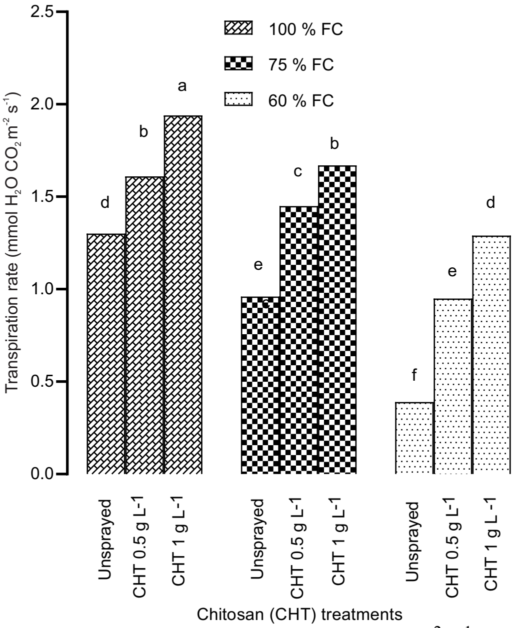

Fig.4. Transpiration rate (mmol H_2O CO_2 m^{-2} s^{-1}) in drought stressed *P. longum* plants

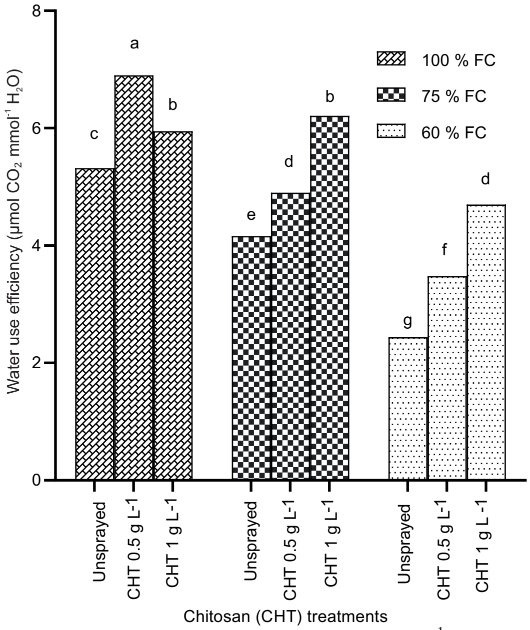

Fig.5. Water use efficiency (µmol CO_2 $mmol^{-1}$ H_2O) in drought stressed *P. longum* plants

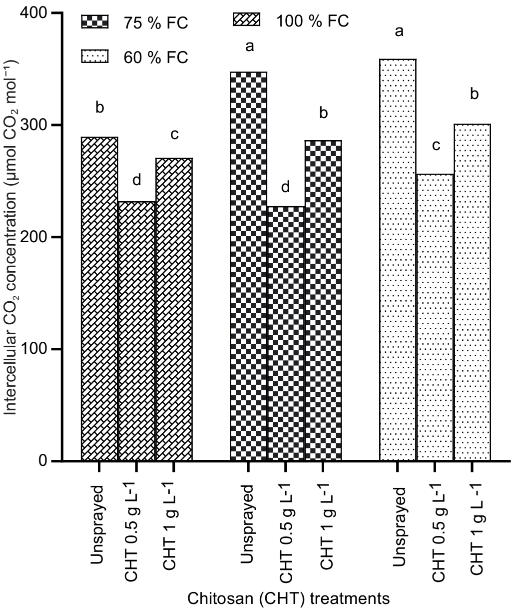

Fig. 6. Intercellular CO_2 concentration (µmol CO_2 mol^{-1}) in drought stressed *P. longum* plants

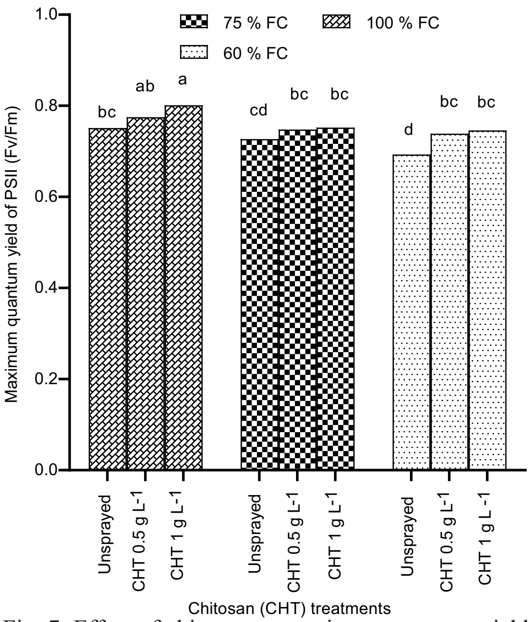

Fig. 7. Effect of chitosan on maximum quantum yield of PSII in drought stressed *P. longum* plants

decrease) and Fv/Fm, 0.69 at 60 % FC (corresponding to a 7.73 % decrease) compared to normal condition (100% FC). The highest Fv/Fm (0.80) was observed at 100 % FC with chitosan 1 g L^{-1}. This was on par with chitosan 0.5 g L^{-1} at the same level of irrigation. The lowest Fv/Fm, 0.69 was observed at 60 % FC. With the application of chitosan 0.5 and 1 g L^{-1}, Fv/Fm enhanced to 0.74 and 0.75, respectively. Severe drought reduced ΦPSII significantly (Fig. 8). At 60 % FC, the lowest value of ΦPSII 0.19, corresponding to a 52.5% decrease was recorded and at 75 % FC, it dropped to 0.29 (27.5% decrease), over the normal condition. However, treatments with chitosan showed improvement, 100 % FC with chitosan 0.5 g L^{-1}, recorded the highest ΦPSII value of 0.56, representing a 40% increase over control, followed by 100 % FC with chitosan 1 g L^{-1} with ΦPSII 0.52, corresponding to 30 % increase over the respective unsprayed treatment. The lowest ΦPSII (0.19) was observed at 60 % FC without chitosan. In the case of drought-stressed plants with chitosan 0.5 g L^{-1} showed a higher increase over control than chitosan 1 g L^{-1}. At 60 % FC, with the application of chitosan 0.5 and 1 g L^{-1}, ΦPSII enhanced to 0.25 and 0.23, respectively.

Total chlorophyll content*:* The effect of chitosan on total chlorophyll content in drought-stressed *Piper longum* plants showed a clear difference among the treatments (Table 2). Drought led to a reduction in total chlorophyll content by 13.49 % at 75 % FC and 26.99 % at 60 % FC over normally irrigated plants (100 % FC). The highest chlorophyll content, 1.975 mg g^{-1}, was observed at 100 % FC with chitosan 1 g L^{-1}, corresponding to a 30.01 % increase over respective unsprayed treatment. The lowest chlorophyll content (1.109 mg g^{-1}) was observed at 60 % FC without chitosan. With the application of chitosan 0.5 and 1 g L^{-1}, the chlorophyll content improved to 11.72 and to 21.37 % over the corresponding unsprayed control.

Yield: The effect of chitosan on spike yield in drought-tressed *Piper longum* plants showed significant variation among the treatments (Table 2). The highest fresh yield was recorded in 100% FC sprayed with 1 g L^{-1} chitosan at 164.29 g, marking a 9.78 % increase over the control. It was observed to be on par with 100% FC sprayed with chitosan 0.5 g L^{-1}. The lowest fresh spike yield, 120.16 g, was observed at 60 % FC, which represents a 19.7 % decrease compared to 100 % FC. With the application of chitosan at 0.5 and 1.0 g L^{-1}, the fresh yield increased to 131.27 and 139.81 g, respectively, showing a 9.24 and 16.35 % increase over respective unsprayed treatment. The highest dry yield (27.56 g) was also observed in 100% FC sprayed with 1 g L^{-1} chitosan at, marking a 42.6% increase over the respective unsprayed treatment. 100% FC sprayed with 0.5 g L^{-1} recorded 23.37 g. The lowest dry spike yield, 15.51 g, was observed at 60% FC without chitosan foliar spray, corresponding to 19.7 % reduction compared to

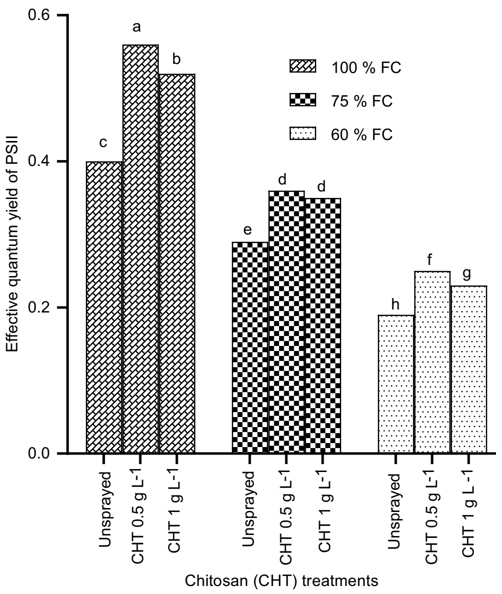

Fig. 8. Effect of chitosan on effective quantum yield of PSII in drought stressed *P. longum* plants

the plants irrigated at 100 % FC. With the application of chitosan at 0.5 and 1.0 g L^{-1}, the dry yield increased to 18.84 and 22.94 g, respectively, showing a 21.47 and 47.90 % increase over respective unsprayed treatment.

Table 2. Effect of chitosan on spikes yield per plant and total chlorophyll content under drought stress

Treatments	Relative water content (%)	Spikes yield per plant		Total chlorophyll (mg g^{-1})
		Fresh yield (g)	Dry yield (g)	
T1 (100% FC)	80.36±0.27^{d}	149.65±3.70^{b}	19.32±0.85^{e}	1.519±0.042^{c}
T2 (100% FC + 0.5 g L^{-1} chitosan)	88.35±0.80^{b}	161.37±1.89^{a}	23.37±0.89^{c}	1.756±0.055^{b}
T3 (100% FC + 1 g L^{-1} chitosan)	95.39±0.17^{a}	164.29±2.67^{a}	27.56±0.97^{a}	1.975±0.016^{a}
T4 (75% FC)	70.25±2.22^{f}	125.34±3.50^{e}	17.64±0.59^{f}	1.314±0.020^{e}
T5 (75% FC + 0.5 g L^{-1} chitosan)	75.36±1.43^{e}	141.27±1.91^{c}	20.61±0.84^{d}	1.436±0.045^{d}
T6 (75% FC + 1 g L^{-1} chitosan)	85.39±0.54^{c}	151.95±2.19^{b}	25.32±0.27^{b}	1.554±0.046^{c}
T7 (60% FC)	61.25±2.43^{h}	120.16±3.47^{e}	15.51±0.14^{g}	1.109±0.013^{g}
T8 (60% FC + 0.5 g L^{-1} chitosan)	66.29±0.06^{g}	131.27±4.73^{d}	18.84±0.49^{e}	1.239±0.016^{f}
T9 (60% FC + 1 g L^{-1} chitosan)	72.59±1.77^{f}	139.81±2.65^{c}	22.94±0.23^{c}	1.346±0.040^{e}
SE (d)	1.22	2.53	0.54	0.029
CV(%)	1.77	2.17	3.09	2.45

Table 3. Correlation among the Pn, Gs, E, Ci, WUE, total chlorophyll content, Fv/Fm and ΦPSII in drought stressed *P. longum* plants

Parameter	Photosynthetic rate	Stomatal conductance	Transpiration rate	Total chlorophyll	Internal Carbon dioxide concentration	Maximum quantum yield of PS II	Effective quantum yield of PS II	Dry yield
Photosynthetic rate	1.00	0.85	0.89	0.95	-0.63	0.80	0.96	0.75
Stomatal conductance		1.00	0.79	0.91	-0.48	0.78	0.82	0.83
Transpiration rate			1.00	0.90	-0.65	0.80	0.80	0.92
Total chlorophyll				1.00	-0.52	0.81	0.92	0.83
Internal CO_2 concentration					1.00	-0.57	-0.60	-0.50
Maximum quantum yield						1.00	0.73	0.76
Effective quantum yield							1.00	0.65

Discussion

In this study, reduction in RWC at lower levels of FC in chitosan unsprayed plants confirmed the actual event of drought stress. In chitosan sprayed drought-stressed *P. longum* plants, RWC was observed to be enhanced. Mirajkar *et al.* (2019) also observed similar findings in *Saccharum officinarum*. They suggested that this rise in RWC was associated with total soluble sugar and proline accumulation. The role of chitosan in osmotic adjustment *via* over accumulation of amino acids, such as proline, glycine-betaine, and soluble carbohydrates was reported in several horticultural and field crops, which regulates water potential in the plant tissues, thus improving RWC.

Drought stress significantly affected the photosynthetic rate decreasing it to an extent of 86.14 % for severe drought at 60 % FC. Application of chitosan under drought stress, both at 60 % and 75 % FC, significantly increased photosynthetic rate and stomatal conductance compared to their respective unsprayed treatments. Stomatal closure is one of the plant's earliest responses to drought stress, driven by the loss of leaf turgor pressure and acoustic emission events in the stem and petiole leading to decreased stomatal conductance. This reduction in CO_2 intake directly lowers the photosynthetic rate. Thus, the plant faces a trade-off between conserving water and maintaining energy production (Hussain *et al.,* 2021). Chitosan application may help plants maintain normal metabolic activity, allowing them to function more effectively under such stress because of increased chlorophyll pigments (Phothi *et al.,* 2017). Chitosan may help plants produce proteins that counteract the negative effects of osmotic stress on photosynthesis. Zhao *et al.* (2019) reported that several genes encoding proteins involved in the photosystem II reaction centre, such as *PsbP*, *Psb27*, *PsaN*, *PetF*, and *PetH*, were upregulated in annual ryegrass seedlings pre-treated with chitosan under osmotic stress. Similarly, Moolphuerk *et al.* (2022) reported an increase in photosynthetic rate and stomatal conductance under drought stress when applied with chitosan. In line with this, Zong *et al.* (2017) reported increased stomatal conductance due to chitosan application in cadmium-stressed plants.

In our study, we reported an increase in stomatal conductance in chitosan applied drought-stressed plants. In these plants, there was an increase in transpiration rate as well. Higher the stomatal conductance and stomatal density, the elevated will be the transpiration rates (Avila *et al.,* 2020). As a result, plants lose water from their aerial parts faster than roots can absorb it, particularly when soil moisture is limited. Although reducing stomatal conductance and transpiration is crucial for plant survival under water stress, this strategy comes with significant metabolic costs. It also restricts carbon dioxide intake, which is essential for the Calvin cycle and slows nutrient transport to aerial parts, as this process is driven by mass flow (Avila *et al.,* 2020). In our study, stomatal distribution and stomatal conductance were enhanced with chitosan application under both water stressed and non-stressed conditions. Stomatal density increased with chitosan application in drought stressed pot marigold and *Salvia abrotanoides* plants (Akhtar *et al.,* 2022; Dowom *et al.,* 2022). Wang *et al.* (2016) suggested that chitosan enhances stomatal conductance by increasing stomatal density and decreasing stomatal aperture ensuring reduce water loss through transpiration and sufficient CO_2 uptake for photosynthesis under drought stress.

Our experiment suggested that chitosan application reduced the damage to water use efficiency in drought stress conditions, by improving the WUE to 92.90 % at 60

% FC and 49.32 % at 75 % FC over the respective unsprayed plants. Akhtar *et al.* (2022) demonstrated that exogenous chitosan application enhances water-use efficiency (WUE) in wheat plants.

The internal CO_2 concentration has significantly increased when induced by drought stress due to a lower metabolism rate. When chitosan was applied, the internal CO_2 concentration decreased both at stressed and non-stressed conditions. This might be because chitosan application reduced the CO_2 accumulation in leaves by allowing normal metabolism. This effect is mainly due to enhanced gas exchange and greater photosynthetic efficiency. According to Ávila *et al.* (2022), chitosan application has been associated with higher stomatal conductance and increased transpiration rates in water-stressed plants, promoting improved gas exchange and hence, higher uptake of CO_2. Moolphuerk *et al.* (2022) reported that chitosan improved CO_2 assimilation under drought stress. It increases the activity of RuBisCO carboxylase, which decreases internal CO_2 concentration by catalyzing the fixation of CO_2 into organic compounds during the Calvin cycle. In contrast to these findings, Iriti *et al.* (2009) reported reduced stomatal conductance and photosynthetic rate with no change in internal CO_2 concentration with chitosan application in *Phaseolus vulgaris*. These contrasting effects could be attributed to its varied modes of application, molecular weights and degrees of deacetylation that determine its functionality.

In non-stressed plants, the maximum quantum yield of photosystem II typically ranges between 0.75 and 0.85. However, these values decrease when plants experience stress conditions like drought (Kalaji *et al.,* 2017) indicating photosystem failure. In line with the above hypotheses, the drought-stressed plants in our study reported values less than 0.75 under 75 % FC and below 0.70 under 60 % FC. Chitosan significantly improved Fv/Fm under drought as well as normal conditions. Similar results have been reported by Oliveira *et al.* (2016) in maize plants. Chitosan treatments have been found to preserve or enhance chlorophyll content, essential for photosynthesis and closely linked to the Fv/Fm ratio. In our study also, chitosan application enhanced chlorophyll in both drought-stressed and normal conditions. Additionally, chitosan boosts the activity of antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase, helping to minimize oxidative stress and lipid peroxidation, thereby safeguarding the photosynthetic apparatus (Wu *et al.*, 2024).

In accordance with the current results, Ali *et al.* (2021) reported that chitosan could enhance chlorophyll content, and thus protect the same under water deficit conditions also. Improvement in chlorophyll content under drought by chitosan also comprehends the enhanced photosynthetic rate. The decrease in chlorophyll under water deficit might be due to the destruction of the pigment-protein complexes which protect the photosynthetic apparatus, or to oxidative damage of chloroplast lipids and proteins (Shao *et al.,* 2007). In our study, the effective quantum yield in drought-stressed plants has been improved more under a lower dose of chitosan 0.5 g L^{-1}. At a higher concentration of chitosan 1 g L^{-1}, the effective quantum yield was higher to the respective unsprayed treatment but lower than that of chitosan 0.5 g L^{-1}.

Drought stress had a significant effect on the dry yield of the spikes costing more than 8.69 % reduction at 75 % FC and 19.72% at 60% FC. The application of chitosan has significantly increased the yield in both stressed and non-stressed plants. According to Pearson's correlation test at P=0.05 significance (Table 3), all the photosynthetic parameters except internal CO_2 concentration positively contributed to the dry yield of the plants. Dry yield had strong correlations with, transpiration rate (0.935), chlorophyll content (0.861), stomatal conductance (0.844) and photosynthetic rate (0.765). Internal CO_2 concentration (-0.500) had a negative correlation with dry yield. This indicates that as those physiological parameters with positive correlation *viz.,* transpiration rate, chlorophyll content, stomatal conductance and photosynthetic rate improve, dry yield tends to increase significantly, as confirmed in our study.

In conclusion, the study demonstrates that foliar application of chitosan enhances photosynthetic efficiency, gas exchange parameters, and water-use efficiency, mitigating the adverse effects of drought by promoting osmotic adjustment, increasing stomatal density, and reducing internal CO_2 concentration. Chitosan-treated plants showed improved stomatal conductance, chlorophyll content, and spike yield compared to respective unsprayed treatments. Overall, chitosan application emerges as a promising strategy to enhance the drought tolerance and productivity of *Piper longum*.

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