EFFECT OF CHITOSAN APPLICATION ON PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERISTICS OF *Piper longum* L.

by

Abhijith K.

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THESIS

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VELLAYANI. THIRUVANANTHAPURAM-695 522

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2022

DECLARATION

I, hereby declare that this thesis entitled **" Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum L.*"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society

Abhijith K.

(2017-09-012)

Vellayani

Date:

CERTIFICATE

Certified that this thesis entitled "**Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L.**" is a bonafide record of research work done independently by Mr. Abhijith K. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Vellayani
Date 1/10/2022

Dr. Deepa S. Nair,
Associate Professor and Head,
Department of Plantation Crops and Spices,
College of Agriculture, Vellayani.
Thiruvananthapuram- 695 522

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Abhijith K.** a candidate for the degree of **B. Sc. – M.Sc. (Integrated) Biotechnology**, agree that this thesis entitled **"Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L."** may be submitted by Mr. Abhijith K., in partial fulfilment of the requirement for the degree.

Deepa 1/10/2022

Dr. Deepa S. Nair,
Associate Professor and Head,
Department of Plantation Crops and Spices,
College of Agriculture, Vellayani.
Thiruvananthapuram-695522

Dr. K. B. Soni,
Professor and Head,
Department of Plant Biotechnology,
College of Agriculture, Vellayani.
Thiruvananthapuram-695522

Swapna

Dr. Swapna Alex
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani,
Thiruvananthapuram-695 522

Dr. R. V. Manju
Professor and Head
Department of Plant Physiology,
College of Agriculture, Vellayani,
Thiruvananthapuram-695522

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Abhijith k.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

@	At the rate of
ANOVA	Analysis of variance
A_{280}	Absorbance at 280
A_{260}	Absorbance at 260
CHT	Chitosan
Ct	Cycle threshold
CRD	Completely randomized design
CD	Critical difference
CAT	Catalase
cm	Centimeter
^{o}C	Degree Celsius
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetate
et al.	And others
Fig.	Figure
g	Gram
g^{-1}	Per gram
H_2O_2	Hydrogen peroxide
MAP	Month after planting
min^{-1}	Per minute
ml^{-1}	Per milliliter
mm	Millimeter
MSL	Mean sea level

ng	nanogram
NTC	Non template control
No.	Number
%	Per cent
POD	Peroxidase
PVP	Polyvinyl pyrrolidone
ppm	Parts per million
$Plant^{-1}$	Per plant
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
rpm	Revolution per minute
SE (m)	Standard error (mean)
SE (D)	Standard error (difference)
Sps.	Species
Viz.	Namely
UV	Ultra violet
V	Volt

INTRODUCTION

1.**INTRODUCTION**

Piper longum L. of the family Piperaceae popularly known as "long pepper," and *Thippali* in Malayalam, is a dioecious perennial climber with separate male and female individuals. Long pepper is native to the Indo-Malayan region. It is distributed naturally in the wild in the tropical and subtropical regions of the world *viz*., the Indian subcontinent, Sri Lanka, Nepal, Myanmar, Indonesia, Bhutan, Bangladesh, the Middle East, and the Americas. The natural distribution of the species is confined to the hotter regions from Central Himalayas, to Assam, Meghalaya, Sikkim, Arunachal Pradesh, Andhra Pradesh, West Bengal, parts of Western Ghats and Andaman & Nicobar Islands (Gajurel *et al.,* 2008; Devi and Das, 2018)

The dried female spikes (pippali) and roots (pipplamool), the economically important parts of the plant, are used in various pharmaceutical preparations. The male and female plants are morphologically similar until the formation of the spike. The female spike is shorter and thicker than the male spike, cylindrical and greenish black when ripe, with an aromatic odor and pungent taste (Oommen *et al*., 2000; Manoj *et al*., 2004; Zaveri *et al*., 2010). The leaves and thick stems are also used in Indian traditional alternative medicine. It is also listed as one of the 52 spices listed by the Spices Board of India. The importance of *P. longum* has been described in the ancient texts of the Indian system of medicine, Ayurveda, such as Charaka Samhita and Sushruta Samhita.

Piperine, piperlongumine, piperlonguminine, pipermonaline, and piperundecalidine are the primary constituents isolated from various parts of *P. longum.* The key constituent of long pepper is the alkaloid, piperine which has antipyretic, analgesic, anti-inflammatory, antioxidant, anti-Alzheimer's, anti-larvicidal and hepatoprotective activities (Santosh *et al*., 2005). It has tremendous potential for the development of drugs for respiratory disorders, viral hepatitis and tumors (Kumar *et al*., 2011). Piperine is said to be a bioavailability enhancer (Tiwari *et al*., 2020). It is also the main constituent of anti-sciatica Chinese medicine, Naru Sanwei Pill (Yu *et al*., 2021)

The plant is in great demand in both national and international markets due to its ayurvedic and pharmaceutical applications. Singh *et al*. (2004) reported that the plant is an ingredient in over 320 classical compound medicinal and herbal formulations. West Bengal and Assam are the major sources of Indian long pepper; small amounts are also obtained from the evergreen forests of Kerala.

Currently, Vietnam is the world's largest exporter of *P. longum* with a share of 22.70 per cent in export followed by Brazil with 7.30 per cent and Indonesia with 5.60 per cent. Among the countries exporting *P. longum*, India is in the fourth position with 1.90 per cent share. USA, Austria, Australia, Africa, Canada, Russia, Philippines, Singapore, etc., are some of the countries where *P. longum* is being exported regularly (Gajurel *et al*., 2021).

The major share of traded thippali is obtained from the wild. Hence, there is a great scope for the cultivation of this plant to meet the growing demand. The land availability and preference for growing medicinal plant species over other food crops on existing land are very limited in Kerala. These challenges in crop production could be overcome only by taking up alternative strategies for enhanced production and productivity. This necessitates the use of agrochemicals for improving the production and productivity. The search for replacing toxic chemical inputs to agricultural land, has led to the use of safe, ecofriendly, biopolymer-based compounds like chitosan. Chitosan is the second most common polysaccharide available on earth. It is a component of the exoskeleton of insects and crustaceans, cell walls of many fungi and some algae.

Chitosan has been used to replace chemical products in agriculture and to boost crop production in recent years. It is a non-toxic, biodegradable and biocompatible substance (Pilehram *et al.,* 2018), produced by the deacetylation of chitin. It is classified as a plant elicitor that activates the genes underlying the biosynthetic pathway of secondary metabolites and plant defense (Bistgani *et al*., 2017; Sharif *et al*., 2018). Chitosan effectively improves physiological mechanism of plant growth. It also enhances fertilizer use efficiency without affecting soil natural micro flora. When applied to

various crop plants, chitosan was observed to have a positive impact on plant growth and development, secondary metabolite production and in the alleviation of biotic and abiotic stresses.

Hence, the study entitled "Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L. " has been proposed with the following objective

- Evaluation of physiological, biochemical and molecular responses to foliar application of chitosan in *P. longum.*

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Piper longum L. also known as "thippali" or long pepper, a member of the Piperaceae family is commonly used as an effective medicine for a variety of pathological conditions, in general and respiratory disorders, in particular (Vedhanayaki *et al*., 2003). Production of long pepper is quite insufficient to meet the increasing demand of the Ayurvedic industry. The plant being extensively sourced from their natural habitat to meet the pharmaceutical demand, it has become very rare in the forests of Kerala (Nair, 2000). Initiatives have to be undertaken for the commercial cultivation and area expansion of the plant. Viswam, a high-yielding variety selected from the geographical race, Cheema thippali, has been released by the Kerala Agricultural University for encouraging its commercial cultivation.

Limited land availability and increased demand in pharmaceutical industry calls in for enhanced productivity synchronized with increased production of the active principle of the plant. Chitosan is a biopolymer-based nontoxic, decomposable and biocompatible substance that has been established as safe and effective to improve plant growth and development, secondary metabolite production, alleviation of biotic and abiotic stresses. The present study 'Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L.' was proposed to study the effect of foliar application of different concentrations of chitosan on plant growth, physiological, biochemical and molecular characteristics of *P. longum*.

In this chapter, literature on the effect of chitosan on growth and development, physiological, biochemical and molecular characteristics of plants are reviewed.

2.1 Long pepper (*Piper longum L.)*

P. longum is a dioecious climber with perennial woody roots and numerous creeping, jointed stems that are thickened at the nodes. The roots clasp at nodes, assisting in the attachment to the host trees. The leaves are alternate, spreading, without stipules and blades varying in size. The male and female plants have similar morphologies, until

the spike is formed. The female spike is erect and yellow and the male spike is greenish-yellow, fleshy and cylindrical with minute flowers. F**e**male spikes also known as long peppers, are shorter and thicker than male spikes. The fruits are tiny, ovoid berries with a lustrous blackish green color with fleshy spikes that have an aromatic scent and pungent taste when it matures (Williamson, 2002; Manoj *et al*., 2004). Because fruiting is apomictic, pollination does not necessitate a male plant. The spike grows in opposite direction of active leaves. The different plant parts including roots, shoots, and berries are used for medicinal purpose (Sujatha and Nybe, 2007). Piperine, piperlongumine, piperettine, piperlonguminine, methyl piperine, pipermonaline, and piperundecalidine are the primary constituents isolated from various parts of *P. longum*. Among these, the most abundant is piperine which is responsible for the pungency of fruits and followed by methyl piperine (Kumar *et al*., 2011).

Bhargava and Chauhan (1968) reported that dried spike on steam distillation produces 0.70 per cent essential oil. The maximum piperine content reported was 3–5 per cent (Madhavi *et al*., 2009). Khushbu *et al*. (2011) isolated piperine (0.02 per cent) and piper longumine (0.20-0.25 per cent) from the roots of the plant. Nair (2015) investigated 41 *P. longum* accessions and discovered that oleoresin, volatile oil and piperine levels ranged from 3.21 to 20.21 per cent, 0.50 to 1.60 per cent and 0.24 to 1.10 per cent, respectively.

P. longum is extensively used in traditional medicine, especially in Ayurvedic medicine. It has a wide range of applications in the medical industry. Chronic bronchitis, asthma, constipation, gonorrhea, tongue paralysis, diarrhea, cholera, chronic malaria, viral hepatitis, respiratory infections, stomachaches, bronchitis, spleen illnesses, cough and tumors are the most prevalent conditions treated with *P. longum* (Khushbu *et al.*, 2011; Gani *et al*., 2019).

Khushbu *et al*. (2011) reported that an extract of the fruits in milk protected guinea pigs from antigen-induced bronchospasm and decreased passive cutaneous anaphylaxis in rats. The *P. longum extract is* observed to have anti-allergic (Kaushik *et*

al., 2012), anti-inflammatory (Kumari *et al*., 2012) and anti-viral (Priya and Kumari (2017) activities.

2.2 Chitosan

Chitosan is a naturally occurring active polysaccharide (Jeraj *et al*., 2006) found in the cell wall of fungus, exoskeletons of crabs, shrimps and insects, parasitic nematode eggs and stomach linings. Chitosan is a deacetylated form of chitin with acids and then deproteinizes with a base. It is a linear copolymer of 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose. It is the second most prevalent polymer in nature after cellulose (Piras *et al*., 2014; Kaya *et al*., 2015). Liang *et al*. (2017) reported that chitosan is the only alkaline natural polysaccharide, which is non-toxic with good biological inter-miscibility, biocompatibility and biodegradability making it suitable for a wide range of applications. It has an effect on the stress transduction system including secondary messengers. It improves physiological responsiveness and reduces the negative effects of abiotic stress (Hidangmayum *et al*., 2019). Chitosan nanoparticles (NPs) are also employed in a variety of applications in agriculture, due to their unique qualities such as, biocompatibility, biodegradability, hydrophilicity, safety, and nontoxicity (Saharan *et al*., 2013)

2.2.1 Growth parameters

The application of chitosan has been found to influence plant growth and development in various plant species. The stimulatory effect of chitosan on physiological, biochemical, and molecular attributes might have had an influence on plant growth and development. This section summarizes the effect of exogenous chitosan application on plant growth characteristics of various plant species.

Chitosan 0.10 –0.50 per cent enhanced the yield and rosmarinic acid production in sweet basil (Kim *et al*., 2005). Uthairatanakij *et al*. (2006) reported that foliar spray with chitosan 600 mg improved fresh weight (29 g) in *Dendrobium Sonia*, compared to

untreated control (26.8 g). Limpanavech *et al.* (2008) observed early and enhanced flowering in *Dendrobium* orchids exposed to chitosan 100 ppm.

According to Farouk *et al*. (2008), chitosan 0.05 per cent significantly increased cucumber growth parameters (plant height, number of branches, shoot dry weight and leaf area), physiological aspects (nitrogen, potassium, phosphorus, calcium, total phenols and photosynthetic pigments in the shoot), yield and quality.

Gladiolus (*Gladiolus communis* L.) corm treatments with chitosan prior to planting enhanced percentage emergence, number of blooms and number of corm lets. It also prolonged vase life of flowers (Ramos-García *et al*., 2009).

El-Tantawy (2009) reported that Chito-care (chitosan) spraying significantly increased vegetative parameters (plant height and number of branches and leaves per plant), fresh and dry weight of roots, total biomass, photosynthetic pigments and yield per plant in tomato. Ghoname *et al*. (2010) reported that chitosan (2, 4, and 6 cm^3 L^{-1}) greatly enhanced plant height, number of leaves, number of branches, biomass, fruit weight and number of fruits in sweet pepper.

Mondal *et al*. (2012) reported that chitosan 25 ppm significantly increased morphological (plant height, leaf number $plant^{-1}$), growth (total dry mass $plant^{-1}$, absolute growth rate and relative growth rate), biochemical (nitrate reductase and photosynthesis) parameters and yield attributes (number of fruits $plant^{-1}$ and fruit size) in okra. Chitosan application also gave 27.90 per cent yield increase over the control.

Chookhongkha *et al.* (2012) reported that application of chitosan 1.00 per cent in soil significantly enhanced plant height, leaf width, leaf length, chlorophyll content, fruit fresh weight, seed number, seed weight and fruit number in chilli. Shehata *et al*. (2012) observed that foliar application of chitosan 4 ml L^{-1} recorded the highest vegetative growth, quality and yield in cucumber.

According to Farouk and Amany (2012), chitosan 250 mg L^{-1} increased plant growth, yield, mineral constituents, chlorophyll and carbohydrate contents in cowpea. However, these parameters were found to decline at higher concentration, chitosan 500 mg L^{-1}.

El-Miniawy *et al*. (2013) reported that foliar applications of chitosan extract (2.5 to 5.0 ml L^{-1}) with varying numbers of applications (once, twice, or three times) increased vegetative growth characteristics (plant length, number of leaves per plant, leaf area, root and vegetative growth fresh and dry weights), as well as yield attributes (fruit weight, total yields per plant) in strawberry.

A study by Salachna and Zawadzinska (2014) observed that freesia corms were soaked in chitosan 0.50 per cent for 20 min recorded more shoots, flowers and corms, early flowering and higher corm weight. Malekpoor *et al*. (2016) reported that chitosan (4 g L^{-1}) spray significantly enhanced plant growth and yield in basil (*Ocimum basilicum*).

Gomez *et al*. (2017) reported that chitosan-polyvinyl alcohol hydrogel increased stomatal width, primary stem, and root length in grafted "Jubilee" watermelon (*Cucurbita maxima x Cucurbita moschata).*

Falcon-Rodriguez *et al*. (2017) reported that foliar application of chitosan at 200 and 325 mg ha^{-1} increased potato yield by 15-30 per cent.

Mukta *et al.* (2017) observed chitosan at 250 and 500 ppm when applied at 7-days intervals from pre-flowering to post flowering stage on strawberry plants, yielded 56 per cent and 43 per cent greater fruit yield, respectively compared to non-treated control.

Mahmood *et al*. (2017) opined that when chitosan 0.50 per cent was applied to the leaves of bell pepper (*Capsicum annuum*), there was significant increase in fruit weight, diameter and yield.

Uge *et al*. (2018) reported that chitosan treatment had a substantial impact on plant height and leaf diameter in black pepper (*Piper nigrum* L.). On application of chitosan 1 per cent, plant height and leaf diameter increased by 58.12 and 54.74 per cent, respectively.

According to Rahman *et al.* (2018), foliar application of chitosan 500 ppm on strawberry significantly enhanced plant growth and fruit yield (up to 42 per cent higher). It also improved carotenoids, anthocyanins, flavonoids and phenolics compared to untreated control.

Foliar application of chitosan 0.5 g L^{-1} improved fresh and dry weights of roots, while chitosan at 1 g L^{-1} recorded the highest value of plant height and chlorophyll content in leaves of *Dracaena surculosa* (El-Khateeb *et al*., 2018).

El-Serafy (2020) observed that foliar spray of oligo-chitosan 50 mg L^{-1} significantly enhanced plant growth and root development in cordyline, which reflected on higher plant biomass when compared to control.

According to Hassnain *et al*. (2020), foliar spray with chitosan 100 mg L^{-1} improved plant height, number of leaves, leaf area, chlorophyll content, relative water content, excised leaf water retention, fruit per plant, fruit weight and yield in tomato. Morovvat *et al*. (2020) observed that foliar spray of chitosan 2 g L^{-1}at flowering stage and at mid-growth stage of tubers enhanced the yield of potatoes (45.68 t per ha).

Abd-El-Hady (2020) reported that foliar spray of chitosan 60 ppm significantly enhanced plant height, number of spikes, spike length and spike fresh weight in *Polyanthes tuberosa.*

Alkharpotly and Abdelrasheed (2021) found that chitosan 300 mg L^{-1} increased yield and quality of 'Balady' globe artichoke plants. Salachna and Lopusiewicz (2022) reported that in Perilla (*Perilla frutescens*), chitosan oligosaccharide at 50 and 100 mg L^{-1} enhanced plant height (14.6 per cent and 13.2 per cent), fresh weight of the aboveground

section of the plants (17.1 per cent and 26.7 per cent), leaves (21.8 per cent and 35.5 per cent) and roots (52.20 per cent and 52.20 per cent).

Faqir *et al.* (2022) reported that chitosan microspheres-based controlled-release of nitrogen fertilizer showed greater plant height (17.04 cm), number of leaves (11.0) and leaf length (62.19 mm) in Chinese cabbage over the untreated control.

Ahmed *et al*. (2022) reported an increase in plant height (49.00 per cent), plant fresh weight (62.70 per cent) and plant dry weight (78.90 per cent) in Java citronella (*Cymbopogon winterianus* Jowitt) on application of chitosan.

2.2.2 Physiological parameters

Chitosan functions as a regulator of photosynthesis and enzyme activities, depending on its concentration, analog, manner of application and plant type. Application of chitosan has been observed to enhance the enzyme activities and influence factors associated with photosynthesis (Mondal *et al*., 2013; Phothi and Theerakarunwong, 2017). The function of catalase and peroxidase enzymes in plants is to protect the cells from the toxic effect of reactive oxygen species (Zandi and Schnug, 2022).

Meng *et al*. (2008) reported in grapes, that chitosan 10 g L^{-1} enhanced polyphenol oxidase, peroxidase and phenylalanine ammonia-lyase. Zeng *et al.* (2010) reported that chitosan 2 per cent enhanced the activities of peroxidase (POD), superoxide dismutase (SOD), glutathione (GSH) and hydrogen peroxide (H_2O_2) in naval oranges.

According to Sheikha *et al*. (2011), chitosan 0.50 per cent significantly increased chlorophyll in the bean. Chitosan 0.0625 and 0.125 per cent enhanced chlorophyll content, photosynthetic rate, stomatal conductance, SOD, CAT and POD activities in wheat (Ma *et al*., 2014). Zou *et al.* (2017) reported that exogenous chitosan treatment increased chlorophyll content, and antioxidant activities of SOD, POD, CAT and APX and also the expression of a number of antioxidant enzyme genes.

Mandal (2010) observed enhanced peroxidase (POD), catalase (CAT) and polyphenol oxidase (PPO) activity at 24h after elicitation using chitosan in *Solanum melongena*. According to Jiao *et al*. (2012), chitosan 100 mg L^{-1} reduced drought stress in potatoes by improving their antioxidation capacity and protective enzyme activities, as well as by controlling the amount of osmotic regulatory substances.

Enhanced activities of antioxidant and defense-related enzymes, such as β-1,3-glucanase (GLU), catalase (CAT) and peroxidase (POD) were observed in peach, post chitosan application by upregulating the associated genes (Ma *et al*., 2013)

In a study conducted by Anusuya and Sathiyabama (2016), it was observed that foliar application of chitosan 0.10 per cent, at monthly intervals, improved defense enzymes activities such as peroxidase (POD) and polyphenol oxidases (PPO) in the leaves and rhizomes of turmeric plants.

Li *et al*. (2017) reported that *Trifolium repens* were pretreated with chitosan 1 mg ml^{-1} led to the development of organic acids, and other metabolites important for osmotic adjustment, antioxidant defense and stress signaling. It also upregulates genes related to amino acid and carbohydrate metabolism, energy synthesis and conversion and ascorbate–glutathione and flavonoid metabolism.

Shabani *et al*. (2019) reported that chitosan 100 mg L^{-1} significantly enhanced the activities of phenylalanine ammonia-lyase (PAL), catalase (CAT), lipoxygenase (LOX), and guaiacol peroxidase (GPX) by upregulating PAL1, TAT and RAS gene in *Melissa officinalis* L.

Enhanced chlorophyll content in response to foliar application of chitosan was observed in freesia (Salachna and Zawadzinska, 2014), thyme (Bistgani *et al*., 2017), grapes (Singh *et al*., 2020) and bent grass (Geng *et al*., 2020). According to Fouda *et al*. (2022), a foliar spray of chitosan 750 ppm on faba bean (*Vicia faba* L) improved total chlorophyll, 63.83 per cent higher than that of untreated control.

Geng *et al.* (2020) observed reduced oxidative damage to leaves and roots on exogenous chitosan application due to enhanced antioxidant enzyme (SOD, POD and CAT) activities. Arshad *et al.* (2022) reported that chitosan 7.5 mg L^{-1} showed enhanced enzymatic activity, including total antioxidant (35.48 per cent), CAT (9.94 per cent), SOD (83.87 per cent), phenolics (7.41 per cent) and POD (64.54 per cent) in rose. Chitosan foliar spray also improved chlorophyll concentration which might be due to increased enzyme activity and gaseous exchange. Higher chlorophyll pigments might have increased the photosynthetic activity, which in turn increased the plant biomass in rose.

Gursoy (2022) observed a boost in enzyme activity including SOD and CAT, as well as chlorophyll and carotenoid level, when Safflower (*Carthamus tinctorius*) under salt stress, was exposed to chitosan 0.60 per cent foliar spray. Mukarram *et al.* (2022) reported that chitosan 120 mg L^{-1} stimulated lemongrass metabolism by increasing dry biomass (37 per cent), enzymatic antioxidant systems such as catalase (22 per cent), peroxidase (18 per cent) and superoxide dismutase activities (19 per cent). They also observed similar elicitation in case of chlorophyll content (25 per cent) stomatal conductance (39 per cent) and photosynthetic rate (41 per cent).

Sara *et al.* (2012) and Shehzad *et al*. (2020) opined that chitosan might have improved uptake and retention of water by osmotic adjustment and maintaining membrane integrity. El-Serafy (2020) also reported higher relative water content, which is indicative of higher water use efficiency, in cordyline leaves in response to application of chitosan. Arshad *et al.* (2022) reported significant increase in water use efficiency due to application of chitosan in rose.

Chitosan application improved stomatal distribution according to Dowom *et al*. (2022). They explained that increased stomatal density and reduced aperture size of stomata on chitosan application facilitated its rapid opening and where by enhancing stomatal conductance, reducing water evaporation and providing CO_2 required for photosynthesis.

Hasanah and Sembiring (2018) reported higher chlorophyll b content and stomatal distribution in soyabean cultivar Devon. Application of chitosan to *Salvia abrotanoides* plants resulted in higher stomatal distribution (6.60 per cent) and chlorophyll (63 per cent) compared to untreated control (Dowom *et al*., 2022).

Al-Ghamdi (2019) observed that foliar application of chitosan 50, 200 and 500 ppm for 8 weeks in *Origanum majorana*, increased stomatal conductance and photosynthetic rate. Lin *et al*. (2020) reported that chitosan (2 g kg^{-1}) significantly enhanced the net photosynthesis and water use efficiency in lettuce. Foliar application of chitosan 100 ppm improved the stomatal conductance, chlorophyll content and activities of antioxidant enzyme, catalase in soyabean (Sadeghipour, 2021)

Chitosan oligosaccharide in tea plants increased antioxidant activities of enzymes (superoxide dismutase (SOD) and peroxidase (POD)), chlorophyll and soluble sugar content in tea plants (Ou *et al*., 2022)

Foliar application of chitosan 7.5 mg L^{-1}, increased photosynthetic rate (72.98 per cent), transpiration rate (62.11 per cent), stomatal conductance (59.54 per cent) and water use efficiency (82.93 per cent) in *Calendulaa officinalis* (Akhtar *et al*., 2022).

2.2.3 Biochemical characteristics

The exogenous application of chitosan has been reported to have significant effect on biochemical constituents of crops. This section summarizes the effect of chitosan application on biochemical characteristics of various plant species.

Chitosan treatment increased total protein and phenolic compounds in tomato fruit (Badawya and Rabeab, 2009). Srisornkompon *et al*. (2014) observed that pre-harvest treatment of chitosan 25 mg L^{-1} significantly increased phenolic content by 57 per cent in the older leaves of tea compared to control plants.

Foliar application of chitosan 0.10 per cent enhanced the production of curcumin in rhizomes (100 per cent) and activities of the defense enzymes like peroxidase and polyphenol oxidase in turmeric (Sathiyabama *et al*., 2016).

Liu *et al.* (2016) reported that chitosan and oligo chitosan 5 g L^{-1} enhanced total phenolic compounds in ginger rhizomes. The activities of enzymes β-1, 3-glucanase and phenylalanine ammonia-lyase were also enhanced, by upregulating the associated genes.

Foliar application of chitosan 0.1, 0.2 and 0.5 per cent significantly improved secondary metabolites like phenols and glycosides in *Stevia rebaudiana* (Mehregan *et al*., 2017). Foliar application of chitosan 0.4 g L^{-1} increased essential oil and phenolic content in basil (Pirbalouti *et al*., 2017). Jaleel *et al*. (2017) observed that chitosan 80 mg L^{-1} enhanced the essential oil content and essential oil yield by 36.70 per cent and 98.00 per cent, respectively in lemongrass. It was observed that foliar spray with chitosan 0.2 g L^{-1} and 0.4 g L^{-1} enhanced the essential oil content and improved its constituents in savory (*Satureja isophylla* L.) under salt stress (Salehi and Rezayatmand, 2017). Malayaman *et al*. (2017) reported that chitosan (50, 100, 150 and 200 mg L^{-1}) enhanced the concentration of hydrolysable tannins, in cells of *Phyllanthusdebilis* in *invitro* culture.

A study conducted by Bistgani *et al*. (2017) revealed significant increase in oil and dry matter production in *Thymus daenensis*, when sprayed with chitosan 400 µl L^{-1}. The foliar application of chitosan enhanced essential oil yield and improved essential oil content, such as α-pinene, β-pinene, α-thujone, β-thujone, limonene,camphor and 1,8-cineole in sage (*Salvia officinalis* L) (Vosoughi *et al*., 2018). Al-Ghamdi (2019) observed that foliar spray of chitosan 50, 200 and 500 ppm increased essential oil content, thymol and cis-sabinene content in *Origanum majorana*.

Silva *et al*. (2021) observed that low concentrations of chitosan showed biostimulating action than higher concentration. Foliar application of chitosan 0.25 and 0.50 per cent recorded higher menthol content in *Mentha arvensis* L, compared to chitosan 0.75 per cent.

Giglou *et al*. (2022) reported that foliar application of soluble chitosan hydrochloride (1 per cent) improved essential oil and biomass yield in peppermint, especially in drought stress conditions, and has partially mitigated the negative effects of drought stress.

Ahmed *et al*. (2022) observed that chitosan increased the essential oil content (35.40 per cent) in Java citronella (*Cymbopogon winterianus* Jowitt). A study by Mukarram *et al.* (2022) demonstrated that chitosan 120 mg L^{-1} enhanced the lemon grass essential oil by 40 per cent, and the phytoconstituents, neral (72 per cent) and citral (26 per cent).

2.2.4 Molecular characteristics

The plant characteristics vary with the molecular level expression of the associated genes. Chitosan application has a significant effect on the expression of genes. Schnabel *et al*. (2021) opined that piperine and piperamide synthase a member of small gene family in *P. nigrum* took part in enzymatic production of piperine from piperoyl coenzyme A and piperidine. These genes are expected to take part in the synthesis of piperine, the key alkaloid constituent in *P. longum*, as well. The gene expression of various traits in plant species in response to chitosan application is summarized below.

Chen and Xu (2005) identified a chitosan binding glycoprotein (lectin family) from mustard (*Brassica campestris*) leaves. Chitosan induced activation of plasma membrane H-ATPase, and fast membrane transient depolarization of motor cells in a dose-dependent manner, indicating the existence of chitosan receptor molecule, was observed in isolated vesicles of *Mimosa pudica* and *Cassia fasciculate* (Amborabe *et al*., 2008).

Studies had shown that chitosan induced activation of mitogen-activated protein kinases (MAPKs) (Yin *et al*., 2010), and jasmonic acid–ethylene signaling in rapeseed (Yin *et al*., 2013).

Chitosan stimulated a receptor-like kinase gene, MAP kinase pathway, lysin motif receptor-like kinase, and chitin elicitor receptor kinase 1 (CERK1), which could bind with chitosan, according to Petutschnig *et al*. (2010) in an experiment done on *Arabidopsis thaliana* knockout mutants.

Dzung *et al*. (2011) observed drought tolerance and phytoalexin production in coffee on application of chitosan. Lei *et al.* (2011) reported that foliar application of chitosan 100 mg L^{-1} increased dihydroartemisinic acid (72 per cent) and artemisinin (53 per cent) content in *Artemisia annua*. PCR analysis showed increased levels of ADS, CYP71AV1 and DBR2 gene expression, which could be attributed to the increased level of metabolites.

According to Katiyar *et al*. (2015), chitosan promotes the expression of many genes involved in plant development and protection, including defensive response genes producing PAL (phenylalanine ammonia-lyase) enzyme and protease inhibitors. It might engage with particular receptors and elicit a defensive response by directly influencing the chitosan-responsive differential gene expression profile interacting with chromatin (Hadwiger, 2015). In spinach, chitosan 0.01 mg L^{-1} stimulated phenyl propanoid pathway, which in turn triggers the PAL activity resulting in enhanced production of cinnamic acid (Singh, 2016).

Petriccione *et al*. (2017) reported that strawberry fruits dipped in chitosan 1 per cent resulted in fruit development, flavonoid biosynthesis and fruit ripening by upregulating Fra a1, Fra a3 and Fra a4 allergens genes. Winkler *et al*. (2017) reported that chitosan oligosaccharide enhanced vegetative growth, carbon and nitrogen metabolism in *Arabidopsis thaliana* by upregulating genes, MAPK3 and PR3. The exposure to chitosan oligosaccharide resulted in increased fresh weight (10 per cent), radicle length, and total carbon and nitrogen content of the plant.

According to Sun *et al*. (2018) chitosan enhanced expression of defense-related enzymes like superoxide dismutase, catalase, peroxidase, chitinase and the associated

genes in tomatoes. Jiao *et al.* (2018) opined that chitosan 150 mg L^{-1} enhanced flavonoid content in hairy root culture of *Isatis tinctoria*, by up-regulating expression of flavanoid biosynthetic genes. According to Ahmad *et al*. (2019), chitosan oligosaccharides influences the genes expression linked to synthesis of vital enzymes involved in the biosynthesis of terpenes in *Mentha piperita* L.

Shabani *et al*. (2019) reported that treatment of chitosan 100 mg L^{-1} significantly enhanced the activities of phenylalanine ammonia-lyase (PAL), catalase (CAT), lipoxygenase (LOX) and guaiacol peroxidase (GPX) by upregulating PAL1, TAT and RAS genes. It also boosted H_2O_2 generation, rosmarinic acid accumulation, expression of RBOH and OPR genes, the key genes involved in the formation of reactive oxygen species and regulation of methyl jasmonate, respectively in *Melissa officinalis* L.

A study by Al-Ghamdi (2019) demonstrated that foliar spray of chitosan 50, 200 and 500 ppm increased essential oil content and thymol and cis-Sabinene content in *Origanum majorana*, by elevated expression of CYP71D179/182, CYP71D178 PII and other essential oil composition-related genes. Transcriptome analysis showed that chitosan oligosaccharides were responsive to the expression of genes for plant growth in tea plants (Ou *et al*., 2022)

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The current study “Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L.” was conducted in the Department of Plant Biotechnology and Department of Plantation Crops and Spices, College of Agriculture, Vellayani, Trivandrum, Kerala from 2021 to 2022. The study aims to investigate the physiological, biochemical and molecular responses of *Piper longum* to foliar application of chitosan.

3.1 EXPERIMENTAL DETAILS

3.1.1 Location

The field experiment was conducted in the area, near the Department of Plant Biotechnology College of Agriculture, Vellayani. The location is situated at 8° 27' 59.0256" North latitude and 76° 58' 3.27" East longitude at an attitude of 26m above MSL.

3.1.2 Planting material

The two-month-old rooted cuttings of variety Viswam procured from the Department of Plantation Crops and Spices, College of Agriculture, Vellanikkara were used for the study. The rooted cuttings were planted in polybag containing soil, sand and cow dung (1:1:1). Staking was done at three months after planting.

3.1.3 Design of Experiment

The experiment was laid in a completely randomized design (CRD) with five treatments and four replications (5 plants per replication).

3.1.4 Treatment details

Chitosan (Plate 1) at four different concentrations was applied as foliar spray at 2, 4 and 6 months after planting. The foliar solution was prepared by dissolving chitosan in

distilled water along with 10ml of acetic acid, made up to 1 L. The spray volume for the plant varied with each foliar application is 30, 40 and 50 ml at 2, 4 and 6 months after planting respectively. The treatments are given in Table 1.

Table 1. Treatment details of chitosan foliar spray

Treatment	Concentration of chitosan
T1	1 g L^{-1}
T2	2 g L^{-1}
T3	3 g L^{-1}
T4	4 g L^{-1}
T5(Control)	0 g L^{-1}

3.2 OBSERVATIONS

3.2.1 Plant growth parameters

The following observations were recorded one month after each foliar application (at 3MAP, 5MAP and 7MAP) of chitosan (Plate 2 and Plate 3).

3.2.1.1 Plant height

The length of the vine from ground level to the tip of the shoot was measured using a meter scale to determine plant height. The mean value was worked out and expressed in centimeters.

3.2.1.2 Number of primary branches per plant

The number of primary branches per plant was recorded by counting the number of primary branches in the selected plants and the mean value was worked out.

3.2.1.3 Number of spike-bearing branches per plant

The number of spike-bearing branches per plant was obtained by counting the spike-bearing branches in the selected plants. The mean value was worked out.

3.2.1.4 Days to emergence of spike

The number of days from the day of planting to the emergence of the first spike (Plate 4a) was recorded from the selected plants and the mean value was worked out.

3.2.1.5 Days to flowering

The number of days taken from the emergence of the spike to the initiation of the flower (Plate 4b) on the spike was recorded from the tagged spikes (ten numbers) of the selected plants and the mean value was worked out.

3.2.1.6 Days from emergence to maturity of spike

The number of days from the spike emergence to its maturation as indicated by dark green color of the spike (Plate 4c) was recorded from the tagged spikes (ten numbers) of the selected plants. The mean value was worked out.

3.2.2 Yield parameters.

The following yield parameters of the plant are recorded up to one year after planting (Plate 5).

3.2.2.1 Number of spikes per plant

Spikes at greenish black stage (Plate 5) are picked from tagged plants and counted for up to one year and expressed as the total number of spike per plant. The mean value worked out.

3.2.2.2 Length of spike

The length of greenish black mature spike was measured by using a centimeter scale and the mean length is recorded in centimeters.

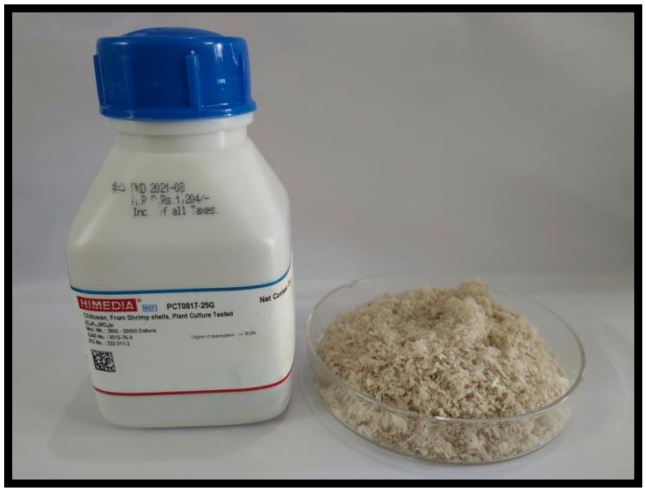

Plate 1. Chitosan (Hi-Media)

Plate 2. Field view of *Piper longum*

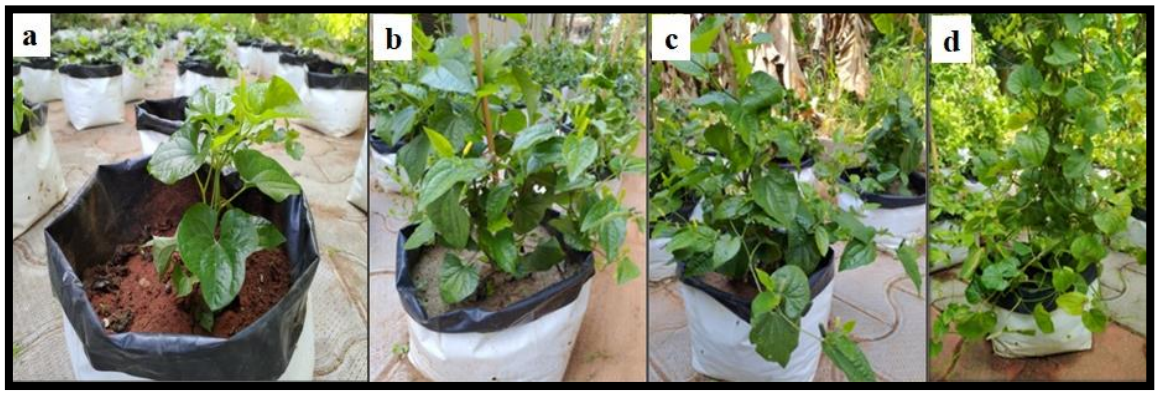

Plate 3. *Piper longum* at a) 2 MAP, b) 3 MAP, c) 5 MAP and d) 7 MAP

Plate 4. a) Emerging spike, b) flowered spike and C) mature spike of *Piper longum*

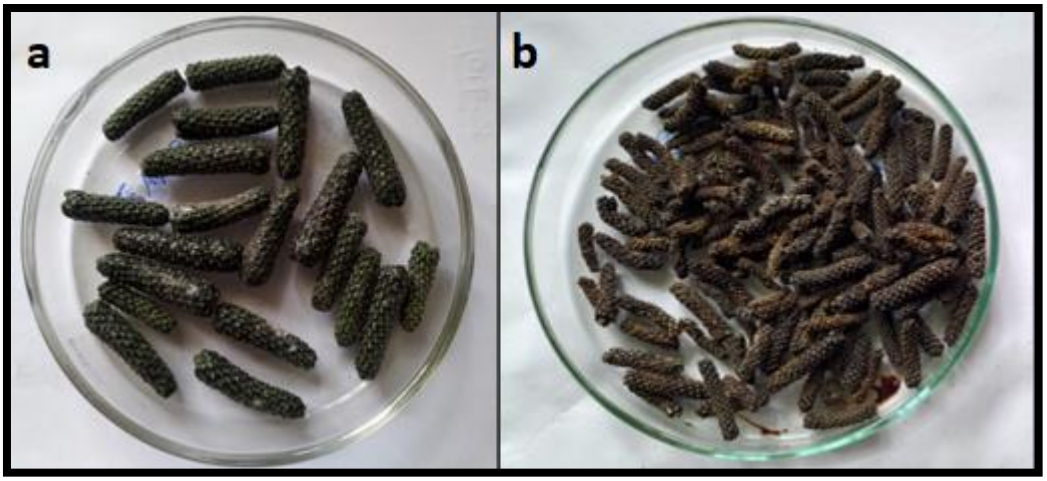

Plate 5. a) Fresh and b) dried spikes of *Piper longum*

3.2.2.3 Girth of spike

The girth of greenish black mature spike was measured by using a micrometer screw gauge and the mean girth is recorded in cm.

3.2.2.4 Spike yield per plant

The greenish black mature spikes were picked and the weight of the freshly harvested spike was taken at each picking up to one year using an electronic balance and recorded in grams. These spikes were dried at 60^{o}C for four days until consistent weights were obtained and the dry weight of the spikes was recorded in grams (g) and the mean value worked out.

3.2.2.5 Root yield

One year after planting, the whole plant was uprooted and roots collected. The weight of freshly collected roots was measured using an electronic balance and recorded in g. The roots were then dried at 60^{o}C until consistent weights were obtained and the dry weight of the roots was recorded in g and the mean value worked out.

3.2.2.6 Driage

The driage of spikes and root was calculated using the equation below, based on fresh and dry weights. The mean value worked out.

$$\text{Driage (per cent)} = 100 - \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \times 100$$

3.2.3 Physiological parameter

The following physiological parameters were recorded one month after each foliar application (at 3MAP, 5MAP and 7 MAP) of chitosan.

3.2.3.1 Stomatal distribution

The stomatal distribution (stomatal density) was recorded by epidermal imprinting technique as described by Aharoni *et al*. (2004). Xylene-thermocol mixture was prepared by dissolving thermocol in xylene until the solution became viscous. This mixture was applied uniformly on the abaxial surface of the leaf and allowed to dry. A cello tape was placed on the abaxial surface and subsequently, the imprints were detached from the leaf surface by removing the cellotape from the surface. Cello tape with imprint was mounted on a microscope glass slide and observed under 40X magnification using digital microscope (Motic BA310). The stomata per unit area were counted and recorded in mm^{-2}.

3.2.3.2 Stomatal conductance

Stomatal conductance was measured between 9 and 11 a.m. using a portable photosynthetic system (Model: CIRAS-3 Ver. 1.06, Amesbury, U.S.A) and expressed in mmoles m^{-2} s^{-1}.

3.2.3.3 Photosynthetic rate

The photosynthetic rate of the plant was measured between 9 and 11 a.m. using a portable photosynthetic system (Model: CIRAS-3 Ver. 1.06, Amesbury, U.S.A) and expressed in µmoles CO_2 m^{-2} s^{-1}.

3.2.3.4 Water use efficiency

Water use efficiency of the plant was measured between 9 and 11 a.m. using a portable photosynthetic system (Model: CIRAS-3 Ver. 1.06, Amesbury, U.S.A) and expressed in mmol CO_2 mol^{-1} H_2O.

3.2.3.5 Chlorophyll content

The chlorophyll content of the leaf sample was determined by the procedure described by Arnon (1949). The leaves were sliced into small pieces. Leaf sample 0.5 g

was weighed and added to DMSO: acetone (80 per cent) mixture (1:1v/v) 10ml taken in a test tube and was incubated overnight at room temperature. The colored solution was then transferred to a measuring cylinder and volume made up to 25ml with DMSO-acetone mixture. The absorbance was measured at 663 and 645nm, using an ELICO-SL 218 Double beam spectrophotometer.

The chlorophyll content was calculated by using the absorbance value in the formula below and expressing it in mg $g^{-1}$of fresh leaf.

$$\text{Total Chlorophyll} = 20.2(A_{645}) + 8.01(A_{663}) \times \frac{\text{Volume}}{1000 \times \text{Fresh weight}} \text{ mg g}^{-1}$$

3.2.3.6 Peroxidase activity

The activity of peroxidase in plants was measured using the technique reported by Reddy *et al*. (1995). Leaf samples (200 mg) were homogenized in 1 ml of 0.1 M phosphate buffer (pH 6.5) and centrifuged for 15 min at 4°C at 5000 rpm. 0.1 g of this extract was mixed with 3.0 ml of pyrogallol solution and adjusted to a spectrophotometer (ELICO-SL 218 Double Beam) reading of zero at 430 nm. The enzyme reaction began with the addition of 0.5 ml of 1 per cent hydrogen peroxide (H_2O_2) to sample cuvettes, and the change in absorbance was monitored every 30 s for the next 3 min. The change in absorbance per minute at 430 nm corresponds to one unit of peroxidase activity g^{-1} min^{-1}.

3.2.3.7 Catalase activity

The catalase activity in plants was determined by using the Luke method (1947). Leaf sample 200 mg in phosphate buffer was ground in a mortar and pestle. The homogenate was then centrifuged for 15 min at 40°C at 5000 rpm, and the supernatant was collected and used for the enzyme test. H_2O_2 phosphate buffer 3.0 ml was taken in an experimental cuvette to which enzyme extract 40 µl was added and properly mixed. The time required for 0.05 unit drop in absorbance at 240 nm, was recorded using a spectrophotometer (ELICO-SL 218 Double Beam). The quantity of enzyme required to reduce the absorbance at 240 nm by 0.05 unit was calculated as one enzyme unit.

3.2.4 Biochemical parameters

3.2.4.1 Estimation of Piperine

The piperine content of the dried spike was determined by spectrophotometric method reported by Sowbhagya *et al*. (1990). Dried thippali spikes (10 mg) were powdered fresh and the sample was extracted in a volumetric flask with acetone100 ml. The flasks were kept at room temperature for 2 h and gently shaken. 0.25 ml of this clear solution from the flask was transferred to a cuvette and diluted with acetone 4.75 ml and made up to 5 ml. The solution was well shaken, and the absorbance of the solution was measured at 337 nm in a UV spectrophotometer (Biospectrometer-Eppendorf) using acetone as a blank.

Preparation of the standard curve.

Standard piperine solutions were produced at concentrations of 0.4, 0.8, 1.2, 1.6, and 2 mg L^{-1}, and their absorbance values at 337 nm were measured. These data were plotted against the concentration on a graph. The concentration corresponding to the absorbance of the sample was determined and the piperine content of the samples was calculated.

The formula was used to calculate the piperine yield per plant

$$\text{Piperine yield per plant} = \text{Dry matter yield } \left(\text{g plant}^{-1}\right) \times \text{piperine content (per cent)}$$

3.2.4.2 Oleoresin

The oleoresin content of *Piper longum* spikes was determined using the Soxhlet apparatus with acetone as the solvent [AOAC, 1980]. Powdered dry spikes were put in a timble and placed in a soxhlet extraction tube. The solvent, acetone150 ml, is then heated to reflex in the round bottom flask. The extraction cycle was repeated several times in three to four hours. A fraction of the non-volatile compound was dissolved in the solvent at the end of each cycle. The non-soluble portion of the extracted material remaining in

the timble was discarded after the extractions were completed (until no color was detected in the extraction tube). Distillation process was repeated to remove the solvent. Following the distillation, the solution in the round bottom flask was transferred to a weighed beaker and allowed overnight to vaporize any remaining acetone. The next day, the beaker and the leftover contents were weighed. The difference in weights equals the amount of oleoresin, which was given as per cent.

The formula was used to calculate the oleoresin yield per plant.

$$\text{Oleoresin oil yield per plant} = \text{Drymatter yield}\left(\text{g plant}^{-1}\right) \times \text{Oleoresin content(per cent)}$$

3.2.4.3 Volatile oil

The hydro-distillation method was used to extract volatile oil using a modified Clevenger apparatus [AOAC, 1980]. Dried spikes 20 g were ground and taken in a round-bottomed flask with distilled water 200 ml. Start the heating process using the heating mantle. The top layer of condensed volatile oil, which is lighter than water, was collected in the graduated tube. The volume of essential oil obtained was recorded and reported as per cent of volume per unit mass.

$$\text{Volume of essential oil (per cent)} = \frac{\text{Volume of the volatile oil collected}}{\text{Total weight of the sample}} \times 100$$

The formula was used to determine the volatile oil yield per plant.

$$\text{Volatile oil yield per plant} = \text{Dry matter yield}\left(\text{g plant}^{-1}\right) \times \text{Volatile oil content (per cent)}$$

3.2.5 Molecular analysis

3.2.5.1 RNA isolation

RNA was isolated using a modified TRIzol reagent technique (Chomczynski *et al*., 1987) from leaf samples of *P. longum.*

Leaf samples 100 mg, from different treatments were collected and wrapped in aluminium foil before being transferred to liquid nitrogen. Collected leaf samples were ground into fine powder in a pre-chilled mortar and pestle using liquid nitrogen and polyvinyl pyrrolidone (PVP) 1 g. The powdered samples were transferred into 1ml TRIzol-containing centrifuge tubes of volume 2 ml and shaken vigorously for 15 s followed by 5 min incubation at room temperature. The mixture was vigorously shaken again for 20 sec after the addition of chloroform 0.2 ml (for every 1 ml of TRIzol reagent) and β-mercaptoethanol 0.05 ml and incubated at room temperature for 5 min. The sample is then centrifuged at 12000 rpm for 15 min at 4°C and the aqueous phase were transferred into a fresh centrifuge tube of volume 1.5 ml. An equal volume of isopropanol was added into the aqueous phase and gently mixed by inversion. The mixture was incubated at room temperature for 10 min and centrifuged at 12000 rpm for 10 min at 4°C. The pellet was recovered from the mixture and washed twice with ethanol (75 per cent). The pellet was air-dried and resuspended in RNase-free water 30 µl and then stored at -80°C.

3.2.5.2 Agarose gel electrophoresis (AGE)

Agarose (2 per cent) was prepared by melting 2 g of agarose in 100 ml of 1X TBE buffer. Ethidium bromide was added to the agarose solution after cooling it at about 50^{o}C. The mixture was then poured into a comb containing gel casting tray. The comb was removed after gel was set and the gel was placed on an electrophoresis unit. The tank was filled with 1X TBE buffer. Extracted RNA sample 5 µl was loaded into the gel along with 1 µl of 6X gel loading dye. Electrophoresis was carried out at 50 V. The gel bands were

evaluated using the Gel Doc XR+ Gel Documentation system (Bio-Rad) after the loading dye reached three fourth of the gel.

3.2.5.3 Quantification of RNA

Quantification of RNA was carried out using a Spectrophotometer (Biospectrometer-Eppendorf). The optical density of isolated RNA was recorded at A_{260} and A_{280}. The concentration and purity of RNA were calculated using the following equations.

$$\text{Concentration of RNA} = 40 \times OD260 \times \text{Dilution factor}$$

$$\text{Purity of isolated RNA} = \frac{A_{260}}{A_{280}}$$

3.2.5.4 cDNA synthesis

cDNA was synthesized using RT Easy 1 cDNA synthesis Kit (G Bioscience), according to the manufacture's protocol. All the steps were carried out in an RNase-free environment to reduce the risk of RNase attacks. The details of reaction mix and thermal cycler conditions are provided in Table 2 and Table 3. The reaction was performed in a thermal cycler (Bio-Rad).

Conversion of RNA to cDNA was confirmed by carrying out PCR using primers for house-keeping genes. The PCR product was analyzed by gel electrophoresis using agarose gel 1 per cent as per the section 3.2.5.2.

Table 2. Reaction mix used for the cDNA synthesis

Reagent	Volume (µl)
2X RT Easy Mix	10 µl
Oligo (dT) 18 Primer (50 µM)	1 µl
Template (RNA)	X µl (variable)
RNase-Free ddH_2O	(9-X) µl
Total	20 µl

Table 3. Thermal cycler condition for reverse transcription

Steps	Temperature(^{0}C)	Time (min)
Reverse transcription	42^{o}C	20
Inactivation	85^{o}C	5

3.2.5.5 Primer for RT-PCR

Reported primers for piperine synthase and actin were used and details are provided in Table 4 (Schnabel *et al*., 2021; Alex *et al*., 2008). The primers were rechecked for self-dimer, cross-dimer, and hairpin structure using Primer Express Software Version 3.0.

Table 4. List of primers for Real-time PCR.

Gene	Primers		Amplicon size
Actin	F	5'-ACATCCGCTGGAAGGTGC-3'	198
	R	5'-TCTGTATGGTAACATTGTGCTC-3'	
Piperine synthase	F	5´- TTGGCGATATCGGAGCACTC-3´	132
	R	5´- CGATCCCGCCGCAAATAAAG -3´	

3.2.5.6 Standardization of annealing temperature

Temperature gradient CFX 96 Real-time PCR (Bio-Rad) was used to standardize the annealing temperature of the piperine synthase and actin primers. The optimum annealing temperature was chosen as the one with the lowest Ct value. The thermal cycler condition and standardization mix for qRT-PCR are given in Tables 5 and 6.

Table 5. Reaction mix for standardization of annealing temperature

Reagent	Volume (µl)
2x AB HS SYBR Green qPCR mix	5 µl
Forward primer	1 µl
Reverse primer	1 µl
Template(cDNA)	1 µl
RNase-Free H_2O	2 µl
Total volume	10 µl

Table 6. Thermal cycler condition for annealing temperature standardization

Stages	Repeat	Temperature (^{0}C)	Time (s)
Initial denaturation	1	95^{0}C	300
Denaturation	35	95^{0}C	45
Annealing		47, 47.5, 48.6, 50.2, 53.7, 54.6, and 55^{0}C	30
Primer extension		72^{0}C	45
Final extension	1	72^{0}C	300

3.2.5.7 Expression analysis of piperine synthase gene

qRT-PCR was carried out for the expression analysis of piperine synthase genes. Real-time PCR analysis was done in CFX96 Real-time PCR (Bio-Rad) using 1 µl of cDNA as a template with a total of 10 µl reaction mixture. The fluorescence dye SYBR GREEN (G-bioscience) was used for performing Real-time PCR. Each reaction was performed in triplicates and the gene expression was normalized using the actin gene (reference gene). In addition, an NTC (no template control) was also set for each primer by replacing the cDNA with DNase-free water 1 µl. Components for reaction mix and thermal cycler conditions are given in Tables 7 and 8 respectively.

Primer-template specificity was monitored by analyzing the melt curve directly after the amplification on the qRT-PCR. The relative expression values of piperine synthase gene were analysed using the comparative Ct method (Livak and Schmittgen, 2001). Relative fold change from Ct values were calculated using MS Excel software

2007. The gene expression of piperine synthase gene in each treatment were expressed as relative fold change over the control plant.

Table 7. Reaction mix for qRT- PCR

Reagent	Volume (µl)
2x AB HS SYBR Green qPCR mix	5 µl
Forward primer	1µl
Reverse primer	1µl
Template (cDNA)	1 µl
Rnase-Free H_2O	2 µl
Total volume	10 µl

Table 8. Thermal cycler condition for qRT-PCR

Stages	Repeat	Temperature (^{0}C)	Time (s)
Initial denaturation	1	95^{0}C	300
Denaturation	35	95^{0}C	45
Annealing		51.2 (for actin) 47.5 (for piperine synthase)	30
Primer extension		72^{0}C	45
Final extension	1	72^{0}C	300

RESULTS

4. RESULTS

An investigation entitled “Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L." was carried out during 2021-2022 at the Department of Plant Biotechnology and Department of Plantation Crops and Spices, College of Agriculture, Vellayani. The project studied the effect of foliar application of chitosan at varying concentrations on plant growth, physiological, biochemical and molecular characteristics in *P. longum.*

The data collected from the field experiment and laboratory analyses were statistically analyzed and the results of the study are presented in this chapter.

4.1 PLANT GROWTH PARAMETERS

The plants were exposed to foliar spray of different concentrations of chitosan at 2, 4, and 6 MAP. The observations on plant growth parameters were recorded after one month of each foliar spray application at 3, 5 and 7 MAP.

4.1.1 Plant height

The effect of chitosan foliar spray on plant height at 3, 5 and 7 MAP is shown in Table 9. At all stages of observation, significant variation was observed with plant height.

At 3 MAP, the highest (59.57 cm) plant height was recorded in T1, followed by T2 (56.73 cm). The shortest plant height was observed in T4 (50.87 cm). The control treatment recorded a plant height of 54.12 cm, which was observed to be higher than T3 and T4.

At 5 MAP, the highest plant height (124.62 cm) was recorded in T1 and the shortest plant height (100.50 cm) in T4. The control treatment recorded a higher plant height, 108.75 cm, compared to T3and T4, but lower than T2.

At 7 MAP, maximum plant height 194.75 cm was recorded in T1 and the lowest (150.25 cm) in T4. The control plant recorded a plant height of 162.62cm, which was higher than T3 and T4.

An increase in plant height at 10.06, 14.59 and 19.75 per cent was observed in T1, over the control treatment at 3, 5 and 7 MAP, respectively.

4.1.2 Number of primary branches

The effect of different concentrations of chitosan foliar spray on the number of primary branches at 3, 5 and 7 MAP shown in Table 10. The parameter exhibited significant variation at all stages of observation among the treatments tried.

At 3 MAP, the highest (4.75) number of primary branches was recorded in T1, followed by T2 (4.0). The lowest number of primary branches was observed in T4 (3.25), while the control treatment recorded a higher value (3.75).

At 5 MAP, the highest (5.75) number of primary branches was recorded in T1, which was on par with T2 (5.25) and was followed by control, T5 (4.50). The lowest number of primary branches was observed in T4 (3.75).

At 7 MAP, the highest (7.75) number of primary branches was recorded in T1, followed by T2 (7.00). The lowest number of primary branches was observed in T4 (4.75), which was on par with T3 (5.00). The control treatment recorded a higher value (5.75) compared to T3 and T4.

The number of primary branches exhibited 26.67, 27.77 and 34.78 per cent increase at 3, 5, and 7 M AP, respectively, when the plants were sprayed with chitosan, CHT @ 1 g L^{-1}.

Table 9. Effect of chitosan foliar spray on plant height

Treatment	Plant height (cm) on 3 MAP	Percentage change over the control	Plant height (cm) on 5 MAP	Percentage change over the control	Plant height (cm) on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	59.57±0.612^{a}	10.06	124.62±0.479^{a}	14.59	194.75±0.645^{a}	19.75
T2 (CHT 2 g L^{-1})	56.73±0.459^{b}	4.82	115.37±0.479^{b}	6.092	181.00±0.816^{b}	11.29
T3 (CHT 3 g L^{-1})	52.75±0.645^{d}	-2.54	105.00±0.913^{d}	-3.44	155.00±0.913^{d}	-4.68
T4 (CHT 4 g L^{-1})	50.87±0.75^{e}	-6.00	100.50±0.7.7^{e}	-7.58	150.25±0.289^{e}	-7.61
T5 (CHT 0 g L^{-1})	54.12±0.323^{c}	0	108.75±0.645^{c}	0	162.62±0.750^{c}	0
SE(d)	0.409		0.470		0.506	
SE(m)	0.289		0.332		0.358	
CV (%)	1.054		0.600		0.424	
CD(P=0.05)	0.871		1.002		1.079	

Table 10. Effect of chitosan foliar spray on number of primary branches

Treatment	Number of primary branches on 3 MAP	Percentage change over the control	Number of primary branches on 5 MAP	Percentage change over the control	Number of primary branches on7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	4.75±0.5^{a}	26.67	5.75±0.5^{a}	27.77	7.75±0.5^{a}	34.78
T2 (CHT 2 g L^{-1})	4.00±0.0^{b}	6.66	5.25±0.5^{a}	11.11	7.00±0.0^{b}	21.73
T3 (CHT 3 g L^{-1})	3.50±0.57^{bc}	-6.66	4.00±0.0^{bc}	-11.1	5.00±0.0^{d}	-13.04
T4 (CHT 4 g L^{-1})	3.25±0.50^{c}	-13.33	3.75±0.50^{c}	-16.66	4.75±0.5^{d}	-17.39
T5 (CHT 0 g L^{-1})	3.75±0.50^{bc}	0	4.50±0.57^{b}	0	5.75±0.5^{c}	0
SE(d)	0.329		0.329		0.274	
SE(m)	0.233		0.233		0.194	
CV (%)	12.09		10.01		6.402	
CD(P=0.05)	0.702		0.702		0.584	

4.1.3 Number of spike-bearing branches

The effect of different concentrations of chitosan foliar spray on the number of spike-bearing branches at 3, 5, and 7 MAP is shown in Table 11. Significant variation was observed among the treatments at all stages of observation.

At 3 MAP, the highest (3.75) number of spike-bearing branches was recorded in T1, followed by T2 (3.5). The lowest number of spike-bearing branches was observed in T4 (2.5), and was on par with the control treatment, which recorded a value of 3.25.

At 5 MAP, the highest (7.00) number of spike-bearing branches was recorded in T1, which was on par with T2 (6.50). The lowest number of spike-bearing branches was observed in T4 (3.75), while the control treatment recorded a higher value (6.00).

At 7 MAP, the highest (8.50) number of spike-bearing branches was recorded in T1, followed by T2 (7.75). The lowest (4.25) number of spike-bearing branches was observed in T4. The control treatment recorded a higher value (7.00) compared to T3 (5.00) and T4.

The number of spike bearing branches exhibited 15.38, 16.67 and 21.42 per cent increase at 3, 5, and 7 M AP, respectively, when the plants were sprayed with chitosan, CHT @ 1 g L^{-1}.

4.1.4 Days to emergence of spike

The effect of different concentrations of chitosan foliar spray on the days to emergence of the spike is shown in Table 12. The parameter exhibited a significant difference among the treatments tried.

The early spike emergence (73.38 days) was recorded in T1 which was followed by T2 (75.51 days). The more number of days to spike emergence was observed in T4 (85.87 days) followed by T3 (80.32 days). Control treatment recorded a lower number of days (78.39 days) compared to T3 and T4.

4.1.5 Days to flowering

The effect of different concentrations of chitosan foliar spray on the number of days to flowering is presented in Table 12. This parameter also exhibited significant variation among the treatments tried.

The minimum number of days for flowering was recorded in T1 (20.40 days) which was on par with T2 (21.51 days). Late flowering was recorded in T4 (31.58 days) followed by T3 (25.95days). Control treatment recorded early flowering (24.39days) compared to T3 and T4.

4.1.6 Days from emergence to maturity of spike

The effect of different concentrations of chitosan foliar spray on the number of days from spike emergence to maturity is shown in Table 12. A significant difference was observed among all the treatments tried.

The lowest number of days for spike emergence to maturity (59.83 days) was observed in T1 which was on par with T2 (60.13 days). The highest number of days for spike emergence to maturity was observed in T4 (63 days) which was on par with T3 (62.60 days). Control treatment recorded 61.98 days from spike emergence to maturity and was on par with T3.

At all stages of observation, it was found that chitosan foliar application, T1 (CHT @ 1 g L^{-1}) exhibited the best performance with respect to all plant growth parameters studied. The control (T5), without chitosan application gave better results compared to T3 (CHT @ 3 g L^{-1}) and T4 (CHT @ 4 g L^{-1}).

Table 11. Effect of chitosan foliar spray on number of spike bearing branches

Treatment	Number of spike-bearing branches on 3 MAP	Percentage change over the control	Number of spike-bearing branches on 5 MAP	Percentage change over the control	Number of spike-bearing branches on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	3.75 ± 0.50^{a}	15.38	7.00 ± 0.0^{a}	16.67	8.50 ± 0.57^{a}	21.42
T2 (CHT 2 g L^{-1})	3.50 ± 0.57^{ab}	7.69	6.50 ± 0.57^{ab}	8.33	7.75 ± 0.5^{b}	10.71
T3 (CHT 3 g L^{-1})	2.75 ± 0.5^{bc}	-15.38	4.50 ± 0.57^{c}	-25.00	5.00 ± 0.0^{d}	-28.57
T4 (CHT 4 g L^{-1})	2.5 ± 0.57^{c}	-23.0	3.75 ± 0.5^{d}	-37.5	4.25 ± 0.50^{e}	-39.29
T5 (CHT 0 g L^{-1})	3.25 ± 0.5^{abc}	0	6.00 ± 0.0^{b}	0	7.00 ± 0.0^{c}	0
SE(d)	0.376		0.303		0.289	
SE(m)	0.266		0.214		0.204	
CV (%)	16.898		7.715		6.281	
CD(P=0.05)	0.802		0.645		0.615	

Table 12. Effect of chitosan foliar spray on days to emergence of spike, days to flowering, and days from emergence to maturity of spike.

Treatment	Days to emergence of spike	Days to flowering	Days from emergence to maturity of spike
T1 (CHT 1 g L^{-1})	73.38 ± 0.883^{e}	20.40 ± 1.115^{d}	59.83 ± 0.596^{c}
T2 (CHT 2 g L^{-1})	75.51 ± 2.091^{d}	21.51 ± 1.103^{d}	60.13 ± 0.163^{c}
T3 (CHT 3 g L^{-1})	80.32 ± 0.196^{b}	25.95 ± 0.196^{b}	62.60 ± 0.563^{ab}
T4 (CHT 4 g L^{-1})	85.87 ± 0.669^{a}	31.58 ± 0.579^{a}	63.00 ± 0.445^{a}
T5 (CHT 0 g L^{-1})	78.39 ± 1.116^{c}	24.39 ± 1.198^{c}	61.98 ± 0.816^{b}
SE(d)	0.620	0.653	0.396
SE(m)	0.438	0.462	0.280
CV (%)	1.114	3.730	0.909
CD(P=0.05)	1.321	1.392	0.843

4.1.7 Yield parameters

The plants are subjected to different concentrations of chitosan at 2, 4, and 6 MAP. The observations on plant yield parameters were recorded.

4.1.7.1 Number of spikes per plant

The effect of different concentrations of chitosan foliar spray on the number of spikes per plant of *P. longum* is presented Table 13. The parameter exhibited significant variation among the treatments tried.

The highest (181.50) number of spikes was recorded in T1 followed by T2 (175.50). The lowest (154.50) number of spikes were recorded in T4. The control treatment recorded a higher value (167.75) compared to T3 and T4.

T1 recorded 8.19 per cent increase over the control treatment with respect to the number of spikes per plant

4.1.7.2 Length of spike

The effect of different concentrations of chitosan foliar spray treatments on length of spikes of *P. longum* is shown in Table 13. The parameter exhibited a significant difference among the treatments tried.

T1 recorded the longest (3.25 cm) spike, followed by T2 (3.10 cm). The shortest spike was observed in T4 (2.75 cm), which was on par with T3 (2.85 cm). The control treatment recorded a higher value (2.97 cm) compared to T3 and T4.

In the case of spike length, T1 recorded 9.24 per cent increase over the control treatment.

Table 13. Effect of chitosan foliar spray on number of spikes, length and girth of spikes.

Treatments	Number of spikes per plant	Percentage change over the control	Length of spikes (cm)	Percentage change over the control	Girth of spikes (mm)	Percentage change over the control
T1(CHT 1 g L^{-1})	181.50±1.29[a]	8.19	3.25±0.058[a]	9.24	9.97±0.40[a]	11.45
T2(CHT 2 g L^{-1})	175.50±1.29[b]	4.62	3.10±0.082[b]	4.20	9.30±0.18[b]	3.91
T3(CHT 3 g L^{-1})	161.50±1.29[d]	-3.72	2.85±0.058[d]	-4.20	6.05±0.05[d]	-3.24
T4(CHT 4 g L^{-1})	154.50±1.29[e]	-7.89	2.75±0.058[d]	-7.56	6.02±0.05[d]	-3.2
T5(CHT 0 g L^{-1})	167.75±0.95[c]	0	2.97±0.096[c]	0	8.95±0.129[c]	0
SE(d)	0.871		0.051		0.148	
SE(m)	0.616		0.036		0.104	
CV (%)	0.732		2.408		2.593	
CD(P=0.5)	1.856		0.108		0.315	

Table 14. Effect of chitosan foliar spray on fresh and dry spike yield and driage per cent of spike.

Treatments	Spike fresh yield (g $plant^{-1}$)	Percentage change over the control	Spike dry yield (g $plant^{-1}$)	Percentage change over the control	Driage
T1(CHT 1 g L^{-1})	163.35±1.162[a]	21.72	29.40 ±0.209[a]	21.73	19.47±0.05
T2(CHT 2 g L^{-1})	149.17±1.097[b]	11.15	26.85 ±0.198[b]	11.11	19.52±0.05
T3(CHT 3 g L^{-1})	113.05±0.904[d]	-15.76	20.34 ±0.163[d]	-15.77	19.55±0.058
T4(CHT 4 g L^{-1})	100.42±0.839[e]	-25.17	18.07 ±0.151[e]	-25.17	19.50±0.0
T5(CHT 0 g L^{-1})	134.20±0.766[c]	0	24.15 ±0.138[c]	0	19.47±0.05
SE(d)	0.683		0.123		0.033
SE(m)	0.483		0.087		0.023
CV (%)	0.731		0.731		0.239
CD(P=0.5)	1.455		0.262		NS

4.1.7.3 Girth of spike

The effect of different concentrations of chitosan foliar spray on the girth of spikes of *P. longum* is shown in Table 13. The parameter exhibited significant variation among the treatments tried.

The highest (9.97 mm) spike girth was recorded in T1, followed by T2 (9.30 mm). The lowest (6.02 mm) spike girth was recorded in T4, which was par with T3 (6.05mm). The control treatment recorded a higher value (8.95 mm) compared to T3 and T4

Spike grith was observed to have an increase of 11.45 per cent in T1, over the control treatment.

4.1.7.4 Spike yield per plant

The effect of different concentrations of chitosan foliar spray on the spike yield per plant of *P. longum,* on both fresh and dry weight basis is shown in Table 14. The parameter exhibited a significant difference among the treatments tried.

The highest (fresh-163.35 g plant^{-1}; dry-29.40 g plant^{-1}) yield was recorded in T1 followed by T2 (fresh-149.17 g plant^{-1}; dry-26.85 g plant^{-1}). The lowest fresh yield was recorded in T4 (fresh-100.42 g plant^{-1}; dry-18.076 g plant^{-1}). The control treatment recorded a higher value (fresh-134.20 g plant^{-1}; dry-24.15 g plant^{-1}) compared to T3 and T4.

T1 recorded 21.73 per cent increase over the control treatment with respect to dry spike yield.

4.1.7.5 Root yield per plant

The effect of different concentrations of chitosan foliar spray on root yield per plant of *P. longum,* both on fresh and dry weight basis is shown in Table 15. The parameter exhibited significant variation among the treatments tried.

T1 recorded the highest (fresh-47.08 g plant^{-1}; dry-8.24 g plant-^{1}) yield, followed by T2 (fresh-39.34 g plant^{-1}; 6.87 g plant^{-1}). The lowest (fresh-27.44 g plant^{-1}; dry-4.80 g

plant^{-1}) yield was recorded in T4, which was on par with T3 (fresh-28.29 g plant^{-1}; dry-4.94 g plant^{-1}), while control treatment recorded a higher value (fresh-35.77 g plant^{-1}; dry-6.25 g plant^{-1}).

T1 recorded 3.84 per cent increase over the control treatment in terms of dry root yield.

At all stages of observation, it was found that the foliar spray treatment T1 (CHT @ 1 g L^{-1}) exhibited the highest value for the above said yield parameters. Also, T3 (CHT @ 3 g L^{-1}) and T4 (CHT @ 4 g L^{-1}) showed lower values than the control (T5).

4.1.7.6 Driage

The effect of different concentrations of chitosan foliar spray on the driage of spikes and roots in *P. longum* is shown in Table 14 and 15 respectively. There is no significant variation among the treatments with respect to driage per cent. The driage of spike and root was found to be 19.40 to 19.55 and 17.46 to 17.50 per cent, respectively.

4.2 PHYSIOLOGICAL PARAMETERS

The physiological parameters were recorded at 3, 5 and 7 MAP in plants exposed to foliar spray with varying concentrations of chitosan.

4.2.1 Stomatal distribution

The effect of chitosan foliar spray treatments on the stomatal distribution of *P. longum* is depicted in Table 16. At all stages of observation, significant variation was observed with respect to stomatal distribution or stomatal density.

At 3 MAP, the highest (222.00 mm^{-2}) stomatal distribution was recorded in T1, followed by T2 (219.25 mm^{-2}). The lowest stomatal distribution was observed in T4 (210.75 mm^{-2}), while the control treatment recorded a higher value (215.75 mm^{-2}).

Table 15. Effect of chitosan foliar spray on fresh and dry root yield and driage per cent of root.

Treatments	Root fresh yield (g plant^{-1})	Percentage change over the control	Root dry yield (g plant^{-1})	Percentage change over the control	Driage
T1(CHT 1 g L^{-1})	47.08±0.185^{a}	31.61	8.24±0.031^{a}	31.84	17.50±0.018
T2(CHT 2 g L^{-1})	39.34±0.895^{b}	9.97	6.87±0.080^{b}	9.92	17.48±0.31
T3(CHT 3 g L^{-1})	28.29±0.403^{d}	-20.9	4.94±0.119^{d}	-20.96	17.46±0.018
T4(CHT 4 g L^{-1})	27.44±0.461^{d}	-23.26	4.80±0.076^{d}	-23.20	17.46±0.018
T5(CHT 0 g L^{-1})	35.77±0.9^{c}	0	6.25±0.488^{c}	0	17.49±0.018
SE(d)	0.519		0.091		0.017
SE(m)	0.367		0.064		0.012
CV (%)	1.786		1.792		0.119
CD(P=0.5)	1.156		0.203		NS

Table 16. Effect of chitosan foliar spray on stomatal distribution

Treatment	Stomatal distribution (m.m^{-2}) on 3 MAP	Percentage change over the control	Stomatal distribution (m.m^{-2}) on 5 MAP	Percentage change over the control	Stomatal distribution (m.m^{-2}) on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	222.00±1.82^{a}	2.89	234.75±0.5^{a}	5.14	247.75±0.5^{a}	7.60
T2 (CHT 2 g L^{-1})	219.25±0.95^{b}	1.50	228.25±0.5^{b}	2.23	240.75±0.5^{b}	4.56
T3 (CHT 3 g L^{-1})	214.50±1.29^{d}	-0.0015	219.00±0.81^{d}	-1.90	221.25±0.5^{d}	-3.90
T4 (CHT 4 g L^{-1})	210.75±1.70^{e}	-0.023	216.25±0.5^{e}	-3.13	218.50±0.57^{e}	-5.10
T5 (CHT 0 g L^{-1})	215.75±1.25^{c}	0	223.25±0.95^{c}	0	230.25±0.28^{c}	0
SE(d)	1.021		0.483		0.342	
SE(m)	0.722		0.342		0.242	
CV (%)	0.667		0.305		0.208	
CD(P=0.05)	2.175		1.030		0.728	

At 5 MAP, the highest (234.75 mm^{-2}) stomatal distribution was recorded in T1, which was followed by T2 (228.25 mm^{-2}). The lowest stomatal distribution was observed in T4 (216.25 mm^{-2}), while the control treatment recorded a higher value (223.25mm^{-2}).

At 7 MAP, the highest (247.75mm^{-2}) stomatal distribution was recorded in T1 and was followed by T2 (240.75 mm^{-2}). The lowest stomatal distribution was observed in T4 (218.50 mm^{-2}). The control treatment recorded a higher value (230.25 mm^{-2}) compared to T3 and T4.

It was observed that T1 recorded 2.89, 5.14 and 7.60 per cent increase in stomatal distribution over the control treatment, at 3, 5 and 7 MAP, respectively.

4.2.2. Stomatal conductance

The effect of different concentrations of chitosan foliar spray on stomatal conductance of *P. longum* is shown in Table 17. The parameter exhibited significant difference among the treatments tried.

At 3 MAP, the highest (327.50mmoles m^{-2} s^{-1}) stomatal conductance was recorded in T1, followed by T2 (317.50 mmoles m^{-2} s^{-1}). The lower stomatal conductance was observed in T3 (306.25 mmoles m^{-2} s^{-1}) and T4 (306.00 mmoles m^{-2} s^{-1}), while the control treatment recorded a higher value (309.85 mmoles m^{-2} s^{-1}).

At 5 MAP, the highest (408.75 mmoles m^{-2} s^{-1}) stomatal conductance was recorded in T1, which was followed by T2 (394.62 mmoles m^{-2} s^{-1}). The lowest stomatal conductance was observed in T4 (369.25 mmoles m^{-2} s^{-1}), while the control treatment recorded a higher value (382.50 mmoles m^{-2} s^{-1}).

At 7 MAP, the highest (496.50 mmoles m^{-2} s^{-1}) stomatal conductance was recorded in T1 followed by T2 (482.25 mmoles m^{-2} s^{-1}). The lowest stomatal conductance was observed in T4 (436.25 mmoles m^{-2} s^{-1}). The control treatment recorded a higher value (460.62 mmoles m^{-2} s^{-1}) compared to T3 and T4.

Table 17. Effect of chitosan foliar spray on stomatal conductance

Treatment	Stomatal conductance (mmoles m^{-2} s^{-1}) on 3 MAP	Percentage change over the control	Stomatal conductance (mmoles m^{-2} s^{-1}) on 5 MAP	Percentage change over the control	Stomatal conductance (mmoles m^{-2} s^{-1}) on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	327.50±0.40[a]	5.68	408.75±0.86[a]	6.86	496.50±0.40[a]	7.77
T2 (CHT 2 g L^{-1})	317.50±0.40[b]	2.46	394.62±0.25[b]	3.16	482.25±0.28[b]	4.69
T3 (CHT 3 g L^{-1})	306.25±1.04[d]	-1.17	374.62±0.47[d]	-2.05	444.75±0.28[d]	-3.44
T4 (CHT 4 g L^{-1})	306.00±1.80[d]	-1.25	369.25±0.28[e]	-3.46	436.25±0.28[e]	5.29
T5 (CHT 0 g L^{-1})	309.87±1.031[c]	0	382.50±0.70[c]	0	460.62±0.47[c]	0
SE(d)	0.604		0.403		0.254	
SE(m)	0.427		0.285		0.180	
CV (%)	0.272		0.148		0.077	
CD(P=0.05)	1.287		0.859		0.541	

Table 18. Effect of chitosan foliar spray on photosynthetic rate

Treatment	Photosynthetic rate (μCO_2 moles m^{-2} s^{-1}) on 3 MAP	Percentage change over the control	Photosynthetic rate (μCO_2 moles m^{-2} s^{-1}) on 5 MAP	Percentage change over the control	Photosynthetic rate (μCO_2 moles m^{-2} s^{-1}) on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	7.80±0.1[a]	9.95	8.27±0.04[a]	11.18	8.87±0.08[a]	12.69
T2 (CHT 2 g L^{-1})	7.53±0.24[a]	6.17	8.15±0.01[b]	9.54	8.74±0.045[b]	10.98
T3 (CHT 3 g L^{-1})	6.85±0.24[bc]	-3.32	6.97±0.02[d]	-6.303	7.17±0.05[d]	-8.92
T4 (CHT 4 g L^{-1})	6.61±0.22[c]	-6.79	6.84±0.04[e]	-8.02	7.03±0.1[e]	-10.66
T5 (CHT 0 g L^{-1})	7.09±0.15[b]	0	7.44±0.06[c]	0	7.87±0.09[c]	0
SE(d)	0.142		0.031		0.056	
SE(m)	0.100		0.022		0.040	
CV (%)	2.79		0.581		1.001	
CD(P=0.05)	0.302		0.066		0.120	

T1 recorded 5.68, 6.86 and 7.77 per cent increase over control treatment in stomatal conductance at 3, 5 and 7 MAP, respectively.

4.2.3 Photosynthetic rate

The effect of different concentrations of chitosan foliar spray treatments on the photosynthetic rate of *P. longum* is shown in Table 18. The parameter exhibited significant variation among the treatments tried.

At 3 MAP, the highest (7.80 µmol CO_2 m^{-2} s^{-1}) photosynthetic rate was recorded in T1 and was on par with T2 (7.53 µmol CO_2 m^{-2} s^{-1}). The lowest photosynthetic rate was observed in T4 (6.61 µmol CO_2 m^{-2} s^{-1}), while the control treatment recorded a higher value (7.09 µmol CO_2 m^{-2} s^{-1}).

At 5 MAP, the highest (8.27 µmol CO_2 m^{-2} s^{-1}) photosynthetic rate was recorded in T1, which was followed by T2 (8.15 µmol CO_2 m^{-2} s^{-1}). The lowest photosynthetic rate was observed in T4 (6.84 µmol CO_2 m^{-2} s^{-1}), while the control treatment recorded a higher value (7.44 µmol CO_2 m^{-2} s^{-1}).

At 7 MAP, the highest (8.87 µmol CO_2 m^{-2} s^{-1}) photosynthetic rate was recorded inT1 which was followed by T2 (8.75 µmol CO_2 m^{-2} s^{-1}). The lowest photosynthetic rate was observed in T4 (7.03 µmol CO_2 m^{-2} s^{-1}). The control treatment recorded a higher value (7.87 µmol CO_2 m^{-2} s^{-1}) compared to T3 and T4.

Photosynthetic rate exhibited an increase of 9.95, 11.18, and 12.69 per cent over the control at 3, 5 and 7 MAP, respectively, when the plants were exposed to chitosan spray, T1.

4.2.4 Water use efficiency (WUE)

The effect of different concentrations of chitosan foliar spray treatments on the water use efficiency of *P. longum* is shown in Table 19. At all stages of observation, significant variation was observed with respect to water use efficiency.

At 3 MAP, the highest (5.34 mmol CO_2 mol^{-1} H_2O) water use efficiency was recorded in T1, followed by T2 (5.15 mmol CO_2 mol^{-1} H_2O). The lowest water use efficiency was observed in T4 (4.42 mmol CO_2 mol^{-1} H_2O), while the control treatment recorded a higher value (4.92 mmol CO_2 mol^{-1} H_2O).

At 5 MAP, T1 recorded the highest (6.15mmol CO_2 mol^{-1} H_2O) water use efficiency followed by T2 (5.92 mmol CO_2 mol^{-1} H_2O). The lowest water use efficiency was observed in T4 (4.90 mmol CO_2 mol^{-1} H_2O), while the control treatment recorded a higher value (5.58 mmol CO_2 mol^{-1} H_2O).

At 7 MAP, the highest (6.83 mmol CO_2 mol^{-1} H_2O) water use efficiency was observed inT1 and was followed by T2 (6.63 mmol CO_2 mol^{-1} H_2O). The lowest water use efficiency was observed in T4 (5.16 mmol CO_2 mol^{-1} H_2O). The control treatment recorded a higher value (6.09 mmol CO_2 mol^{-1} H_2O) compared to T3 and T4.

T1 recorded 8.55, 10.22 and 12.23 per cent increase in water use efficiency over control treatment at 3, 5 and 7 MAP, respectively.

4.2.5 Chlorophyll content

The effect of different concentrations of chitosan foliar spray treatments on the chlorophyll content of *P. longum* is presented in Table 20. The parameter exhibited significant variation among the treatments tried.

At 3 MAP, the highest (1.608 mg g^{-1}) chlorophyll content was recorded in T1 followed by T2 (1.586 mg g^{-1}). T4 recorded the lowest (1.533 mg g^{-1}) chlorophyll content, while the control treatment recorded a higher value (1.569 mg g^{-1}).

At 5 MAP, T1 recorded the highest chlorophyll content (1.811 mg g^{-1}), followed by T2 (1.785 mg g^{-1}). The chlorophyll content was observed to be the lowest (1.645 mg g^{-1}) in T4, while the control treatment recorded a higher value (1.727 mg g^{-1}).

Table 19. Effect of foliar spray on water use efficiency

Treatment	Water use efficiency (mmol CO_2 mol^{-1} H_2O) on 3 MAP	Percentage change over the control	Water use efficiency (mmol CO_2 mol^{-1} H_2O) on 5 MAP	Percentage change over the control	Water use efficiency (mmol CO_2 mol^{-1} H_2O) on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	5.34 ± 0.12^{a}	8.55	6.15 ± 0.03^{a}	10.22	6.83 ± 0.05^{a}	12.23
T2 (CHT 2 g L^{-1})	5.15 ± 0.10^{b}	4.77	5.92 ± 0.08^{b}	6.04	6.63 ± 0.04^{b}	8.86
T3 (CHT 3 g L^{-1})	4.62 ± 0.11^{d}	-5.97	5.14 ± 0.04^{d}	-7.97	5.54 ± 0.03^{d}	-8.99
T4 (CHT 4 g L^{-1})	4.42 ± 0.04^{e}	-10.10	4.90 ± 0.05^{e}	-12.25	5.16 ± 0.05^{e}	-15.18
T5 (CHT 0 g L^{-1})	4.92 ± 0.05^{c}	0	5.58 ± 0.05^{c}	0	6.09 ± 0.05^{c}	0
SE(d)	0.068		0.040		0.035	
SE(m)	0.048		0.029		0.025	
CV (%)	1.955		1.032		0.822	
CD(P=0.05)	0.144		0.086		0.075	

Table 20. Effect of chitosan foliar spray on chlorophyll content

Treatment	Chlorophyll content (mg g^{-1}) on 3 MAP	Percentage change over the control	Chlorophyll content (mg g^{-1}) on 5 MAP	Percentage change over the control	Chlorophyll content (mg g^{-1}) on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	1.608 ± 0.004^{a}	2.42	1.811 ± 0.005^{a}	5.20	1.875 ± 0.006^{a}	5.98
T2 (CHT 2 g L^{-1})	1.586 ± 0.017^{b}	1.02	1.785 ± 0.008^{b}	3.40	1.836 ± 0.010^{b}	3.76
T3 (CHT 3 g L^{-1})	1.562 ± 0.012^{c}	-0.50	1.694 ± 0.017^{d}	-2.00	1.718 ± 0.018^{d}	-2.90
T4 (CHT 4 g L^{-1})	1.533 ± 0.024^{d}	-2.30	1.645 ± 0.016^{e}	-4.80	1.684 ± 0.048^{d}	-4.90
T5 (CHT 0 g L^{-1})	1.569 ± 0.003^{bc}	0	1.727 ± 0.009^{c}	0	1.776 ± 0.015^{c}	0
SE(d)	0.010		0.008		0.017	
SE(m)	0.007		0.006		0.012	
CV (%)	0.906		0.687		1.381	
CD(P=0.05)	0.021		0.018		0.037	

At 7 MAP, the highest chlorophyll content (1.875 mg g^{-1}) was recorded in T1 followed by T2 (1.836 mg g^{-1}). The lowest (1.684 mg g^{-1}) chlorophyll content was observed in T4 and was on par with T3 (1.718 mg g^{-1}). The control treatment recorded a higher value (1.776 mg g^{-1}) compared to T3 and T4.

The chlorophyll content was observed to give 2.42, 5.20 and 5.98 per cent increase over control treatment, in plants exposed to T1 at 3, 5 and 7 MAP, respectively.

4.2.6 Peroxidase activity

The effect of different concentrations of chitosan foliar spray treatments on the peroxidase activity of *P. longum* is shown in Table 21. At all stages of observation, significant variation was observed in peroxidase activity.

At 3 MAP, the highest peroxidase activity (18.60 activity g^{-1} min^{-1}) was recorded in T1 followed by T2 (18.19 activity g^{-1} min^{-1}). The lowest value (17.20 activity g^{-1} min^{-1}) was observed in T4, while the control treatment recorded a higher value (17.83 activity g^{-1} min^{-1}).

At 5 MAP, the highest (19.35 activity g^{-1} min^{-1}) peroxidase activity was recorded in T1 followed by T2 (18.67 activity g^{-1} min^{-1}). The lowest value (17.55 activity g^{-1} min^{-1}) was observed in T4, while the control treatment recorded a higher value (18.27 activity g^{-1} min^{-1}).

At 7 MAP, T1 recorded the highest peroxidase activity (19.86 activity g^{-1} min^{-1}) followed by T2 (19.12 activity g^{-1} min^{-1}). The lowest value (18.11 activity g^{-1} min^{-1}) was observed in T4. The control treatment recorded a higher value (18.72 activity g^{-1} min^{-1}) compared to T3 and T4.

T1 recorded 4.32, 5.90 and 6.06 per cent increase over control treatment, in peroxidase activity at 3, 5 and 7 MAP, respectively.

4.2.7 Catalase activity

The effect of different concentrations of chitosan foliar spray treatments on the catalase activity of *P. longum* is shown in Table 22. At all stages of observation, significant variation was observed among the treatments in catalase activity.

At 3 MAP, the highest (773.52 activity g^{-1} min^{-1}) catalase activity was recorded in T1, followed by T2 (739.13 activity g^{-1} min^{-1}). The lowest (647.79 activity g^{-1} min^{-1}) catalase activity was observed in T4 and was observed to be par with T3 (666.92 activity g^{-1} min^{-1}). The control treatment recorded a higher value (731.43 activity g^{-1} min^{-1}) compared to T3 and T4.

At 5 MAP, the highest (861.18 activity g^{-1} min^{-1}) catalase activity was recorded in T1 followed by T2 (819.64 activity g^{-1} min^{-1}). T4 was observed to give the lowest (687.08 activity g^{-1} min^{-1}) catalase activity. Compared to T3 and T4, the control treatment recorded a higher value (809.52 activity g^{-1} min^{-1}).

At 7 MAP, the highest catalase activity was observed in T1 (933.48 activity g^{-1} min^{-1}) followed by T2 (861.18 activity g^{-1} min^{-1}). The lowest (716.03 activity g^{-1} min^{-1}) catalase activity was observed in T4 and was on par with T3 (731.00 activity g^{-1} min^{-1}). The control treatment recorded a higher catalase activity (850.00 activity g^{-1} min^{-1}) compared to T3 and T4.

T1 recorded 5.75, 6.38 and 9.82 per cent increase over control treatment in catalase activity at 3, 5 and 7 MAP, respectively.

At all stages of observation, it was found that the foliar spray treatment T1 (CHT @ 1 g L^{-1}) exhibited the highest value among all physiological parameters tried. Also, foliar spray with a higher concentration of chitosan, T3 (CHT @ 3 g L^{-1}) and T4 (CHT @ 4 g L^{-1}) recorded lower values than the control (T5).

Table 21. Effect of chitosan foliar spray on peroxidase activity

Treatment	Peroxidase activity (activity g^{-1} min^{-1}) on 3 MAP	Percentage change over the control	Peroxidase activity (activity g^{-1} min^{-1}) on 5 MAP	Percentage change over the control	Peroxidase activity (activity g^{-1} min^{-1}) on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	18.60±0.13[a]	4.32	19.35±0.21[a]	5.90	19.86±0.06[a]	6.06
T2 (CHT 2 g L^{-1})	18.19±0.06[b]	2.01	18.67±0.05[b]	2.19	19.12±0.09[b]	2.10
T3 (CHT 3 g L^{-1})	17.49±0.06[d]	-1.89	17.90±0.06[d]	-2.03	18.30±0.05[d]	-2.25
T4 (CHT 4 g L^{-1})	17.20±0.04[e]	-3.51	17.55±0.23[e]	-3.95	18.11±0.10[e]	-3.28
T5 (CHT 0 g L^{-1})	17.83±0.07[c]	0	18.27±0.06[c]	0	18.72±0.12[c]	0
SE(d)	0.058		0.107		0.065	
SE(m)	0.041		0.076		0.046	
CV (%)	0.459		0.826		0.490	
CD(P=0.05)	0.123		0.228		0.138	

Table 22. Effect of chitosan foliar spray on catalase activity

Treatment	Catalase activity (activity g^{-1} min^{-1}) on 3 MAP	Percentage change over the control	Catalase activity (activity g^{-1} min^{-1}) on 5 MAP	Percentage change over the control	Catalase activity (activity g^{-1} min^{-1}) on 7 MAP	Percentage change over the control
T1 (CHT 1 g L^{-1})	773.52±28.75[a]	5.75	861.18±22.3[a]	6.38	933.48±50.15[a]	9.82
T2 (CHT 2 g L^{-1})	739.13±0.0[b]	1.05	819.64±20.23[b]	1.25	861.18±22.36[b]	1.31
T3 (CHT 3 g L^{-1})	666.92±15.1[c]	-8.81	708.33±0.0[c]	-12.5	731.00±15.39[c]	-13.94
T4 (CHT 4 g L^{-1})	647.79±12.10[c]	-11.43	687.08±14.16[c]	-15.12	716.03±15.39[c]	-15.76
T5 (CHT 0 g L^{-1})	731.43±15.39[b]	0	809.52±14.16[b]	0	850.00±0.0[b]	0
SE(d)	11.994		10.539		18.682	
SE(m)	8.481		7.452		13.210	
CV (%)	2.383		1.918		3.228	
CD(P=0.05)	25.564		22.462		39.821	

4.3. BIOCHEMICAL PARAMETERS

The biochemical parameters, piperine, oleoresin and volatile oil content were analyzed using the spikes obtained from the plants exposed to foliar spray with different concentrations of chitosan at 2, 4 and 6 MAP.

4.3.1 Piperine content and yield

The effect of different concentrations of chitosan foliar spray treatments on the piperine content and yield of *P. longum* is shown in Table 23. The parameter exhibited significant variation among the treatments tried.

The highest (1.34 per cent) piperine content was observed in T1, followed by T2 (1.11 per cent). The lowest (0.72 per cent) piperine content was observed in T4, while the control treatment recorded higher value (0.97 per cent).

With regard to piperine yield T_1 recorded the highest yield (0.428 mg plant^{-1}) and was followed by T2 (0.323 mg plant^{-1}). T4 gave the lowest (0.141 mg plant^{-1}) piperine yield. The control treatment recorded a higher value (0.254 mg plant^{-1}) compared to T3 and T4.

An increase of 37.46 and 68.00 per cent was observed in T1 over the control treatment in piperine content and yield, respectively.

4.3.2 Oleoresin content and yield

The effect of different concentrations of chitosan foliar spray on oleoresin content and yield of *P. longum* is shown in Table 24. Significant variation was observed with respect to the parameter, among the treatments tried.

T1 was observed to give the highest oleoresin content (10.70 per cent). This was followed by T2 (9.46 per cent). The lowest (7.16 per cent) oleoresin content was recorded in T4, while control treatment recorded a higher value (8.50 per cent).

Table 23. Effect of chitosan foliar spray on piperine content and piperine yield

Treatment	Piperine content (per cent)	Percentage change over the control	Piperine yield (mg plant^{-1})	Percentage change over the control
T1 (CHT 1 g L^{-1})	1.34±0.02^{a}	37.46	0.428±0.009^{a}	68.00
T2 (CHT 2 g L^{-1})	1.11±0.016^{b}	14.43	0.323±0.002^{b}	29.20
T3 (CHT 3 g L^{-1})	0.81±0.022^{d}	-16.20	0.178±0.003^{d}	-28.80
T4 (CHT 4 g L^{-1})	0.72±0.027^{e}	-25.40	0.141±0.002^{e}	-43.60
T5 (CHT 0 g L^{-1})	0.97±0.020^{c}	0	0.254±0.005^{c}	0
SE(d)	0.015		0.004	
SE(m)	0.011		0.003	
CV (%)	2.155		1.905	
CD(P=0.05)	0.032		0.009	

Table 24. Effect of chitosan foliar spray on oleoresin content and oleoresin yield

Treatment	Oleoresin (per cent)	Percentage change over the control	oleoresin yield (g plant^{-1})	Percentage change over the control
T1 (CHT 1 g L^{-1})	10.70±0.30^{a}	25.86	3.39±0.136^{a}	52.01
T2 (CHT 2 g L^{-1})	9.46±0.053^{b}	11.25	2.73±0.005^{b}	22.42
T3 (CHT 3 g L^{-1})	7.58±0.032^{d}	-10.84	1.66±0.02^{d}	-25.55
T4 (CHT 4 g L^{-1})	7.16±0.085^{e}	-15.75	1.41±0.02^{e}	-36.77
T5 (CHT 0 g L^{-1})	8.50±0.099^{c}	0	2.23±0.043^{c}	0
SE(d)	0.151		0.065	
SE(m)	0.106		0.046	
CV (%)	1.733		2.852	
CD(P=0.05)	0.387		0.168	

With regard to oleoresin yield, T_1 recorded the highest yield (3.39 g plant^{-1}). This was followed by T2 (2.73 g plant^{-1}). The lowest (1.41 g plant^{-1}) oleoresin yield was observed in T4. The control treatment recorded a higher value (2.23 g plant^{-1}) compared to T3 and T4.

T1 recorded 25.86 and 52.01 per cent increase over the control treatment in oleoresin content and yield, respectively.

4.3.3 Volatile oil content and yield

The effect of different concentrations of chitosan foliar spray treatments on the volatile oil content and yield of *P. longum* is shown in Table 25. The parameter exhibited significant variation among the treatments tried.

The highest (1.46 per cent) volatile oil content was observed in T1 followed by T2 (1.40 per cent). The lowest (1.28 per cent) volatile oil content was observed in T4, while the control treatment recorded a higher value (1.38 per cent). The control treatment was observed to be on par with T2.

T_1 recorded the highest volatile oil yield (0.46 g plant^{-1}), followed by T2 (0.40 g plant^{-1}). The lowest (0.25 g plant^{-1}) volatile oil yield was observed in T4. The control treatment recorded a higher value (0.36 g plant^{-1}) compared to T3 and T4.

Volatile oil content and yield, increased by 5.77 and 27.82 per cent, respectively in T1, over the control treatment.

At all stages of observation, it was found that the foliar spray treatment, T1 (CHT @ 1 g L^{-1}) exhibited the highest value among all parameters tried. Also, T3 (CHT @ 3 g L^{-1}) and T4 (CHT @ 4 g L^{-1}) gave lower values than the control (T5).

Table 25. Effect of chitosan foliar spray on volatile oil content and volatile oil yield

Treatment	volatile oil (per cent)	Percentage change over the control	volatile oil yield (g plant^{-1})	Percentage change over the control
T1 (CHT 1 g L^{-1})	1.46±0.007^{a}	5.77	0.464±0.008^{a}	27.82
T2 (CHT 2 g L^{-1})	1.40±0.007^{b}	1.44	0.406±0.00^{b}	11.84
T3 (CHT 3 g L^{-1})	1.34±0.007^{c}	-2.80	0.295±0.001^{d}	-18.73
T4 (CHT 4 g L^{-1})	1.28±0.014^{d}	-7.50	0.252±0.002^{e}	-30.57
T5 (CHT 0 g L^{-1})	1.38±0.007^{b}	0	0.363±0.005^{c}	0
SE(d)	0.009		0.004	
SE(m)	0.006		0.003	
CV(%)	0.650		1.148	
CD(P=0.05)	0.023		0.011	

4.4 MOLECULAR ANALYSIS

The observations on plant molecular characteristics are recorded 24 h after second application of chitosan (at 4 MAP).

4.4.1 RNA isolation

RNA was isolated from leaves of *Piper longum* variety Viswam, 24h after the second application of chitosan using TRIzol reagent method. The RNA sample isolated was stored at -80ºC.

4.4.2 Agarose gel electrophoresis (AGE)

The isolated RNA was observed as three distinct bands (Plate 6) in 2 per cent agarose gel.

4.4.3 Quantification of RNA

The concentration as well as the purity of isolated RNA is given in Table 26. The OD_{260} / OD_{280} ranged from 1.91-2.06 and the concentration ranged from 268.8 - 400.8 ng μl^{-1}.

Table 26. Purity and quantity of isolated RNA

Treatment	Replication	OD_{260}/OD_{280}	Quantity of RNA (ng μl^{-1})
T1 (CHT 1 g L^{-1})	R1	1.931	268.8
	R2	1.912	314.4
T2 (CHT 2 g L^{-1})	R1	2.061	400.8
	R2	2.062	283.8
T3 (CHT 3 g L^{-1})	R1	1.972	379.2
	R2	1.916	376.8
T4 (CHT 4 g L^{-1})	R1	1.962	278.2
	R2	1.943	378.8
T5 (CHT 1 g L^{-1})	R1	1.947	319.2
	R2	1.956	367.2

4.4.4 cDNA synthesis

The isolated RNA samples were converted to cDNA using RT Easy 1 cDNA synthesis kit (G biosciences). For every 20 µl reaction, 2 µl (200 ng) of RNA sample was used. The cDNA thus made was stored at -80^{o}C. The synthesized cDNA was confirmed by using actin gene (housekeeping gene) with amplicon size 198 bp (Plate 7)

4.4.5 Primer for RT-PCR

The primers were reconstructed based on the instruction by the manufacturer, after dissolution it was stored at -20^{o}C and was used as stock. Ten times diluted stocks were used as working standards.

4.4.6 Standardization of annealing temperature

The optimum annealing temperature for actin (52.1^{o}C) and piperine synthase (47.5 ^{o}C) were selected after performing thermal gradient qRT-PCR at 47.0, 47.5, 48.6, 50.2, 52.1 and 53.0^{o}C, as the lowest threshold cycle (Ct) value was observed at these temperatures (Table 27). qRT-PCR product was observed as distinct bands (Plate 8) in agarose gel (1.8 per cent) and the melt curves obtained are given in the Figure 1.

Table 27. Standardized annealing temperature for Real-time PCR

Temperature	Threshold cycle (Ct) values
47.5 ^{0}C	22.45
52.1 ^{0}C	25.24

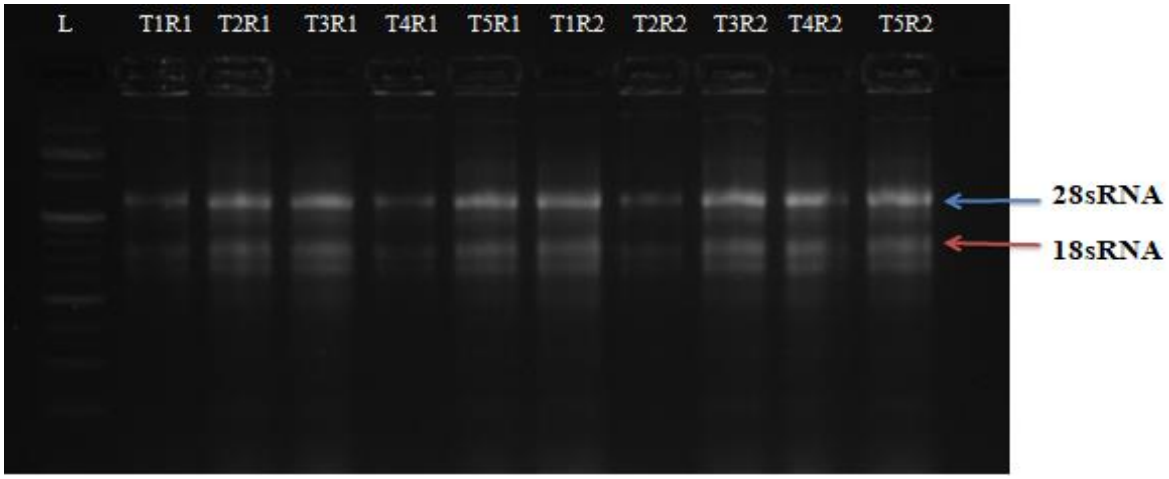

Plate 6. Gel profile of RNA isolated from leaves of *Piper longum*
T1: CHT (1 g L^{-1}), T2: CHT (2 g L^{-1}), T3: CHT (3 g L^{-1}), T4: CHT (4 g L^{-1})
T5: Control, R: Represent replication, L: 100bp ladder

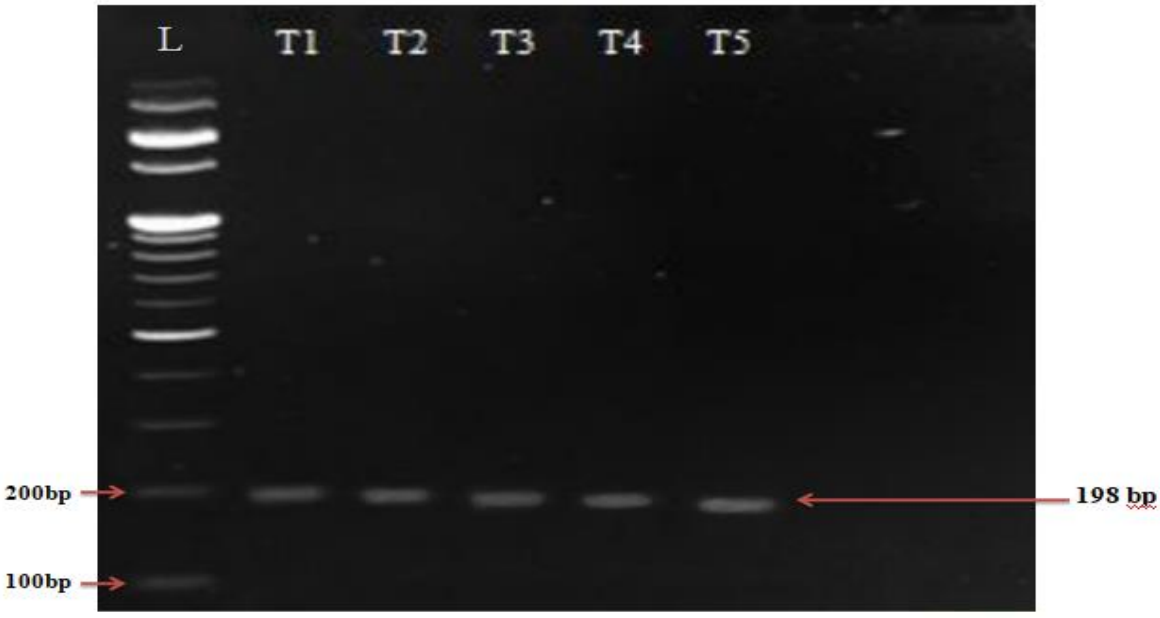

Plate 7. Gel profile of cDNA after amplification with actin primers
Lane T1: CHT (1 g L^{-1}), Lane T2: CHT (2 g L^{-1}), Lane T3: CHT (3 g L^{-1})
Lane T4: CHT (4 g L^{-1}), Lane T5: Control, L: 100bp ladder

4.4.7 Expression analysis of piperine synthase gene

The expression analysis of piperine synthase gene was studied by using actin as reference gene. The synthesized cDNA was subjected to real-time PCR with reference gene actin and piperine synthase. The melt curve analysis showed a single peak in all treatments (Fig 2).

The relative expression of piperine synthase gene at different concentrations of chitosan with respect to the control plant was worked out using the comparative Ct method (Table 28). It was observed that chitosan @ 1 and 2 g L^{-1} increased the expression by 1.8 and 1.33 fold respectively, while the lowest gene expression was observed in plant sample exposed to chitosan foliar spray @ 4 g L^{-1}.

Table 28. Effect of chitosan foliar spray on the expression of piperine synthase gene

Treatments	Relative fold change (RFC)
T1(CHT 1 g L^{-1})	1.873±0.009
T2(CHT 2 g L^{-1})	1.331±0.016
T3(CHT 3 g L^{-1})	0.568±0.008
T4(CHT 4 g L^{-1})	0.440±0.006
T5(CHT 0 g L^{-1})	1±0.00
SE(d)	0.01
SE(m)	0.007
CV (%)	0.922
CD (P=0.05)	0.025

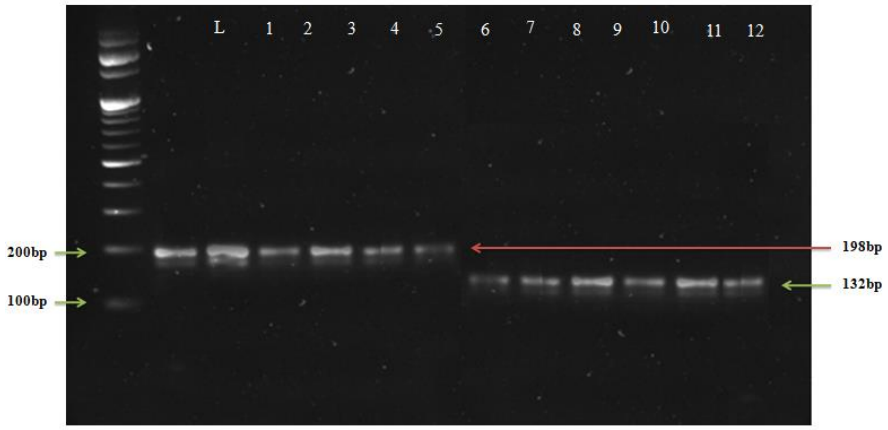

Plate 8. Gel profile of actin and piperine synthase on different annealing temperature. Lane 1-6: Amplification using actin primer, Lane7-12: Amplification using piperine synthase primer, Lane 1 and 7: 47 ^{0}C, Lane 2 and 8: 47.5 ^{0}C, Lane 3 and 9: 48.6 ^{0}C, Lane 4 and 10: 50.2 ^{0}C, Lane 5 and 11: 52.1 ^{0}C, Lane 6 and 23: 53^{0}C, and L: 100bp ladder

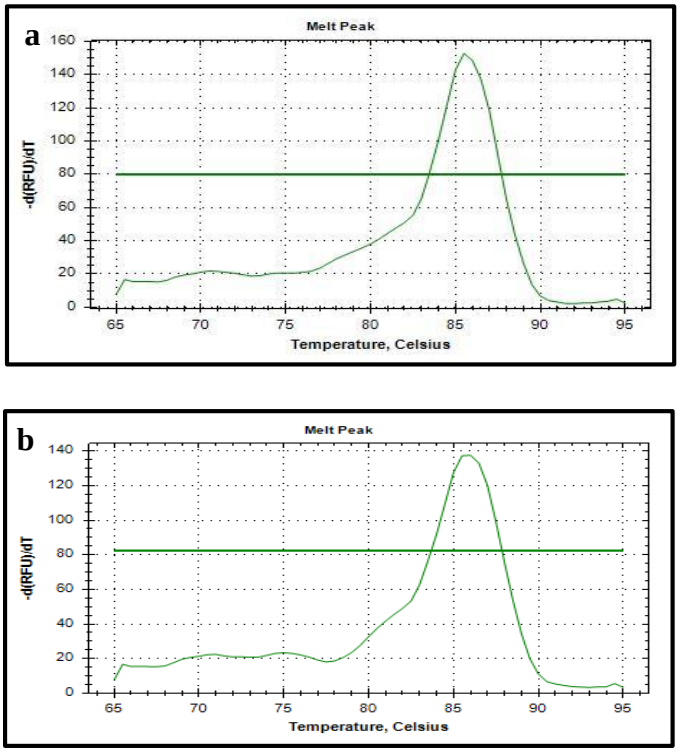

Fig.1. Melt curve of primers a) Piperine synthase gene at 47.5 ^{0}C and b) Actin gene at 52.1 ^{0}C

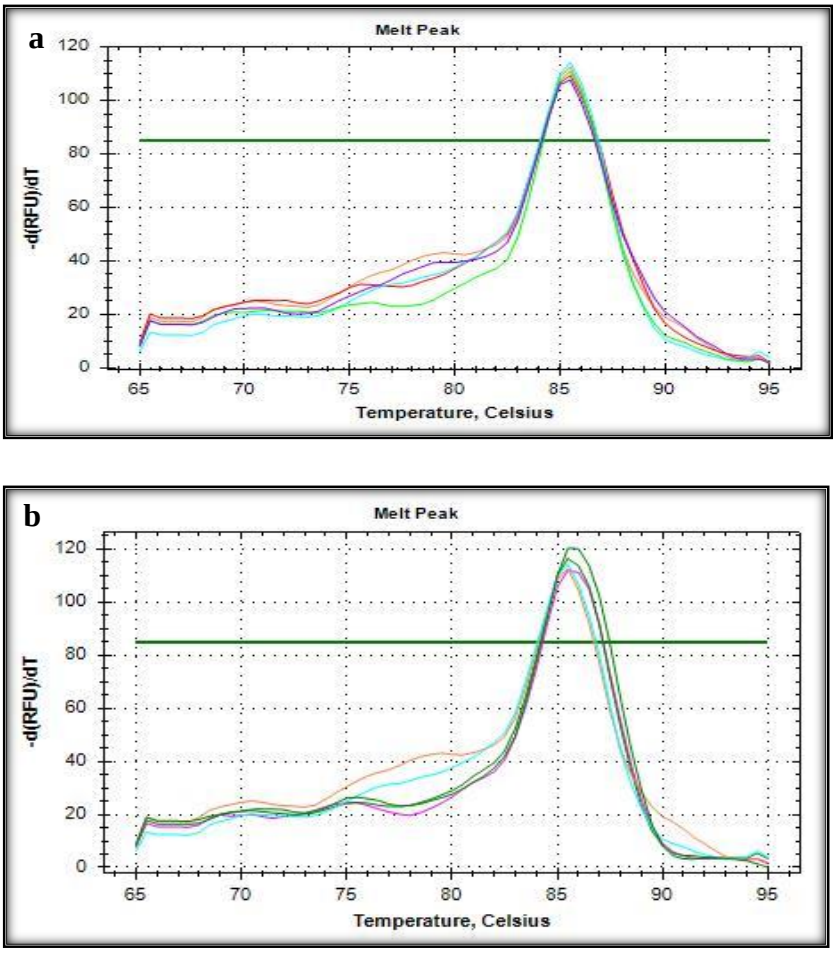

Fig.2. Melt curve of primers a) Actin, b) Piperine synthase

DISCUSSION

5. DISCUSSION

This chapter discusses findings of the current study entitled "Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L." with reference to the observed data and relevant literature.

Chitosan is an alkaline natural polysaccharide with good biological inter-miscibility, biocompatibility and biodegradability. It is a non-toxic polymer that triggers a positive reaction in plants, a defense-related response, accumulation of secondary metabolites and improves plant growth and tolerance to a variety of diseases (Al-Tawaha *et al*., 2005). It also plays a substantial role in the enhancement of physiological responsiveness and mitigation of adverse effects of biotic and abiotic stresses (Hidangmayum *et al*., 2019). The study validates the physiological, biochemical and molecular responses of *P. longum* to foliar application of chitosan.

5.1 EFFECT OF CHITOSAN ON PLANT GROWTH PARAMETERS

In the study, foliar application of CHT at different concentrations significantly influenced the plant growth in terms of plant height, number of primary branches per plant, number of spike-bearing branches per plant, days to emergence of spike, days to flowering and days from emergence to maturity of spike. All these parameters showed an increasing trend with decreasing concentration of CHT.

CHT treated plants (CHT 1 g L^{-1}) showed significantly higher values with respect to plant height (194.75 cm), number of primary branches (7.75) and spike-bearing branches (8.5) at 7 MAP. CHT @ 2 g L^{-1} also showed higher values over the control for these parameters. The same trend was observed in the early stages (3 and 5 MAP) of growth also. However, at higher concentrations (3 and 4 g L^{-1}) the parameters recorded lower values than the control plants. The result obtained in the study is in contradiction with that of Uge *et al*. (2018) where chitosan @ higher concentration (1 per cent) made a substantial improvement on all plant growth indices, compared to lower concentrations (0.50 and 0.75 per cent) in black pepper (*Piper nigrum* L.).

Enhanced plant growth with the application of chitosan has been observed in different crops *viz.*, watermelon (Gomez *et al*., 2017), strawberry (Rahman *et al.*, 2018), tomato (Hassnain *et al*., 2020), cordyline (El-Serafy, 2020), chinese cabbage (Faqir *et al.*, 2022), perilla (Salachna and Lopusiewicz, 2022) etc.

According to Guan *et al.* (2009), chitosan adjusts the cell osmoticum to boost the absorption and availability of water and other nutrients, thus promoting plant development. The activation of plant hormones like IAA (auxin), cytokinin and gibberellic acid by CHT would encourage the proliferation of root cells, which in turn, increases nutrient uptake (Lopez-Moya *et al*., 2017). Muley *et al*. (2019) observed that chitosan when applied is absorbed by the leaves, and provides amino acids, thereby promoting plant metabolic processes, growth and development. Mukarram *et al*. (2022) is of the view that CHT is involved in signal transduction related to various plant growth phenomena. The osmotic adjustment in stomatal cells induced by chitosan leads to enhanced stomatal opening and CO_2 integration. This stimulates plant physiological systems involved in nutrition absorption, cell division, cell elongation, enzyme activation and protein synthesis, which are directly related to plant growth (Arshad *et al*., 2022).

In the study, days to emergence of spike, days to flowering and days from emergence to maturity of spike showed significant variation among the treatments. CHT @ 1g L^{-1} concentration showed early emergence of spike (73.38 days), flowering (20.40 days), and maturity (59.83 days) compared to the higher concentration and control. Improved flowering on CHT application was observed in the following crops, *Dendrobium* (Limpanavech *et al*., 2008), *Gladiolus* (Ramos-Garcia *et al*., 2009), *Freesia* (Salachna and Zawadzinska, 2014) and potatoes (Morovvat*et al*., 2020). Early floral responses due to CHT application could be attributed to better nutrient availability, protein synthesis, cell proliferation, enzyme activity and plant vegetative growth (Limpanavech *et al*., 2008; Arshad *et al*., 2022).

Foliar spray treatment of CHT at lower concentration enhanced yield considerably in terms of spike number and spike yield in our study. CHT 1 g L^{-1} recorded higher

(fresh-163.35 g plant^{-1}and dry-29.40 g plant^{-1}) spike yield among all the treatments tried (Fig.3). Spike (dry) yield accounted for 21.73 per cent increase in spike yield over the control. The increase in yield on chitosan application was reported in various crop plants *viz.*, soybean (Lee *et al*. 2005), sweet basil (Kim *et al*. 2005), sweet pepper (Ghoname *et al*., 2010), bell pepper (Mahmood *et al*., 2017), strawberry (Mukta *et al.*, 2017), potato (Morovvat *et al*., 2020), tomato (Hassnain *et al*., 2020), artichoke (Alkharpotly and Abdelrasheed, 2021) etc. High yield of plant due to exogenous CHT application could be attributed to its impact on photosynthetic parameters, nutrient uptake, cell division and accelerated assimilation transport from source to sink and its final conversion to the reserve food material (Sharma and Sharma, 2021; Arshad *et al*., 2022).

In the study, it was observed that CHT foliar spray could evoke a significant effect on root yield (Fig.4). CHT 1 g L^{-1} recorded higher root yield (fresh-47.08 24 g plant^{-1}; dry-8.24 g plant^{-1}. The root (dry) yield accounted for 31.84 per cent increase, over the control. Sheikha and Al-Malki (2011) indicated that different concentrations of chitosan enhanced root weight in bean. El-Serafy (2020) opined that CHT enhanced root cell development by inducing certain enzymes (chitinases, pectinases and glucanases). Chitosan also induces ion uptake, which might act as a signal for root initiation, which in turn results in higher root biomass. As roots function in water and nutrient absorption, root development caused by CHT treatment would have a significant impact on crop production also.

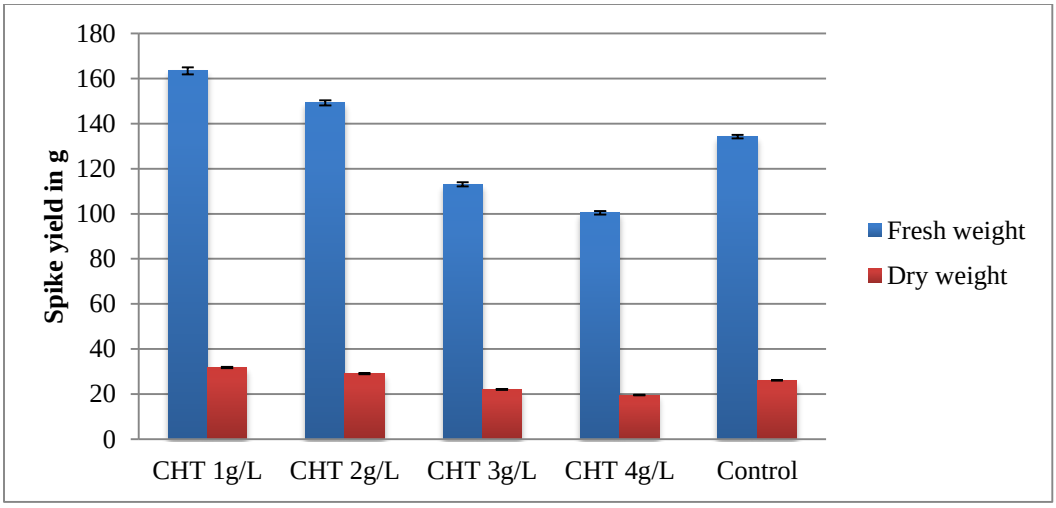

Fig.3. Effect of chitosan foliar spray on spike yield in *P. longum*

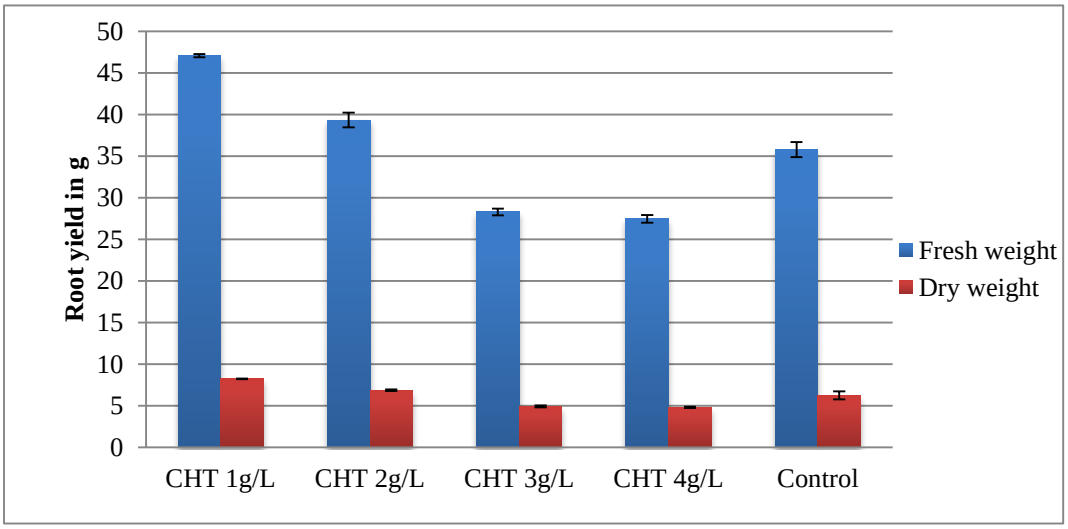

Fig.4. Effect of chitosan foliar spray on root yield in *P. longum*

5.2 EFFECT OF CHITOSAN ON PLANT PHYSIOLOGICAL PARAMETERS

In the study, it was evident that there was significant variation in physiological parameters among the different CHT treatments. The physiological parameters like stomatal conductance, stomatal distribution, photosynthetic rate, water use efficiency, chlorophyll content and enzyme activity show significant differences among treatments, with the highest value recorded at chitosan 1g L^{-1}. According to Xue *et al.* (2017), both stomatal (stomatal distribution, stomatal conductance and water use efficiency) and non-stomatal factors (chlorophyll content), that are modified by chitosan application, significantly influence photosynthesis.

The highest stomatal conductance (496.50 m moles m^{-2} s^{-1}), stomatal distribution (247.75 mm^{-2}), photosynthetic rate (8.87 µmol CO_2 m^{-2} s^{-1}) and water use efficiency (6.83 m mol CO_2 mol^{-1} H_2O) were recorded in chitosan CHT 1g L^{-1} foliar spray. The above finding is in line with that of Hasanah and Sembiring (2018) who observed enhanced stomatal distribution in soybean on foliar application of chitosan. Improved photosynthetic rate and stomatal conductance were reported in lemon grass (Mukarram *et al*., 2022) and *Calendula officinalis* (Akhtar *et al*., 2022) on chitosan application. Stomatal conductance regulates gaseous exchange. Higher stomatal conductance therefore would increase photosynthesis and improves plant yield (Ahmed *et al.*, 2022). Dowom *et al*. (2022) explained that chitosan improved stomatal distribution and reduced stomatal aperture size which facilitated rapid opening and closing of stomata, resulting in enhanced stomatal conductance, reduced water evaporation and provided sufficient CO_2 required for photosynthesis. In *Azadirachta indica*, stomatal distribution was positively correlated with net photosynthesis and biomass production (Kundu and Tigerstedt, 1998). Pearce *et al.* (2006) observed in *Populus* spp. that stomatal distribution was positively correlated with stomatal conductance.

CHT 1 g L^{-1} recorded significantly higher water use efficiency at all stages of observation (3, 5 and 7 MAP). It recorded a water use efficiency of 6.83 mmol CO_2 mol^{-1} H_2O at 7 MAP. In accordance with our study increased water use efficiency were

reported in plants like *Helianthus annuus* (Shehzad *et al.*, 2020) and *Calendula officinalis* (Akhtar *et al*., 2022) due to chitosan application. El-Serafy (2020) and Arshad *et al*. (2022) reported higher relative water content indicative of higher water use efficiency in cordyline and rose plants, respectively. CHT is observed to promote uptake and retention of water, by preserving membrane integrity and osmotic regulation (Sara *et al*., 2012; Shehzad *et al*., 2020). It also improves root growth and slows down transpiration, thereby improving plant water intake and water use efficiency (Bittelli *et al*., 2001; Mondal *et al*., 2016).

CHT enhances the concentration of photosynthesis-related chlorophyll a, b and carotenoid pigments in leaves. This boosted the formation of organic molecules and sugar by enhancing the fixation of carbon dioxide and water, thus improved photosynthesis (Dzung *et al*., 2011). Chitosan oligomer in the cell can enhance the enzyme activity by moving into the nucleus and chloroplast, thereby improving antioxidative and photosynthetic activities (Pichyangkura and Chadchawan, 2015). According to Mukarram *et al*. (2022) through the upregulation of Fv/Fm, Fv′/Fm′, and PSII genes, chitosan-induced PSII stabilization might have increased photosynthetic activity. Higher chlorophyll content and enzyme activity in photosynthetic cells might be the cause of enhanced stomatal conductance, CO_2 absorption and increased photosynthetic rate in plant leaves (Arshad *et al*., 2022).

Chlorophyll content was significantly enhanced by the application of CHT 1 g L^{-1}, with the highest value (1.875 mg g^{-1}) (Fig 5) observed at 7 MAP. The findings were synchronized with those of Chookhongkha *et al.* (2012) in chilli, El-Khateeb *et al*. (2018) in *Dracaena surculosa*, Hassnain *et al*. (2020) in tomato and Mukarram *et al*. (2022) in lemon grass. Effects of chitosan on chlorophyll content have been related to its ability to block chlorophyllase, which is engaged in the catabolic route for chlorophyll, and stimulate the expression of genes involved in the chlorophyll biosynthesis process (Ahmed *et al*., 2019). Significant enhancement of chlorophyll and carotenoids due to CHT foliar spray could be attributed to increased enzymatic activity and gaseous

exchange. It also provides extra amino groups to chlorophyll synthesis and prevents the degradation of chlorophyll (Arshad *et al*., 2022; Muley *et al*., 2019). Zhang *et al*. (2018) opined that chlorophyll degradation is also delayed by CHT application through scavenging ROS in thylakoid membranes. CHT increased endogenous cytokinins, which further stimulates chlorophyll content (Sadeghipour, 2021). These factors prevent the breakdown of chlorophyll pigment and thus enhance photosynthesis, on application of chitosan.

Antioxidant enzymes such as CAT (catalase) and peroxidase (POD) were significantly varied by the application of CHT in all three stages (Fig 6 and 7). CAT (933.48 activity g^{-1} min^{-1}) and POD (19.86 activity g^{-1} min^{-1}) recorded the highest value in CHT 1 g L^{-1}at 7 MAP. The above results were in line with the findings of Zeng *et al.* (2010) in orange, Ma *et al*. (2013) in peach, Anusuya and Sathiyabama (2016) in turmeric, Sun *et al*. (2018) in tomato and Mukarram *et al.* (2022) in lemon grass. The enhancement in antioxidant activity (POD, SOD and CAT) on exposure to CHT might be due to enhanced photosynthetic pigments and stomatal conductance, brought about by regulating genes of nucleus and chloroplast (Choudhary *et al*., 2017; Singh *et al*., 2019; Silva *et al*., 2020). CHT treatment generates oligomers in the cell that migrates to the nucleus and chloroplast to synthesize enzymes as well as increase antioxidative activity (Pichyangkura and Chadchawan, 2015). Shehzad *et al*. (2020) opined that CHT treatment protects plants from lipid peroxidation and oxidative damage by detoxifying H_2O_2 and superoxide radicals.

5.3 EFFECT OF CHITOSAN ON PLANT BIOCHEMICAL PARAMETERS

The chitosan foliar spray treatments showed significant variation among different CHT foliar treatments with the highest piperine content (1.34 per cent), oleoresin content (10.70 per cent) and volatile oil (1.46 per cent) recorded at CHT 1 g L^{-1}. A 37.46 per cent increase in piperine content was observed in foliar spray treatment CHT 1 g L^{-1} over the control (Fig 8). Oleoresin and volatile oil content in plants sprayed with chitosan1g L^{-1},

increased by 25.86 and 5.77 per cent, respectively over the control plants. The higher oleoresin and volatile oil content were observed in CHT 1 g L^{-1} (Fig 9 and 10).

CHT has been observed to enhance the production of secondary metabolites *viz,* flavonoids in *Hypericum perforatum* (Brasili *et al*., 2014), phenolics in *Origanum vulgare* (Yin *et al*., 2012), curcumin in *Curcuma longa* (Sathiyabama *et al*., 2016), phenols and glycosides in *Stevia rebaudiana* (Mehregan *et al*., 2017), menthol in *Mentha piperita* (Ahmad *et al*., 2017) and Xanthone in *Gentiana dinarica* (Krstić-Milosevic *et al*., 2017). Essential oils in basil (Ghasemi *et al*. 2014), *Thymus daenensis* (Bistgani *et al*., 2017), lemon grass (Jaleel *et al*., 2017), peppermint (Giglou *et al*., 2022) and Java citronella (Ahmed *et al*., 2022) were recorded to improve on chitosan application.

Chitosan act as elicitors involved in certain signal transduction systems that induce gene expression of enzymes in the secondary metabolic pathway. Yin *et al*. (2013) reported the influence of chitosan on jasmonic acid–ethylene signaling in rapeseed. The biosynthesis of piperine, the secondary metabolite in *P.longum* follows phenylpropanoid pathway. The phenylalanine ammonia-lyase (PAL)is the first enzyme in phenylpropanoid pathway (Liu *et al*., 2016). The phenylpropanoid pathway is probably where the aromatic portion of piperine originates. (Schnabel *et al*., 2021). CHT might have stimulated the production of piperine by activating genes associated with this pathway. Lopez-Moya *et al*. (2019) opined that CHT can stimulate the formation of secondary metabolites in plants by activating response genes that synthesise PAL.CHT also enhanced the salicylic acid synthesis, which can stimulate the development of PAL, thereby enhancing secondary metabolite production (Wiesel *et al*., 2014).

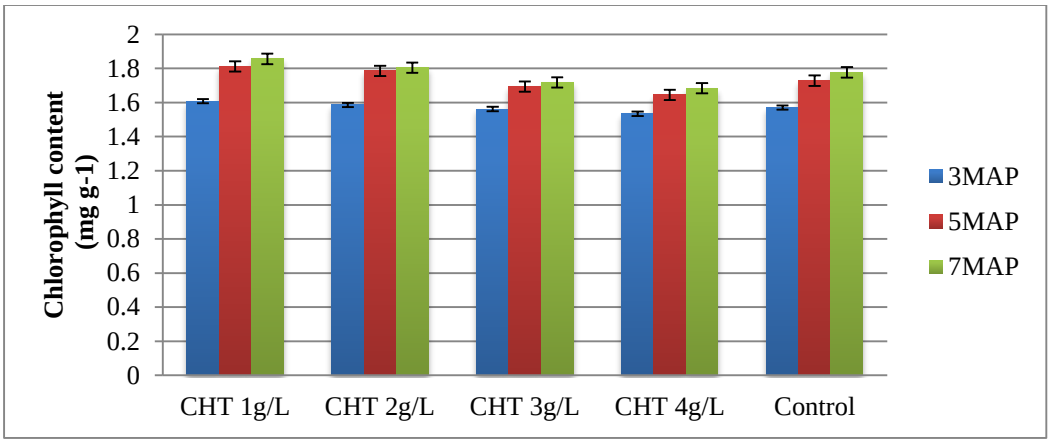

Fig.5. Effect of chitosan foliar spray on chlorophyll content in *P. longum*

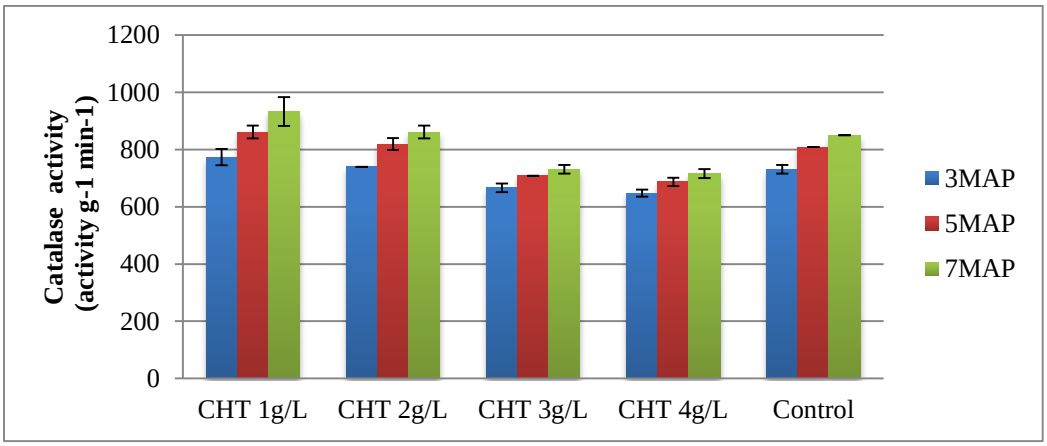

Fig.6. Effect of chitosan foliar spray on catalase activity in *P. longum*

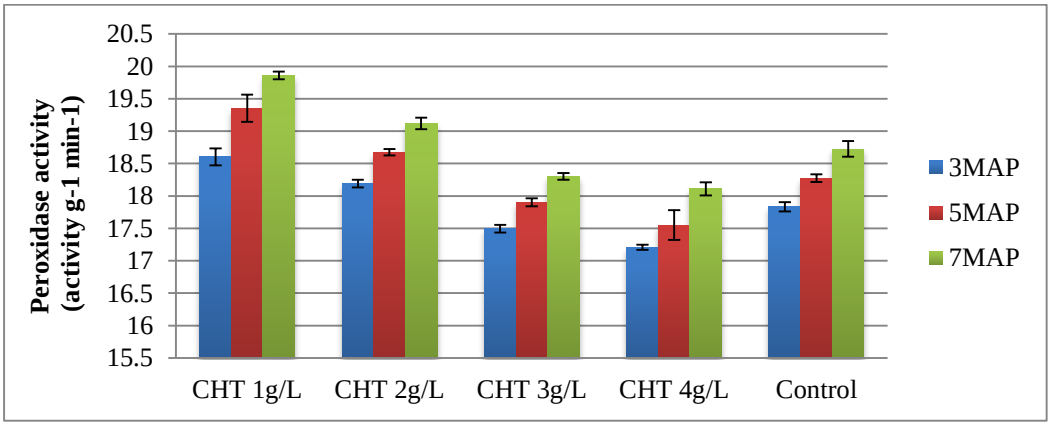

Fig.7. Effect of chitosan foliar spray on peroxidase activity in *P. longum*

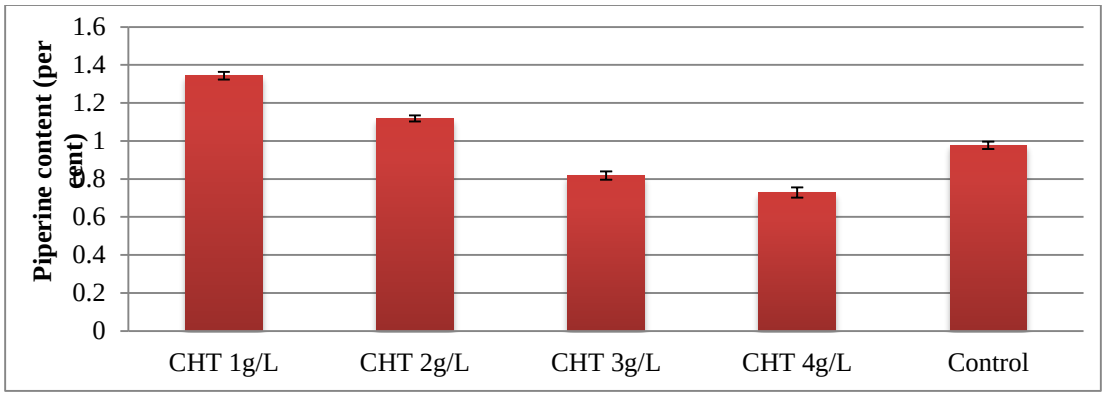

Fig.8. Effect of chitosan foliar spray on piperine content (per cent) in *P. longum*

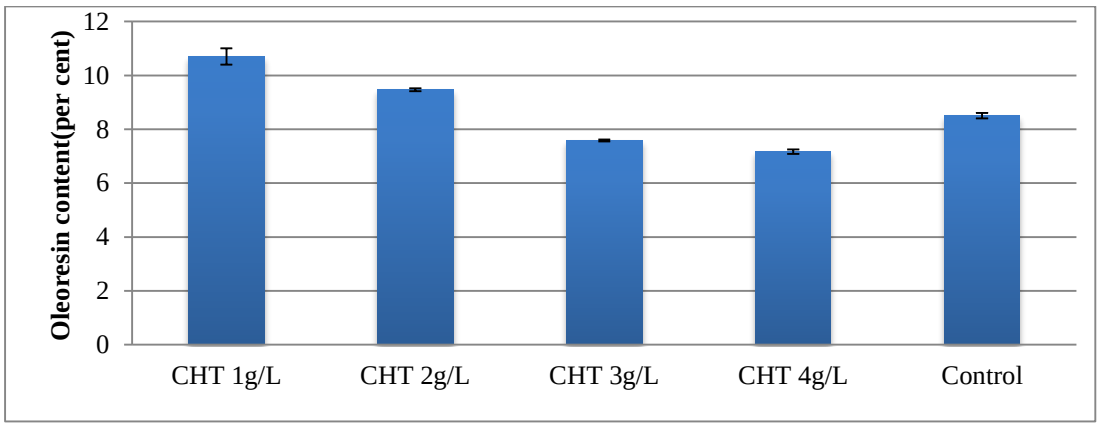

Fig.9. Effect of chitosan foliar spray on oleoresin content in *P. longum*

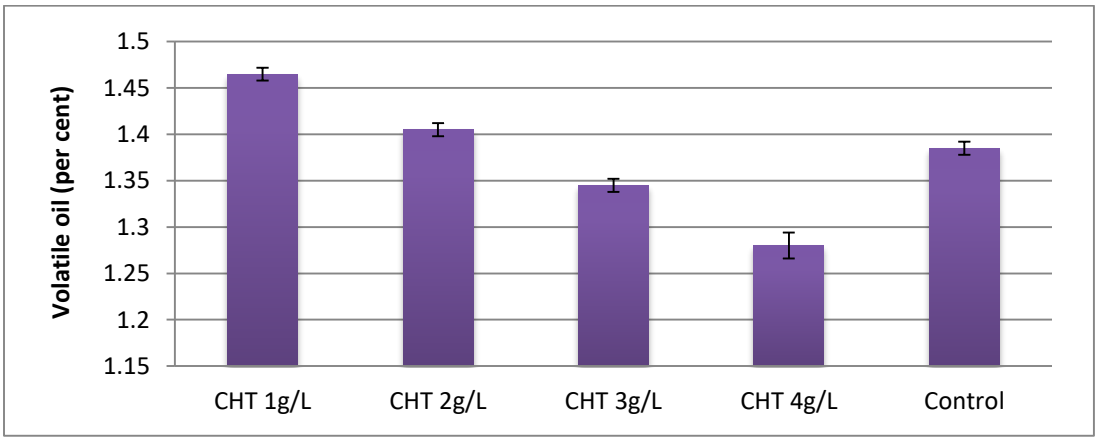

Fig.10. Effect of chitosan foliar spray on volatile oil content in *P. longum*

In the study it was observed that chitosan treatments at lower concentrations enhanced both volatile oil and oleoresin, while at higher concentrations, both these were observed to be reduced, even lower than the control treatment. Both volatile oil and oleoresin content showed a similar trend, with varying concentrations of chitosan application. In line with this, Kurian *et al.* (2002) observed that volatile oil and oleoresin is positively correlated in black pepper. Lei *et al.* (2011) reported higher artemisinin biosynthesis in *Artemisia annua* with foliar spray of chitosan at lower concentration of 100 mg L^{-1} compared to higher concentrations upto 300 mg L^{-1}. According to them, chitosan induced the transcription of many genes in artemisinin biosynthesis pathway and increased artemisinin content in *A. annua.*

CHT 1 g L^{-1} recorded the highest yield in terms of piperine (0.428 mg $plant^{-1}$), oleoresin (3.39 g $plant^{-1}$) and volatile oil (0.464 g $plant^{-1}$). The increase in piperine (37.46 per cent), oil (25.86 per cent) and oleoresin (5.77 per cent) content was observed in the study. However, the yield increase in terms of these biochemical parameters accounted for 68.00, 52.01 and 27.82 per cent. This could be attributed to increase in spike yield to the tune of 21.73 per cent over the control. The application had brought about a substantial increase in spike yield, which reflected with a significant improvement in piperine, oil and oleoresin yield. This is in line with the observation of Jaleel *et al.* (2017) in lemongrass. The treated lemon grass plants exhibited increased nutrient uptake, enzyme activities and cholorophyll, which in turn, improved nutrient levels, efficient assimilation and translocation of photosythates, improved growth and yield in plants and greater yield of oil.

5.3 EFFECT OF CHITOSAN ON GENE EXPRESSION

Chitosan treatment induces modifications in target genes encoding key enzymes and transporters involved in secondary metabolic pathways. Schnabel *et al.* (2021) identified and characterized two major biosynthetic branches of piperine biosynthetic genes piperine synthase (piperoyl-CoA:piperidinepiperoyltransferase) and piperamide

synthases from *Piper nigrum* L. by differential RNA-Seq approach. In our study, the expression of piperine synthase genes due to chitosan application has been elucidated.

RNA for gene analysis were isolated from leaves 24h after the second stage of chitosan application, shown distinct bands on the agarose gel. The purity of isolated RNA ranged from 1.91-2.06 and the concentration of isolated RNA ranges between 268.80 and 400.80 ng μl^{-1}. This implies that the RNA isolation protocol used in the study resulted in good quality and quantity of isolated RNA. A distinct band of 198 bp on agarose gel after PCR reaction using actin primer confirmed the synthesis of cDNA. This synthesized cDNA were used for the expression analysis. Using real-time PCR analysis, the impact of exogenous chitosan on the stimulation of gene expression was studied. The expression levels of the piperine synthase gene could be correlated with the production of piperine in *P.longum* in response to CHT application. Analysis of the gene expression revealed that piperine synthase expression was significantly increased during foliar spray treatment of CHT 1 g L^{-1} (1.87 fold) and CHT 2 g L^{-1} (1.33 fold) when compared to the control (Fig.11). According to Kim *et al.* (2005), chitosan might affect the function of the genes and enzymes responsible for producing secondary metabolites. Several studies had reported the influence of chitosan on the expression of genes associated with secondary metabolite production. Lei *et al*. (2011) reported that chitosan induces artemisinin biosynthesis by upregulating ADS and DBR2 gene in *Artemisia annua*. Similarly, Shabani *et al*. (2019) reported jasmonic acid biosynthesis by upregulation of OPR gene in *Melissa officinalis* L.

According to Singh (2016), chitosan activated the phenolpropanoid pathway, targeting various classes of secondary metabolites. This might be the reason for the enhanced production of secondary metabolites like piperine due to the exogenous application of chitosan. The enzyme, piperine synthase catalyzed the conversion of piperidine and piperoyl-CoA into piperine in the terminal stage of the synthesis pathway (Schnabel *et al*., 2021). The plant responses to chitosan might be due to its effect on several signaling pathways, including those involving various secondary messengers, transcription factors (TFs) and an oxidative burst by releasing H_2O_2. Chitosan activates

mitogen-activated protein kinase (MAPK) that moves into the nucleus, where it phosphorylates transcription factors and increases gene expression. It turns NADPH oxidase and triggers an oxidative burst that results in the production of H_2O_2. H_2O_2 activates the enzyme PAL; which in turn stimulates the phenylpropanoid pathway (Ahmed *et al*., 2019). Chitosan is said to be a signaling molecule, which stimulates different enzyme activities. It also interacts with chromatin and directly affects gene expression.

In the study, the varied expression of piperine synthase gene has been observed due to varying concentrations of chitosan. This might be due to the effect of chitosan on the enzymes associated with the phenylpropanoid pathway, which is the biosynthetic pathway for piperine synthase. Among the treatments, CHT 1 g L^{-1} gave the highest piperine content which could be attributed to the highest (1.87 fold) increase in the expression level of piperine synthase gene.

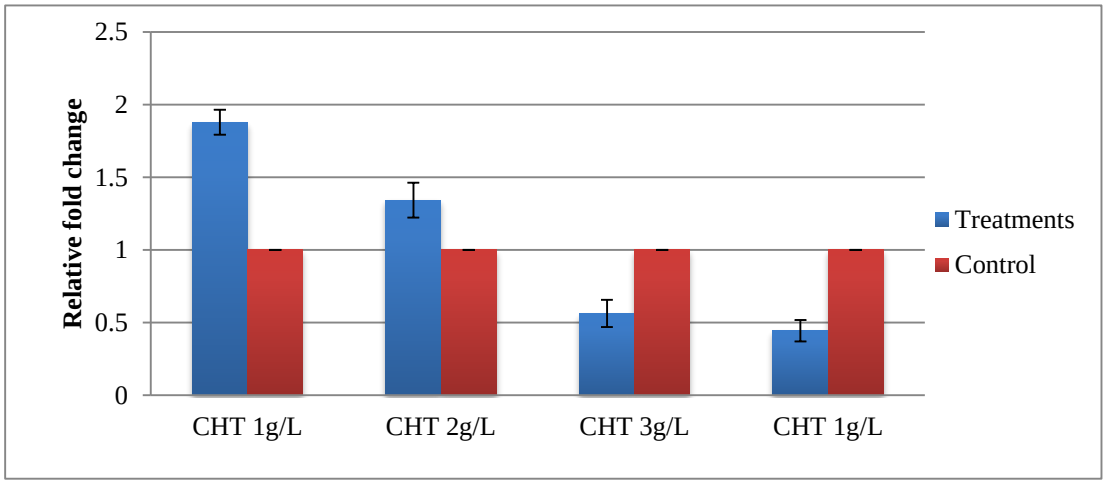

Fig.11. Relative expression of piperine synthase gene on foliar application of chitosan

The study confirms that the foliar application of chitosan at a lower concentration of 1 g L^{-1}, could give enhanced yield and quality in terms of piperine, oleoresin and oil content in *P. longum*. The same treatment recorded better physiological and molecular responses, which might have led to the enhanced yield and quality of *P. longum*. It was

also observed in the study that higher concentrations of chitosan, 3 and 4 g L^{-1} were inhibitory for plant growth and production of secondary metabolites. This elucidates the fact that the optimization of the concentration of chitosan is in evitable for its utilization in plant growth promotion.

Future line of work

With this basic study of the effect of chitosan on physiological, biochemical and molecular characteristics of *P. longum*, the following future lines of work could be initiated.

1. Effect of lower concentrations of chitosan on yield and quality of *P.longum*
2. Mechanism of action of chitosan on *P. longum*
3. Effect of chitosan in combination with other biostimulants in *Piper* spp
4. Effect of nano chitosan on *Piper* spp

SUMMARY

6. SUMMARY

The study entitled “Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L.” was conducted in the Department of Plant Biotechnology and Department of Plantation Crops and Spices, College of Agriculture, Vellayani, Trivandrum, Kerala from 2021 to 2022. The study aims to investigate the physiological, biochemical and molecular responses of *Piper longum* to foliar application of chitosan.

The two-month-old rooted cuttings of variety Viswam procured from the Department of Plantation Crops and Spices, College of Agriculture, Vellanikkara were used for the study. The rooted cuttings were planted in polybag containing soil, sand and cow dung (1:1:1). Staking was done three months after planting.

Chitosan at four different concentrations (1 g L^{-1}, 2 g L^{-1}, 3 g L^{-1}, 4 g L^{-1}) at 2, 4 and 6 months after planting (MAP). The control plants were devoid of chitosan application. The experiment was laid in a completely randomized design (CRD) with five treatments and four replications (5 plants per replication). The observations were taken on plant growth, physiological, biochemical and molecular parameters of P. *longum* in response to foliar application of CHT.

The plant growth parameters *viz,* plant height, number of primary branches, number of spikes bearing branches, days to emergence of spike, days to flowering and days from emergence to maturity of spike were recorded at 3, 5 and 7 MAP. The parameters varied significantly among the treatment tried, the foliar spray treatment with CHT 1 g L^{-1} recorded the highest plant height (59.57 cm, 124.62 cm and 194.75 cm), number of primary branches (4.75, 5.75 and 7.75), and number of spikes bearing branches (3.75, 7.00 and 8.50) at 3, 5 and 7MAP. At 7 MAP, these plants recorded 19.75, 34.78, and 21.42 per cent increase in plant height, number of primary branches, and number of spikes bearing branches, respectively. It was observed that CHT 1 g L^{-1} showed earliness with respect to emergence of spike (73.38 days), flowering (20.40 days) which

was on par with CHT 2 g L^{-1} (21.51 days), and emergence to maturity of spike (59.83 days) which was on par with CHT 2 g L^{-1} (60.13 days) with respect to control. The study demonstrated that higher concentrations of CHT 3 g $L^{-1}$and 4 g L^{-1} were inhibitory, for plant growth parameters in comparison to the control treatment. These treatments also recorded lateness in spike emergence, flowering and reaching spike maturity.

The yield parameters *viz*., number of spikes per plant, length of spike, girth of spike, spike yield per plant, and root yield per plant varied significantly with different CHT foliar spray treatments. With respect to the number of spikes per plant (181.5), length of spike (3.25 cm), girth of spike (9.97 mm), spike yield (fresh-163.35 g $plant^{-1}$ and dry-29.40 g $plant^{-1}$) and root yield (fresh-47.08 g $plant^{-1}$ and dry-8.24 g $plant^{-1}$), T1 recorded significantly higher values followed by T2. T1 recorded 21.73 and 31.84 per cent increase in spike yield (dry) and root yield (dry) per plant, respectively. The higher concentrations of CHT 3 g L^{-1} and 4 g L^{-1} were detrimental with respect to yield parameters. The least values in terms of spike (dry) yield (18.07 g $plant^{-1}$) and root (dry) yield (4.80 g $plant^{-1}$) were recorded in plants subjected to CHT 4 g L^{-1}. This accounted for 25.17 and 23.20 per cent decrease in spike yield and root yield, respectively. The parameter, driage in terms of spike yield and root yield, did not show any significant variation among the treatments.

The plant physiological parameters *viz*., stomatal distribution, stomatal conductance, photosynthetic rate, water use efficiency, chlorophyll content and enzyme activities (CAT and POD) gave significantly higher values in plant subjected to foliar spray treatment with CHT 1 g L^{-1}, at all stages of observation. The treatment CHT 1 g L^{-1} obtained the highest value with respect to stomatal distribution (222.00, 234.75 and 247.75 mm^{-2}), stomatal conductance (327.50, 408.75 and 496.50 mmoles m^{-2} s^{-1}), photosynthetic rate (7.80, 8.27 and 8.87 µmol CO_2 $m^{-2}s^{-1}$), water use efficiency (5.34, 6.15 and 6.83 mmol CO_2 mol^{-1} H_2O), chlorophyll content (1.608, 1.811 and 1.875 mg g^{-1}), POD (18.60, 19.35 and 19.86 activity g^{-1} min^{-1}) and CAT (773.52, 861.18 and 933.48 activity g^{-1} min^{-1}) at 3, 5 and 7 MAP, respectively. This was followed by CHT 2 g L^{-1}. At 7 MAP, these

plants recorded 7.60, 7.77, 12.69, 12.23, 5.98, 6.06 and 9.82 per cent increase in stomatal distribution, stomatal conductance, photosynthetic rate, water use efficiency, chlorophyll content, POD and CAT, respectively. The higher concentrations of CHT 3 g L^{-1} and 4 g L^{-1}, recorded lower values with respect to these parameters compared to the control treatment.

The mature dark green oven-dried spikes were analyzed for piperine, oleoresin and volatile oil content, which varied significantly among the treatments tried. The treatment, CHT 1 g L^{-1} reported the highest values with respect to piperine (1.34 per cent and 0.428 mg $plant^{-1}$), oleoresin (10.70 per cent and 3.39 g $plant^{-1}$), and volatile oil (1.46 per cent and 0.464 g $plant^{-1}$) content and yield, respectively, followed by CHT 2 g L^{-1}. CHT 1 g L^{-1}gave 37.46 and 68.00 per cent, 25.86 and 52.01 per cent, and 5.77 and 27.82 per cent increase in piperine, oleoresin and volatile oil content and yield, respectively, over the control treatment. However, at higher concentrations of CHT (3 g $L^{-1}$and 4 g L^{-1}) these parameters found to be significantly lower than control.

The molecular analysis of *Piper longum* was done using the leaf samples collected 24 h after the application of foliar spray at 4 MAP. RNA isolated from the sample was used for cDNA synthesis followed by RT-qPCR. The relative fold change analyzed using Ct values showed upregulation of the piperine synthase gene in treatment CHT 1 g L^{-1} (1.87 fold) and CHT 2 g L^{-1} (1.34 fold) with respect to control treatment. However, at higher concentrations, CHT 3 g L^{-1} and 4 g L^{-1}, the piperine synthase gene was observed to be downregulated.

The study confirmed that foliar spray of CHT at concentrations 1 g L^{-1} and 2 g L^{-1}, at 2, 4, and 6 MAP could effectively enhance plant growth, physiological, biochemical, molecular and yield characteristics. However, the best performance was observed in the lowest concentration of chitosan tried, 1 g L^{-1}. The foliar application of CHT 1 g L^{-1} gave maximum enhancement in piperine, oleoresin, volatile oil, spike yield and root yield with an increase of 37.46, 25.86, 5.77, 21.72, and 31.6 per cent, respectively over the control treatment. The molecular basis of increase in piperine content studied by RT-PCR using

piperine synthase primer showed upregulation of piperine synthase gene under CHT application at 1 g L^{-1}.

The results of the study indicated that chitosan, a non-toxic and eco-friendly biopolymer has the potential for substantial enhancement of yield and quality of the commercially exploited medicinal plant, *Piper longum.* The mechanism of action of chitosan to this effect has yet to be clearly elucidated.

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EFFECT OF CHITOSAN APPLICATION ON PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERISTICS OF *Piper longum* L.

by

Abhijith K.

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ABSTRACT

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Faculty of Agriculture

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COLLEGE OF AGRICULTURE

VELLAYANI. THIRUVANANTHAPURAM-695 522

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8. ABSTRACT

The study on the "Effect of chitosan application on physiological, biochemical and molecular characteristics of *Piper longum* L." was carried out in the Department of Plant Biotechnology and Department of Plantation Crops and Spices, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala during 2021-2022. The objective of the study was to investigate the physiological, biochemical and molecular responses of *Piper longum* to foliar application of chitosan (CHT).

The plants raised from the rooted cuttings of long pepper variety, Viswam were exposed to foliar spray with varying concentrations of chitosan (1 g L^{-1}, 2 g L^{-1}, 3 g L^{-1}, 4 g L^{-1}) at 2, 4, and 6 months after planting (MAP). The control plants were devoid of chitosan application. The study was carried out in a completely randomized design (CRD) with four replications. The observations were taken on plant growth, physiological and biochemical parameters and molecular characteristics.

The plant growth parameters recorded at 3, 5 and 7 MAP, showed significant variation among the treatments tried. CHT 1 g L^{-1} recorded significantly higher values for the growth parameters at all stages of observation. At 7 MAP, the foliar treatment with CHT 1 g L^{-1} recorded the highest values for plant height (194.75 cm), number of primary branches (7.75), number of spike-bearing branches (8.5), days to emergence of the spike (73.78 days), days to flowering (20.40 days) and days from emergence to maturity of the spike (59.83 days).

The physiological parameters at 3, 5, and 7 MAP, showed significant variation among the chitosan foliar spray treatments. The data on physiological parameters showed a similar trend at all stages of observation with CHT 1 g L^{-1}, recording the highest values. At 7 MAP, CHT 1 g L^{-1} showed significantly higher values (496.50 m moles m^{-2} s^{-1}, 247.75 mm^{-2}, 8.87 µmol CO_2 m^{-2} s^{-1}, and 6.83 mmol CO_2 mol^{-1} H_2O, respectively) for stomatal conductance, stomatal distribution, photosynthetic rate and water use efficiency. With respect to enzyme activity, the highest peroxidase (19.86 activity g^{-1} min^{-1}) and

catalase (933.48 activity g^{-1} min^{-1}) activities were observed at 7 MAP, in plants subjected to chitosan foliar spray with CHT 1 g L^{-1}. At 7 MAP, the highest value (1.87 mg g^{-1}) of chlorophyll content was also observed in the treatment with CHT 1 g L^{-1}.

The oleoresin, volatile oil and piperine contents obtained from mature dark green dried and powdered spike showed significant differences among the chitosan foliar spray treatments tried. The foliar spray with CHT 1 g L^{-1} gave significantly higher values with respect to oleoresin (10.70 per cent), piperine (1.34 per cent) and volatile oil (1.46 per cent) content.

The yield parameters *viz.,* the number of spikes per plant, girth and length of the spike, spike yield per plant and root yield per plant significantly varied with respect to various chitosan foliar spray treatments. Treatment with CHT 1 g L^{-1} was observed to be significantly superior with respect to the number of spikes per plant (181.5), girth of the spike (9.997 mm), length of the spike (3.25 mm), spike yield (fresh-163.35 g $plant^{-1}$; dry-31.81g $plant^{-1}$) and root yield (fresh- 47.08g $plant^{-1}$; dry-8.24g $plant^{-1}$), among all the treatments tried. The driage however, did not show any significant variation among the treatments.

The molecular analysis of *Piper longum* was done using the leaf samples collected 24 h after the application of foliar spray at 4 MAP. RNA isolated from the sample was used for cDNA synthesis followed by RT-qPCR. The relative fold change analyzed using Ct values showed upregulation of the piperine synthase gene in treatment CHT 1 g L^{-1} (1.87 fold) and CHT 2 g L^{-1} (1.34 fold) with respect to control treatment.

The chitosan foliar spray at higher concentrations of CHT (3 g L^{-1} and 4 g L^{-1}) was observed to be inhibitory, giving a lower performance, even lower than the control treatment, with respect to the various parameters studied.

The study confirmed that foliar spray of CHT at concentrations 1 g L^{-1} and 2 g L^{-1}, at 2, 4 and 6 MAP could effectively enhance plant growth, physiological, biochemical, molecular, and yield characteristics. However, the best performance was observed in the

lowest concentration of chitosan tried, 1 g L^{-1}. The foliar application of CHT 1 g L^{-1} gave maximum enhancement in piperine content, oleoresin, volatile oil, spike yield, and root yield with an increase of 37.46, 25.86, 5.77, 21.72, and 31.6 per cent, respectively over the control treatment. The molecular basis of increase in piperine content was studied by RT-PCR using piperine synthase primer showed upregulation of piperine synthase gene under CHT application at 1 g L^{-1}.

The results of the study indicated that chitosan, a non-toxic and ecofriendly biopolymer has the potential for substantial enhancement of yield and quality of the commercially exploited medicinal plant, *Piper longum*. The mechanism of action of chitosan to this effect is yet to be clearly elucidated.