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Journal homepage: <http://www.plantarchives.org>

DOI Url : <https://doi.org/10.51470/PLANTARCHIVES.2026.v26.no.1.120>

## EFFECT OF EXOGENOUS APPLICATION OF SALICYLIC ACID ON BIOCHEMICAL PARAMETERS, ANTIOXIDANT ENZYMES AND SECONDARY METABOLITES IN *PIPER LONGUM* L.

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(Date of Receiving : 04-01-2026; Date of Revision : 27-02-2026; Date of Acceptance : 16-03-2026)

### ABSTRACT

The present study investigated the effect of exogenous application of salicylic acid on the biochemical parameters and secondary metabolite production in *Piper longum* L. The rooted cuttings from the promising variety of long pepper, Viswam, were exposed to foliar spray treatments with varying salicylic acid (SA) concentrations (0.1 mM, 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM and 2.5 mM) and control (SA 0.0 mM) at 2, 4 and 6 months after planting (MAP). The results revealed that total chlorophyll, total proteins, antioxidant enzyme activity, piperine, volatile oil and oleoresin contents were significantly higher in plants treated with SA at 0.5 mM. This treatment gave approximately 31 % increase in piperine content, 11.86 % increase in volatile oil and 11.97 % increase in oleoresin content over the control. However, a declining trend in these parameters was observed with further increase in salicylic acid concentration in *P. longum*.

**Keywords:** *Piper longum*, Salicylic acid, piperine, elicitation.

**Abbreviations:** CAT: catalase, MAP: month after planting, PGPR: plant growth promoting rhizobacteria, POD: peroxidase, SA: Salicylic acid, SOD: superoxide dismutase

### Introduction

*Piper longum* L., popularly known as long pepper, belonging to the family Piperaceae, is an economically important medicinal plant well adapted to the agro-climatic situations prevailing in humid tropics. Long pepper of commerce is the dried, fully mature but unripe female spikes. Long pepper is used for the treatment in Ayurveda, Siddha, and Unani systems of medicine, especially in the treatment of bronchial disorders. Apart from the spikes, roots, and thicker part of the stem are also medicinally important and are dried and used as an important drug called piplamool. Trikadu, an ayurvedic formulation, is a blend of black

pepper, long pepper and ginger prescribed against several respiratory complaints (Sivarajan and Balachandran, 1994; Kumar *et al.*, 2011). *P. longum* is an ingredient in classical herbal formulations. Because of its great demand in the pharmaceutical industry, plants are being overexploited, especially from the wild, resulting in their extinction (Nair, 2000). As the possibility of area expansion under medicinal plants is limited, strategies for improving their production and quality have to be resorted to. The use of plant growth regulators is gaining popularity in agriculture for this purpose. Salicylic acid is an endogenous phenolic growth regulator that regulates plant growth and

development as well as a signalling molecule that evokes physiological and biochemical responses in plants (Klessig 2018; Guo *et al.*, 2019). SA is chemically 2-hydroxy benzoic acid, with an aromatic ring bearing a hydroxyl group or its functional derivative, which is synthesised by plants (Hayat and Ahmad 2007). According to Vicente and Plasencia (2011), SA influences physiological and biochemical processes throughout the life period of the plants. Previous literature has confirmed the positive influence of salicylic acid foliar spray on physiological and biochemical parameters in medicinal and aromatic plant species (Li *et al.*, 2014; Aftab 2010). Hence, this study was proposed to evaluate the physiological and biochemical responses in *P. longum* on foliar application of salicylic acid.

### Materials and Methods

The rooted cuttings from the promising variety of long pepper, Viswam plants, were planted in polybags filled with soil supplemented with 500g vermicompost. PGPR (Plant growth promoting rhizobacteria) mix I was applied at 100 g per plant at the time of planting. Cow dung slurry was applied at two months' interval. Staking was provided to the plants two weeks after planting. These plants exposed to foliar spray treatments with varying salicylic acid (SA) concentrations and control treatments *viz.*, SA 0.1 mM (T1), SA 0.5 mM (T2), SA 1.0 mM (T3), SA 1.5 mM (T4), SA 2.0 mM (T5), SA 2.5 mM (T6) and control (0.0 mM SA) at 2, 4 and 6 months after planting (MAP). The plants were maintained for up to one year after planting. The study was conducted in completely randomized block design (CRD) with three replications.

The biochemical parameters *viz.*, total chlorophyll, total proteins, and antioxidant enzymes, were recorded at 3, 5 and 7 months after planting by analysing the leaves. The piperine, volatile oil and oleoresin were recorded by analysing the dried fully matured but unripe spikes.

#### Total chlorophyll content

Chlorophyll content of leaf samples was determined using the procedure described by Arnon (1949). The leaves were chopped into small bits, and 0.5 g of the leaf sample was weighed out. The leaf bits were incubated overnight at room temperature in 10 ml DMSO (Dimethyl Sulphoxide): 80% acetone mixture (1:1 v/v) in test tubes. The coloured solution was then transferred into a measuring cylinder and made up to 25ml with a DMSO-acetone mixture. The absorbance was measured using spectrophotometer (ELICO-SL 218 Double Beam) at 663 and 645 nm.

The chlorophyll content was determined by substituting the absorbance values in the formula given below and expressed in  $\text{mg g}^{-1}$  of fresh leaf.

Total Chlorophyll =  $20.2(A_{645})$

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

#### Total Proteins

The total soluble proteins of leaf samples were estimated using a simple protein dye binding assay (Bradford, 1976) using bovine serum albumin (BSA) as the standard. Coomassie brilliant blue G 250 (100 mg) was dissolved in 50 ml of 95 per cent ethanol. 100 ml of concentrated (ortho) phosphoric acid 85% (w/v) was added to the above solution. The solution was then diluted to a final volume of 200 ml with distilled water. Leaf sample (0.1 g) was taken and was grounded to a thin paste, and soluble protein was extracted with 10 ml of phosphate buffer (pH 7.8). The extract was centrifuged at 5000 rpm for 10 min. A known volume (5 ml) of diluted dye binding solution was added to 20  $\mu\text{l}$  of the supernatant. The solution was thoroughly mixed and allowed the colour to develop for at least 5 min, but no longer than 30 min, a blue colour developed and the absorbance was measured at 596 nm. The protein content was calculated using the BSA standard in the range of (10–100  $\mu\text{g}$ ). A standard curve was plotted using standard protein absorbance vs concentration. The protein content was expressed as  $\text{mg g}^{-1}$  on fresh weight basis.

#### Peroxidase

The peroxidase activity in plants was determined following the method described by Reddy *et al.* (1995). Leaf samples (200 mg) were homogenised in 1 ml of 0.1 M phosphate buffer (pH 6.5) and centrifuged at 5000 rpm for 15 min at 4°C. 0.1 g of this extract was added to 3.0 ml of pyrogallol solution, and adjusted to give a spectrophotometer (ELICO-SL 218 Double Beam) reading of zero at 430 nm. The enzyme reaction started on adding 0.5 ml of one per cent hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) into the sample cuvettes, and the change in absorbance was measured every 30 s up to 3 min. One unit of peroxidase is defined as the change in absorbance  $\text{minute}^{-1}$  at 430 nm.

#### Catalase

The catalase (CAT) activity in plants was estimated following the method described by the Luck (1974). Leaf sample (200 mg) was ground in phosphate buffer using mortar and pestle. The homogenate thus obtained was centrifuged at 5000 rpm for 15 min at 4°C, and the supernatant was collected and used for the enzyme assay. The  $\text{H}_2\text{O}_2$  phosphate buffer (3.0 ml) was

taken in an experimental cuvette. This was followed by the rapid addition of 40  $\mu\text{l}$  of enzyme extract, which was mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm. The enzyme solution containing  $\text{H}_2\text{O}_2$  – free phosphate buffer served as a control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

### Superoxide dismutase

Superoxide dismutase (SOD) activity was estimated following the method described by Karkkar *et al.* (1984). Leaf sample (1 g) was ground in a pre-chilled pestle and mortar with 10 ml ice-cold 50 mM potassium phosphate buffer (pH 7.8), centrifuged at 10000 rpm for 10 minutes, and the supernatant was collected and used for estimation. Mix 3 ml of reaction mixture containing 50 mM potassium phosphate buffer, 13 mM methionine, 2  $\mu\text{M}$  riboflavin, 0.1 mM EDTA, 75  $\mu\text{M}$  NBT and 50  $\mu\text{L}$  of crude enzyme extract, in duplicate. Make up the volume equal by adding double-distilled water. Then set the blank without enzyme and NBT to calibrate the spectrophotometer. Set another control having NBT but no enzyme as the reference control. Expose all the tubes to 400 W bulb (4 x 100 W bulbs) for 15 min, and then read the absorbance immediately at 560 nm spectrophotometer and then calculate the percentage inhibition. The 50 per cent inhibition of the reaction between riboflavin and NBT in the presence of methionine was taken as 1 unit in SOD activity.

### Estimation of Piperine

The piperine content in the dried spikes was estimated spectroscopically as per the procedure described by Sowbhagya *et al.* (1990). Dried thippali spikes (10 mg) were powdered afresh, and samples were extracted with 100 ml of acetone in a volumetric flask. The flasks were maintained at room temperature and shaken well for 2 h. Then 0.25 ml of clear solution from the flask was taken in a cuvette and made up to 5 ml with 4.75 ml of acetone. The solution was shaken well, and the absorbance of the solution was read at 337 nm in a UV spectrophotometer, with acetone as blank.

### Preparation of the standard curve

Standard piperine solutions of concentrations *viz.*, 0.4, 0.8, 1.2, 1.6, and 2  $\text{mg l}^{-1}$  were prepared, and their absorbance values at 337 nm were recorded. These values were plotted on a graph against concentration. The concentration corresponding to the absorbance of the sample was determined, and piperine content in the samples was worked out.

### Estimation of volatile oil

Extraction of oil was done by using modified Clevenger apparatus by the hydro distillation method (AOAC, 1980). Twenty grams of dried spikes were powdered and taken in a round-bottomed flask, to which 200ml of distilled water was added. Initiate heating with the heating mantle. The condensed volatile oil was collected in the graduated tube as the top layer, being lighter than water. The volume of essential oil collected was noted and expressed in per cent volume per unit mass of the sample.

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

### Estimation of oleoresin

The oleoresin content in the spikes of *Piper longum* was estimated using Soxhlet apparatus by the solvent extraction method [AOAC, 1980] with acetone as solvent. Two grams of dried spikes were powdered, packed in a timple and placed in Soxhlet extraction tube. The solvent, acetone (150 ml), taken in the round-bottom flask, is then heated to reflux. The extraction cycle was repeated many times over three to four hours. During each cycle, a portion of the non-volatile compound is dissolved in the solvent. After completing the extractions (till no colour was observed in the extraction tube), the non-soluble portion of the extracted sample remaining in the timple was discarded. Again, repeat the distillation process to remove all the solvents. After the distillation, the solution left in the round-bottom flask is transferred to a weighed beaker and kept overnight for vaporising the leftover acetone in the solution. The beaker, along with the remaining contents, is weighed the next day. The difference in the weights gives the quantity of oleoresin, and is expressed in per cent.

### Statistical analysis

The experiment was laid out in completely randomised block design with three replications. Statistical analysis was done using OPSTAT software.

### Result

The total chlorophyll, total proteins, antioxidant enzymes, piperine, volatile oil and oleoresin content exhibited significant variations among the various treatments tried.

Foliar spray treatment with SA 0.5 mM (T2) recorded maximum values with respect to total chlorophyll (1.62  $\text{mg g}^{-1}$ , 1.66  $\text{mg g}^{-1}$  and 1.72  $\text{mg g}^{-1}$ ), total proteins (12.27  $\text{mg g}^{-1}$ , 13.28  $\text{mg g}^{-1}$  and 14.62  $\text{mg g}^{-1}$ ) at 3, 5 and 7 MAP, respectively (Table 1). Total

chlorophyll and total proteins were increased by 21.98 % and 28.24 % as compared to the control at 7 MAP.

The antioxidant enzymes, *viz.*, POD, CAT and SOD were also found to be higher in the plants subjected to foliar spray with SA 0.5 mM (Fig 1, 2, and 3). This treatment was found to give higher activity of POD (19.66 activity  $\text{g}^{-1} \text{min}^{-1}$ , 21.07 activity  $\text{g}^{-1} \text{min}^{-1}$  and 23.93 activity  $\text{g}^{-1} \text{min}^{-1}$ ), CAT (912.33 U  $\text{ml}^{-1}$ , 1027.00 U  $\text{ml}^{-1}$  and 1164.67 U  $\text{ml}^{-1}$ ) and SOD (1.093 activity  $\text{g}^{-1} \text{min}^{-1}$ , 1.201 activity  $\text{g}^{-1} \text{min}^{-1}$  and 1.292 activity  $\text{g}^{-1} \text{min}^{-1}$ ) at 3, 5 and 7 MAP, respectively. The activity of the POD was enhanced by 18.99 %, CAT by 26.70 % and SOD by 26.29 %, over the control treatment, which was devoid of salicylic acid at 7 MAP.

The piperine (Fig. 4), volatile oil (Fig. 5), and oleoresin content (Fig. 6) varied significantly among the different treatments. The treatment, SA 0.5 mM ( $T_2$ ), recorded significantly higher piperine (1.15 %), volatile oil (1.32 %) and oleoresin (14.21 %) contents. This treatment gave an increase of 31 % in piperine content, 11.86 % in volatile oil and 11.97 % in oleoresin content over the control.

Salicylic acid at 0.1 mM immediately followed that at 0.5 mM in all the above-mentioned biochemical parameters. However, at higher concentrations of salicylic acid (1.0 to 2.5 mM), a declining trend was observed in all these parameters.

### Discussion

In the study, it was observed that the chlorophyll pigments were significantly enhanced by the application of SA. Among the treatments, the highest recorded values were obtained with SA concentration of 0.5 mM in all three stages of observation, indicating the stimulatory role of SA in enhancing photosynthetic pigment accumulation. Enhancement in chlorophyll content by the foliar application of SA has been widely reported in several other plant species. Similar observations were reported by Yildirim *et al.* (2008) in *Cucumis sativus*, Li *et al.* (2014) in *Torreya grandis*, Manaa *et al.* (2014) in *Solanum lycopersicum* and Al-Rubaye and Atia (2016) in *Cucurbita pepo*, where the SA application resulted in a significant increase in chlorophyll content. According to Sreenivasulu *et al.* (2000), SA plays an important role in protecting the photosynthetic apparatus under stress conditions. It acts as an antioxidant molecule concentrated in chloroplasts and helps scavenge the excess reactive oxygen species (ROS) generated during stress, thus helping in maintaining chlorophyll stability and photosynthetic efficiency. The beneficial effects of SA may also be attributed to its ability to enhance

endogenous antioxidant systems, which improve resistance to oxidative stress and regulate the accumulation of free radicals within plant tissues. Supporting these observations, foliar application of SA at 1.0 mM was reported to increase chlorophyll content in *Artemisia annua* (Aftab *et al.*, 2010). These findings suggest exogenous application of SA helps improve chlorophyll biosynthesis and stability, thereby enhancing the photosynthetic capacity of plants.

Total protein content in the leaves was significantly enhanced by the exogenous application of salicylic acid, and the highest values were recorded with SA at a concentration of 0.5 mM at all three stages of observation. Similar enhancement in total protein content due to salicylic acid application has been reported in *Ocimum basilicum* and *Origanum majorana* by Gharib (2006), in *Cyclamen persicum* by Farjadi-Shakib *et al.* (2012) and in *Phaseolus vulgaris* by Hadi *et al.* (2014). The increase in protein content might be attributed to the stimulatory effect of salicylic acid on nitrogen uptake and assimilation, which ultimately enhances protein biosynthesis in plant tissues (Gharib, 2006).

The activity of antioxidant enzymes such as CAT, POD and SOD was significantly enhanced by the exogenous application of salicylic acid at all three stages of observation in a concentration-dependent manner. Among the treatments, the highest enzyme activities were recorded with SA at a concentration of 0.5 mM. Similar results were reported by Hayat *et al.* (2008) in *Lycopersicon esculentum*, Patel *et al.* (2011) in *Cicer arietinum* and Qudos (2015) in *Capsicum annum*, where salicylic acid application resulted in increased activity of antioxidant enzymes. The increase in antioxidant enzyme activity might be due to the regulatory role of salicylic acid in the plant antioxidant defence system. Exogenous application of SA is known to induce the production of  $\text{H}_2\text{O}_2$ , which acts as a signalling molecule and enhances plant resistance against abiotic and biotic stresses by activating antioxidant enzymes (Ganesan and Thomas, 2000). Salicylic acid also stimulates the expression of antioxidative genes, thereby increasing the activity of enzymatic antioxidants such as CAT, POD and SOD. Furthermore, the stimulation of antioxidant enzymes may also be associated with SA-induced protein synthesis, which contributes to improved antioxidant defence in plants (Kovacik *et al.* 2009).

The piperine content was significantly influenced by the foliar application of salicylic acid. In the present study, plants subjected to foliar spray with SA at a concentration of 0.5 mM recorded a 31 per cent increase in piperine content over the control, with a

value of 1.153 per cent. This indicates that the exogenous application of salicylic acid plays an important role in enhancing the accumulation of secondary metabolites in plants. Similar effects of salicylic acid in promoting the accumulation of secondary metabolites have been reported in other plant species. Exogenous application of SA enhanced the accumulation of resveratrol in *Arachis hypogaea* (Chung *et al.* 2003) and induced the accumulation of herniarin and umbelliferone alkaloids in *Matricaria chamomilla* (Pastirova *et al.* 2004). The increase in piperine content observed in the present study might be attributed to the stimulatory effect of SA on enzyme activities, nutrient uptake, photosynthesis and the translocation of photosynthates and other metabolites within the plant system (Khan *et al.* 2007). In addition, salicylic acid has been reported to induce the expression of genes associated with the biosynthesis of secondary metabolites, thereby enhancing the production of active compounds in plants (Malarz *et al.* 2007). These findings suggest that exogenous application of salicylic acid can effectively promote the accumulation of piperine in *Piper longum*.

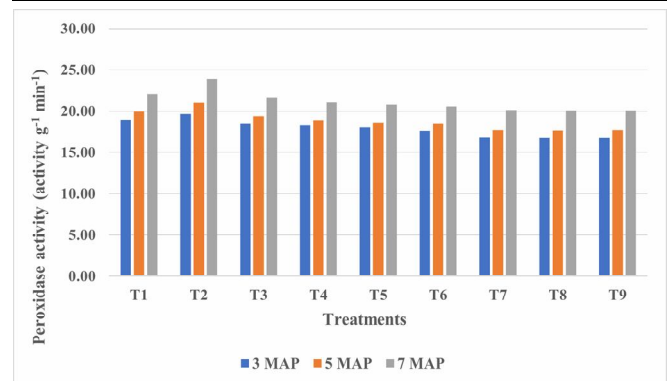
The volatile oil and oleoresin increased by 12 % in plants exposed to foliar spray with SA 0.5 mM over the control. The higher volatile oil content (1.32 %) was obtained with SA at a concentration of 0.5 mM. Similar enhancement in essential oil content and yield per plant following salicylic acid application has been reported in *Ocimum basilicum* and *Origanum majorana* (Gharib 2006). The increase in volatile oil content might be attributed to the stimulatory effect of salicylic acid on plant growth and metabolic activities. According to Idrees *et al.* (2010), improvement in vegetative growth, enhanced nutrient uptake, an increase in leaf oil gland population and stimulation of monoterpene synthesis contribute to higher oil yield in plants such as lemon grass. In the present study, the higher oleoresin content was also recorded in plants exposed to foliar spray with SA at a concentration of 0.5 mM. Similar findings were reported by Rodrigues *et al.* (2009), who observed enhanced oleoresin production in *Pinus elliotti* following the application of salicylic acid-based stimulant paste. However, contrary results have also been reported where SA application at higher concentrations (100 ppm) showed inhibitory effects on herbage and essential oil yields in *Pelargonium graveolens*, *Mentha arvensis* and *Cymbopogon martini* (Ram *et al.* 1997). These observations indicate that appropriate concentrations of salicylic acid can enhance the accumulation of volatile oil and oleoresin in plants.

## Conclusion

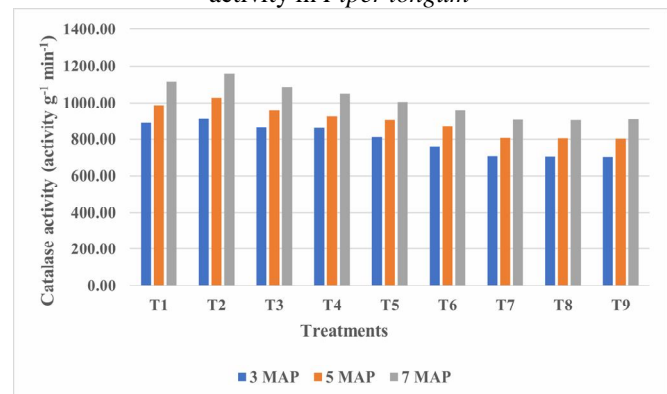
In conclusion, foliar spray with salicylic acid at 0.5 mM recorded higher values for total chlorophyll, total proteins, antioxidant enzyme activity, piperine, volatile oil and oleoresin content in *Piper longum* L., followed by SA 0.1 mM. Higher concentrations of salicylic acid showed a declining trend in these parameters. This study reports the elicitation of biochemical parameters and secondary metabolite production mediated by salicylic acid in *P. longum*. These results confirm the positive influence of salicylic acid on plant growth, development and secondary metabolite production.

**Table 1 :** Effect of foliar spray treatments on total chlorophyll and total protein

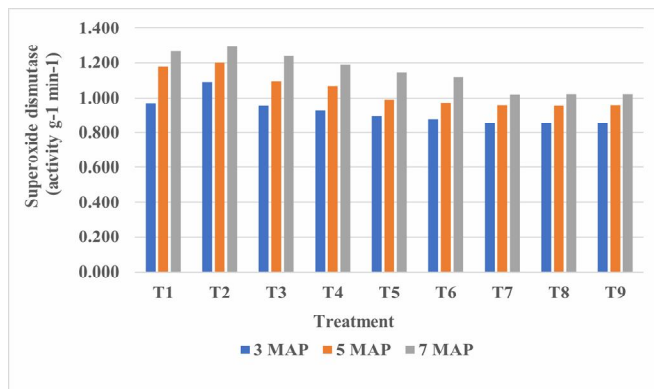
Treatments	Total chlorophyll (mg g <sup>-1</sup> )			Total Proteins (mg g <sup>-1</sup> )		
	3 MAP	3 MAP	3 MAP	3 MAP	5 MAP	7 MAP
T <sub>1</sub> (SA 0.1 mM)	1.561	1.561	1.561	11.72	12.83	13.86
T <sub>2</sub> (SA 0.5 mM)	1.624	1.624	1.624	12.27	13.28	14.62
T <sub>3</sub> (SA 1.0 mM)	1.530	1.530	1.530	11.43	12.57	13.66
T <sub>4</sub> (SA 1.5 mM)	1.497	1.497	1.497	10.90	11.94	13.29
T <sub>5</sub> (SA 2.0 mM)	1.488	1.488	1.488	10.04	11.39	12.83
T <sub>6</sub> (SA 2.5 mM)	1.436	1.436	1.436	9.76	10.46	12.20
T <sub>7</sub> (Ethanol)	1.324	1.324	1.324	9.27	9.94	11.45
T <sub>8</sub> (Water spray)	1.316	1.316	1.316	9.26	9.95	11.39
T <sub>9</sub> (Control)	1.310	1.310	1.310	9.24	9.97	11.40
SEm (±)	0.01	0.01	0.01	0.02	0.03	0.02
CD (0.05)	0.021	0.021	0.021	0.105	0.121	0.106



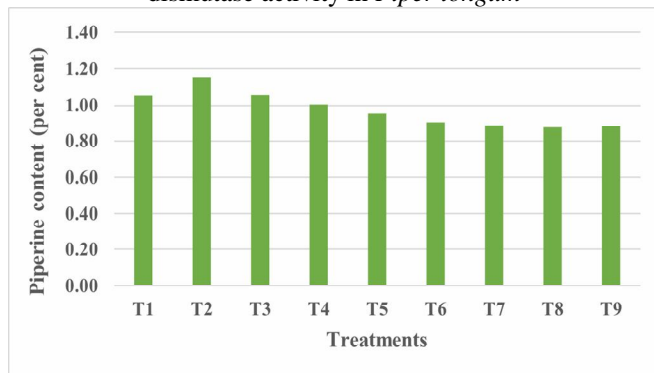
**Fig. 1 :** Effect of foliar spray treatments on peroxidase activity in *Piper longum*



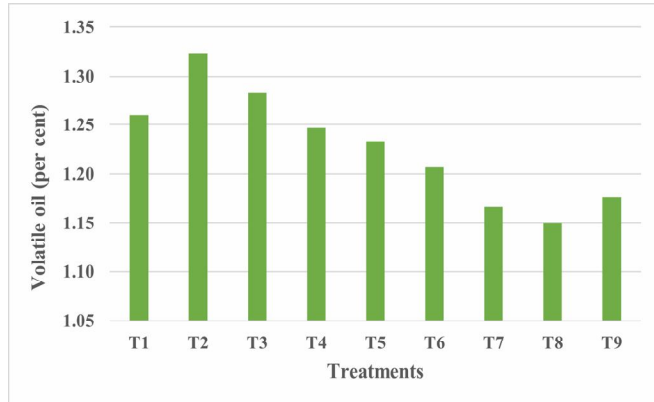
**Fig. 2 :** Effect of foliar spray treatments on catalase activity in *Piper longum*



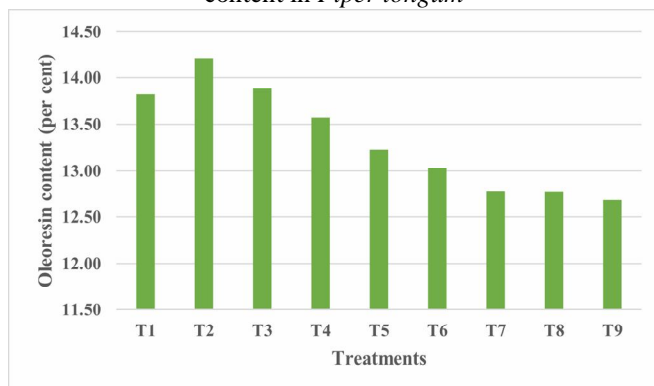
**Fig. 3 :** Effect of foliar spray treatments on superoxide dismutase activity in *Piper longum*



**Fig. 4 :** Effect of foliar spray treatments on piperine content in *Piper longum*



**Fig. 5 :** Effect of foliar spray treatments on volatile oil content in *Piper longum*



**Fig. 6 :** Effect of foliar spray treatments on oleoresin content in *Piper longum*

## Acknowledgement

We sincerely acknowledge the Department of Plantation, Spices, Medicinal and Aromatic Crops, Department of Plant Physiology, and Department of Agronomy of Kerala Agricultural University for providing the necessary laboratory facilities and continuous support throughout the course of our research work.

## Competing interests

Authors have declared that no competing interests exist.

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