



Exogenous Salicylic Acid Induces Metabolite Production Conferring Foot Rot Resistance in Black Pepper

Sincy Joseph ^{a++}, Gleena Mary C. F. ^{a#*},
Reshmy Vijayaraghavan ^{a†}, Shahida K. ^{a#}
and Parvathi M Sreekumar ^{b†}

^a Department of Plant Pathology, KAU, Thrissur, India.
^b Department of Plant Physiology, KAU, Thrissur, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: <https://doi.org/10.9734/ijpss/2025/v37i55445>

Open Peer Review History:

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://pr.sdiarticle5.com/review-history/135723>

Original Research Article

Received: 03/03/2025
Accepted: 05/05/2025
Published: 08/05/2025

ABSTRACT

A study on biochemical factors involved in black pepper defense response against *Phytophthora* sp. on exogenous application of salicylic acid was carried out in *Phytophthora* sp. susceptible variety Panniyur 1. Total phenol content and total flavonoid content were studied under different concentration of salicylic acid to know the performed and induced responses. The pathogen was inoculated (soil inoculation) seven days after treatment application and plants were observed for changes at 24, 48 and 72 HAI. Leaves samples were subjected for biochemical analysis. Although

⁺⁺Research Scholar;

[#]Assistant Professor;

[†]Assistant Professor and Head;

^{*}Corresponding author: E-mail: gleena.m@kau.in;

Cite as: Joseph, Sincy, Gleena Mary C. F., Reshmy Vijayaraghavan, Shahida K., and Parvathi M Sreekumar. 2025. "Exogenous Salicylic Acid Induces Metabolite Production Conferring Foot Rot Resistance in Black Pepper". *International Journal of Plant & Soil Science* 37 (5):212-19. <https://doi.org/10.9734/ijpss/2025/v37i55445>.

total phenolic content remained relatively stable across treatments, the highest levels were observed in plants treated with 1500 ppm SA, while healthy controls exhibited the lowest. A general decline in phenolic content at 48 HAI followed by an increase at 72 HAI was noted. Similarly, flavonoid content showed a decrease at 48 HAI, with a subsequent rise at 72 HAI, with maximum accumulation recorded in plants treated with 1500 ppm SA. Overall, the results suggest that exogenous application of SA enhances phenolic and flavonoid production, thereby strengthening the defense response in black pepper against *Phytophthora* infection.

Keywords: Phenol; flavonoid; black pepper; *Phytophthora* sp.

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), known as "King of spices" is one of the most historically significant and widely used spice crops. Globally, India being the fifth-largest producer of black pepper, contributes around 66,000 metric tonnes to the world market (FAO, 2023). It is referred to as 'black gold' (Nair, 2004), being a valuable export commodity for several spice-producing countries (Ton et al., 2005). Disease and pest incidences are the major constraints in black pepper production globally. Plant pathogens play a significant threat to cultivation of black pepper and is one of the most important factors in agricultural yield losses. The cultivation of black pepper has decreased to one-third due to the destructive disease, like foot rot which remains a major challenge in black pepper production (Hema et al., 2007).

Foot rot, caused by *Phytophthora capsici*, is regarded as one of the most destructive diseases affecting black pepper cultivation in India. About 25-30 per cent vine death has been reported in Kerala (Mammooty et al., 2008). Application of chemical fungicides, against pathogen can generate residual effect, resistance issues and resurgence in future. Salicylic acid (SA) is a key plant hormone involved in activating defense mechanisms against various biotic and abiotic stresses. The SA plays a key role in resistance against microbial pathogens, establishment of local and systemic acquired resistances (LAR and SAR), and the accumulation of pathogenesis-related (PR) proteins for controlling plant pathogens (Ding and Ding, 2020).

Exogenous application of salicylic acid (SA) at low concentrations can trigger defense responses in susceptible host plants. SA initiate signal transduction pathways that lead to transcription of various genes thereby triggering the accumulation of defense molecules such as polyphenols, alkaloids, terpenoids, and steroids involved in defense and resistance of plants

(Pieterse and Van Loon 1999; Hayat et al., 2010; Giri and Zaheer 2016). In addition, elicitors like JA and SA induce the formation of reactive oxygen species (ROS) and hydroxyl radicals ($\cdot\text{OH}$), which can cause oxidative damage and cell death by inducing HR reaction. Besides, plants often respond to pathogens by producing specific phenolic substances, which play a crucial role in pre-invasive structural defenses including the reinforcement of cell walls (e.g., lignin and callose depositions), and productions of toxic compounds (e.g., phytoalexins) at attempted penetration sites (Kumar et al., 2014). A significant increase in the synthesis of flavonoids in response to application of SA was observed in various medicinal plant species like *Matricaria chamomilla*, *Taraxacum officinale* and *Zingiber officinale*. The beneficial roles of phenolics and flavonoids are largely attributed to their antioxidant properties (Ghasemzadeh et al., 2010).

The present investigation was conducted to elucidate the role of exogenous application of salicylic acid in triggering total phenol and flavonoid content in black pepper against *Phytophthora* sp.

2. MATERIALS AND METHODS

Three-month-old black pepper rooted cuttings at three cuttings per bag was used for the experiment. The pathogen was isolated from diseased sample collected from Kottappady, Ernakulam district on potato dextrose agar (PDA) medium and maintained at $25\pm 2^\circ\text{C}$ on carrot agar was used for pot culture studies. Pathogen was mass multiplied on sterile soil with black pepper leaves. The pathogen was challenge inoculated seven days after treatment application. The SA at different concentration 700, 1000, 1500, 1000+ copper hydroxide and copper hydroxide @ 0.2 per cent was applied on the plant as foliar spray at four months after planting (MAP) (Table 1). The soil with pathogen of seven DAI at 10 g/ polybag was used for challenge inoculation.

Table 1. Details of treatments

| Treatments | Concentration |
|----------------|--|
| T ₁ | Salicylic acid 700 ppm |
| T ₂ | Salicylic acid 1000 ppm |
| T ₃ | Salicylic acid 1500 ppm |
| T ₄ | 1000 ppm salicylic acid + copper hydroxide (0.2 %) |
| T ₅ | Copper hydroxide (0.2 %) |
| T ₆ | Control |
| T ₇ | Absolute control |

The experiment was laid out in completely randomized design with four replication and 18 plants per replication. The variety used was Panniyur 1 susceptible to foot rot. In combined application of salicylic acid and copper hydroxide, salicylic acid was applied one month after planting and copper hydroxide (0.2%) was applied after symptom appearance. Changes in the plant metabolites were analysed at 24, 48 and 72 hours after pathogen inoculation (HAI).

2.1 Effect of Different Treatments on Total Phenol Content in Black Pepper

Phenols were extracted and analysed as described by Bray and Thorpe (1954). One gram of leaf tissue was homogenized in 10 ml of 80 per cent methanol and centrifuged at 10,000 rpm for 20 min to collect the supernatant. To the remaining pellet, an additional 5 ml methanol was added and centrifuged again, and the supernatants were collected and evaporated to dryness in a water bath. The dried residue was re-dissolved in 5 ml methanol.

Total phenolic content was then estimated using the Folin-Ciocalteu method (Majeed et al., 2024). Various extract aliquots, each 0.2 ml, were transferred to test tubes, and the volume was adjusted to 3 ml with distilled water. Folin-Ciocalteu reagent (0.5 ml) was added, and the mixture was incubated for 3 min. Subsequently, 2 ml of 20 per cent sodium carbonate solution was added, followed by vortexing. The test tubes were then placed in a water bath for 1 min and kept in the dark for 1 h. Absorbance was measured at 650 nm against a reagent blank. Gallic acid was used as a standard and results were expressed as mg gallic acid equivalent per gram of fresh weight.

2.2 Effect of Different Treatments on Flavonoid Content in Black Pepper

The flavonoid content of the plant extract was measured using a colorimetric method described

by Gupta (2013), with slight modifications. The extraction procedure was same as above.

To estimate flavonoid content, 0.5 ml aliquots of the extract were transferred into separate test tubes. Each tube received 3 ml of five per cent sodium nitrate and 2.5 ml of distilled water, followed by a three min incubation at room temperature. Then, 0.3 ml of 10 per cent aluminium chloride was added and incubated for another 6 min. After that, 2 ml of 1M sodium hydroxide was added, and the volume was adjusted to 10 ml with distilled water. The mixture was kept in the dark for 1 h, and absorbance was recorded at 415 nm. Flavonoid content was quantified using quercetin as a standard and expressed as mg quercetin equivalents per gram of sample.

3. RESULTS AND DISCUSSION

The total phenol content in various treatments varied at different time intervals shown in Fig. 1 and Table 1. At the initial hours of treatment application maximum increase in total phenols was recorded in SA treatment at 1500 ppm ($2.79 \text{ min}^{-1} \text{ g}^{-1}$) followed by 1000 ppm ($2.74 \text{ min}^{-1} \text{ g}^{-1}$) compared to other treatments. A declining trend was recorded in most of SA concentrations up to 48 hours after inoculation which further showed an increasing trend at 72 HAI and 1500 ppm SA ($2.79 \text{ min}^{-1} \text{ g}^{-1}$) maintaining the maximum levels at 72 HAI. Salicylic acid is key signalling molecule in the phenyl propanoid pathway which contributes to the synthesis of phenols and flavonoids. The exogenous application of SA might have contributed to the high levels of SA at the time of pathogen infection which accelerated the production of phenols in the initial hours of infection.

Deenamo et al., (2018) studied the effect of salicylic acid (SA) on induced resistance in rubber tree against *Phytophthora palmivora* and reported a similar finding that total phenolic content was higher in SA treated plants than that of the inoculation with *P. palmivora* (DW + P.

pa). Dong et al., (2010) also observed the effects of salicylic acid (SA) on the accumulation of phenolic compounds in *Salvia miltiorrhiza* cell culture. They reported an increase in phenolic compounds (salvianolic acid and caffeic acid) after 24 hours of application. After 24 h, there is gradual reduction in peak area up to 64 h. Similar elevation of total phenol content by exogenous application of salicylic acid was noticed Blanch et al., (2020) in table grapes, Yin et al., (2024) in barley sprouts. A similar trend of gradual reduction in the phenol content was observed in our study which are in consistent with their observations.

Flavonoid levels in pepper plants subjected to various treatments followed by pathogen inoculation showed notable fluctuations across different time intervals (Fig. 2 and Table 2). At 24 HAI, flavonoid content in various treatments ranged from 4.34 to 6.25 $\text{min}^{-1} \text{g}^{-1}$, with the highest in 1500 ppm SA treatment (6.25 $\text{min}^{-1} \text{g}^{-1}$) followed by 1000 ppm SA (5.58 $\text{min}^{-1} \text{g}^{-1}$) and the lowest in untreated control (1.90 $\text{min}^{-1} \text{g}^{-1}$) (Table 2). A reduction in flavonoid content was noted at 48 HAI in all treatments with T₃ maintaining the highest content (6.11 $\text{min}^{-1} \text{g}^{-1}$) and inoculated control recording the lowest (4.14 $\text{min}^{-1} \text{g}^{-1}$). Further reduction of flavonoid levels was observed during 72 HAI and 1500 ppm SA showed the highest flavonoid content (5.82 $\text{min}^{-1} \text{g}^{-1}$). The flavonoid content of SA 1500 ppm and 1000 ppm was high compared to inoculated control and healthy control throughout the observation intervals.

Exogenous application of salicylic acid has increased the total flavonoid content in *Taraxacum officinale* Wigg (Kim et al., 2009). Tajik et al., (2019) reported that there is induction of phenolic and flavonoid compounds in leaves of saffron (*Crocus sativus* L.) upon salicylic acid application. Shi et al., (2019) reported that exogenous salicylic acid (SA) promotes the accumulation of biomass and flavonoid content in *Phellinus igniarius* (Agaricomycetes). They found that SA induced the biosynthesis of flavonoids not only in a concentration-dependent manner but also in a time-dependent manner. A maximum accumulation was reported in 24 hours in their study which is similar to our findings. Pacheco (2013) studied the induction of growth, flowering and flavonoids production in marigold plants upon salicylic acid spray. They have observed a significant higher total flavonoid content in the marigold inflorescences at 1 mM SA. These results are also in accordance with the findings obtained in our experiments.

In our study there is an increase in total phenol content (TPC) and flavonoid upon spray application of salicylic acid over the control plant. Similar findings has been reported by Perez et al. (2014) who studied effect SA treatment of peppermint. They showed an increase in phenol and flavonoids content of 0.5, 1 and 2 m M concentration of SA compared with the control. Regarding the phenolic composition, the main compounds remained unchanged, although their concentrations increased significantly.

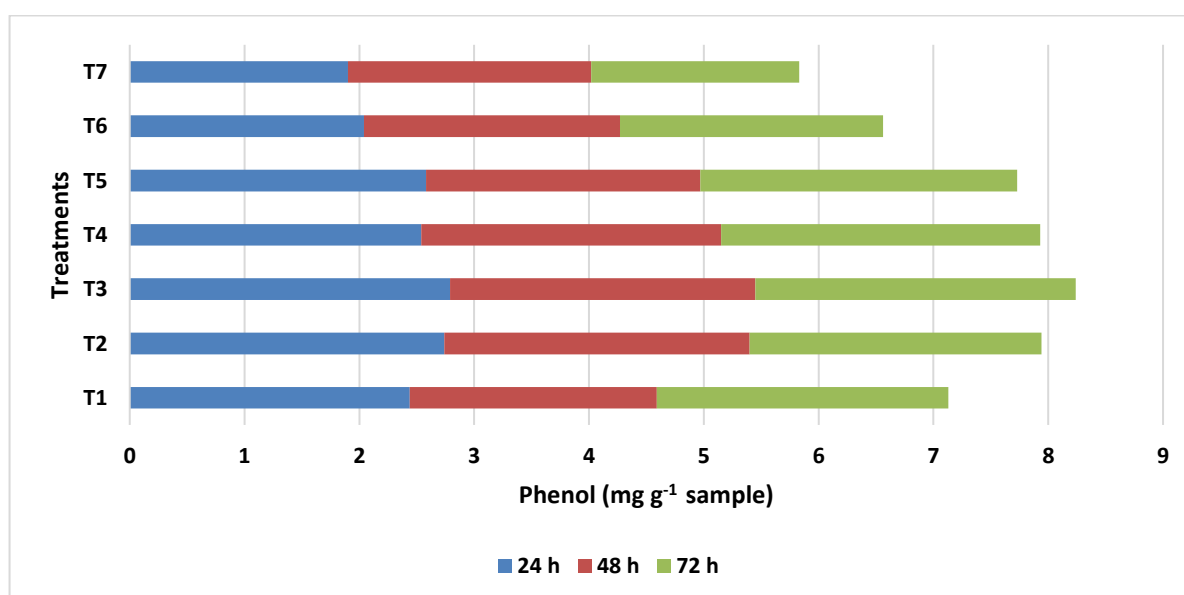


Fig. 1. Total phenol content of different treatments over different time interval

Table 2. Effect of different treatments on total phenol content in black pepper

| Treatments | Phenol content at different intervals (mg g ⁻¹) | | | | | | Treatment mean value |
|---------------------------------------|---|------------------------------|-------------|------------------------------|-------------|------------------------------|---------------------------|
| | 24 h | Per cent +/- over control | 48 h | Per cent +/- over control | 72 h | Per cent +/- over control | |
| T1 (700 ppm) | 2.44 ± 0.26 | -19.60 | 2.15 ± 0.11 | 3.58 | 2.54 ± 0.22 | -10.91 | 2.38 ± 0.25 ^{bc} |
| T2 (1000 ppm) | 2.74 ± 0.40 | -34.31 | 2.66 ± 0.10 | -19.28 | 2.54 ± 0.13 | -10.91 | 2.65 ± 0.24 ^a |
| T3 (1500 ppm) | 2.79 ± 0.27 | -36.76 | 2.79 ± 0.78 | -19.28 | 2.79 ± 0.78 | -21.83 | 2.75 ± 0.44 ^a |
| T4 (1000 ppm SA) +copper hydroxide) | 2.54 ± 0.13 | -24.50 | 2.61 ± 0.34 | -17.04 | 2.78 ± 0.60 | -21.39 | 2.64 ± 0.38 ^a |
| T5 (Copper hydroxide) | 2.58 ± 0.13 | -26.47 | 2.39 ± 0.39 | -7.17 | 2.76 ± 0.27 | -20.52 | 2.57 ± 0.30 ^{ab} |
| T6 (Control) | 2.04 ± 0.09 | | 2.23 ± 0.10 | | 2.29 ± 0.19 | | 2.18 ± 0.16 ^{cd} |
| T7 (Absolute control) | 1.90 ± 0.41 | 6.86 | 2.12 ± 0.06 | 4.93 | 1.81 ± 0.11 | 20.96 | 1.95 ± 0.26 ^d |
| Treatment mean at different intervals | 2.43 ± 0.40 | | 2.40 ± 0.29 | | 2.50 ± 0.49 | | |

In each column figures followed by same letter donot differ significantly according to DMRT
+ - Increase, - - Decrease

Table 3. Effect of different treatments on flavonoid content in black pepper

| Treatments | Flavonoid content at different intervals (mg g ⁻¹) | | | | | | Treatment mean value |
|---------------------------------------|--|------------------------------|----------------------------|------------------------------|----------------------------|------------------------------|--------------------------|
| | 24 h | Per cent +/- over control | 48 h | Per cent +/- over control | 72 h | Per cent +/- over control | |
| T1 (700 ppm) | 4.80 ± 0.51 ^{ef} | 6.25 | 4.68 ± 0.42 ^{efg} | 10 | 4.79 ± 0.22 ^{ef} | -9.86 | 4.76 ± 0.37 ^c |
| T2 (1000 ppm) | 5.58 ± 0.31 ^{bc} | -8.98 | 5.46 ± 0.10 ^{bc} | -5 | 5.38 ± 0.46 ^{bcd} | -23.39 | 5.47 ± 0.31 ^b |
| T3 (1500 ppm) | 6.25 ± 0.56 ^a | -22.07 | 6.11 ± 0.04 ^a | -17.5 | 5.82 ± 0.20 ^{ab} | -33.48 | 6.06 ± 0.36 ^a |
| T4 (1000 ppm SA) + copper hydroxide) | 5.56 ± 0.36 ^{bc} | -8.59 | 5.35 ± 0.08 ^{bcd} | -2.88 | 5.54 ± 0.42 ^{bc} | -27.06 | 5.48 ± 0.31 ^b |
| T5 (Copper hydroxide) | 5.54 ± 0.14 ^{bc} | -8.20 | 4.88 ± 0.28 ^{def} | 6.15 | 5.56 ± 0.06 ^{bc} | -27.52 | 5.32 ± 0.37 ^b |
| T6 (Control) | 5.12 ± 0.15 ^{cde} | | 5.20 ± 0.15 ^{cde} | | 4.36 ± 0.29 ^{fg} | | 4.89 ± 0.44 ^c |
| T7 (Absolute control) | 4.34 ± 0.61 ^{fg} | 15.23 | 4.14 ± 0.56 ^g | 20.38 | 4.16 ± 0.32 ^g | 4.58 | 4.22 ± 0.47 ^d |
| Treatment mean at different intervals | 5.31 ± 0.69 ^a | | 5.12 ± 0.64 ^b | | 5.09 ± 0.67 ^b | | |

In each column figures followed by same letter donot differ significantly according to DMRT
+ - Increase, - - Decrease

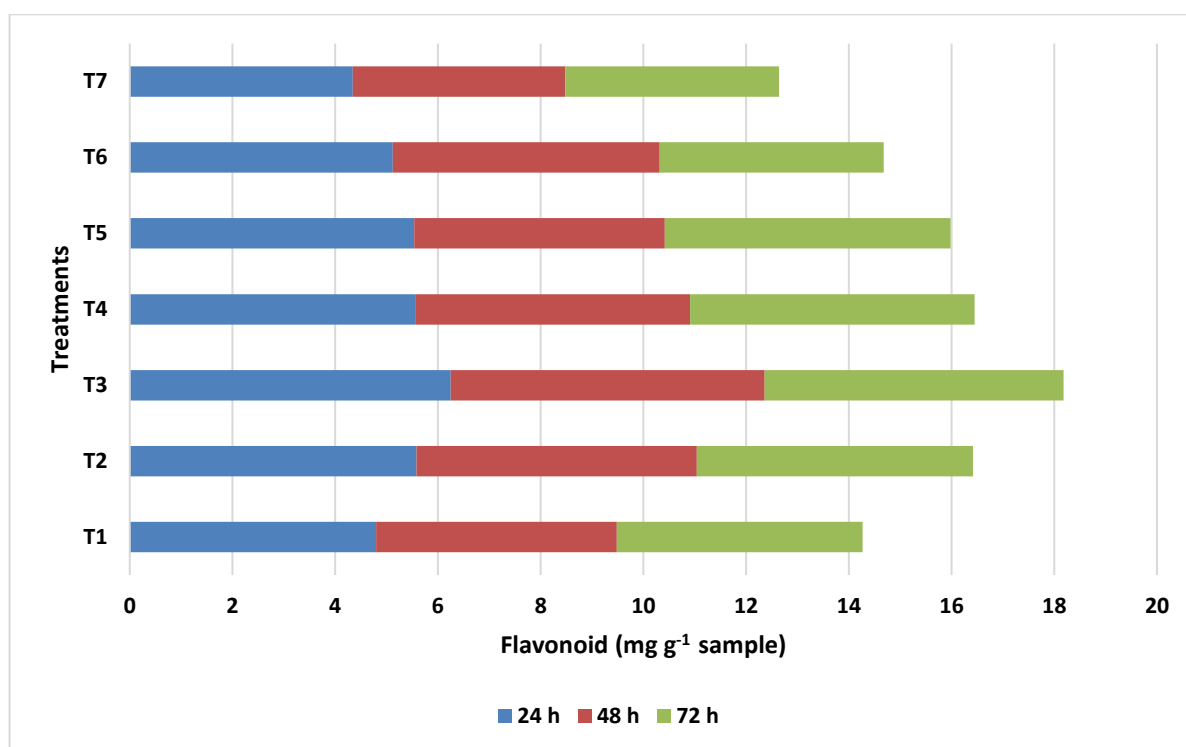


Fig. 2. Total flavonoid content of different treatments over different time interval

4. CONCLUSION

The study demonstrated that salicylic acid treatments significantly influenced the secondary metabolite production in black pepper plants challenged with *Phytophthora* sp., indicating their role in enhancing host defense responses. The study also revealed that total phenol and flavonoid contents exhibited varying trends among treatments following pathogen inoculation. Spray application of SA at 1500 ppm invariably recorded higher levels of phenols and flavonoids across time intervals, indicating a stronger biochemical response over the spray. Least enhancement was noted for healthy control followed by inoculated control. This reveals the potential of exogenous salicylic acid application in enhancing the defense response over pathogen infection. These findings suggest that SA can be effectively used as an alternative for chemical fungicides which enhance host resistance by triggering phenolic and flavonoid content during pathogen infection.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models

(ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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