

Enhancing polyphenol synthesis and antioxidant activity in *Camellia sinensis* using shikimic and salicylic acids

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Abstract

Background: *Camellia sinensis*, widely consumed as tea, is the second most popular beverage globally and is valued for its health benefits. However, environmental stressors pose a significant challenge to the tea industry. This study investigates the potential of shikimic acid (ShA) and its derivative, salicylic acid (SA), as inducers to enhance polyphenol content and antioxidant activity across different growth stages.

Results: Experiments on Biclinal Tea Stock 1 (BTS1) family plants, including 4-month-old seedlings and 2-year-old plants, revealed significant increases in polyphenol levels and antioxidant activity following induction, though effects diminished over time. SA provided a stronger initial boost, whereas ShA sustained longer-lasting effects. Two-year-old plants exhibited greater baseline polyphenol levels and a stronger response to induction than seedlings. A strong correlation between polyphenol accumulation and antioxidant activity was also observed. Molecular docking simulations suggested that SA signaling facilitates NPR1-TGA and NPR1-WRKY binding to the PAL promoter, enhancing PAL gene transcription. Additionally, polyphenols such as flavones, p-coumaric acid, gallic acid, and caffeic acid were found to interact with the PAL enzyme through mechanisms similar to that of trans-cinnamic acid and may, therefore, act as feedback inhibitors in a manner analogous to trans-cinnamic acid.

Conclusion: These findings provide novel insights into optimizing tea plant defenses and enhancing polyphenol-related health benefits, offering potential strategies for improving tea quality and resilience against environmental stress.

KEYWORDS

antioxidant activity, *Camellia sinensis*, NPR1, polyphenols, salicylic acid, shikimic acid

INTRODUCTION

Camellia sinensis, commonly known as tea, was discovered by Emperor Shennong in 2737 BCE when some leaves from a nearby tree fell into his boiling water, according to Chinese legend.¹ Tea is rich in polyphenols, including flavonols, flavonoids, and phenolic acids. Catechins, the main tea polyphenols, are flavonoids with an α -phenyl-benzopyran

structure.² They can help prevent cardiovascular diseases like atherosclerosis and coronary heart disease. They also have anti-aging properties and can aid in diabetes management.³ EGCG, a key tea polyphenol, regulates MAP-kinase, NF- κ B, and IGF/IGF-1 pathways, enhancing tea's anti-cancer effects. Combining tea polyphenols with chemotherapy improves efficacy and reduces side effects.^{4,5} Polyphenols support antioxidant and anti-inflammatory mechanisms, enhance natural killer cell

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activity, promote T lymphocyte differentiation, inhibit inflammatory signaling, and aid antigen presentation by dendritic cells.^{6,7}

Polyphenols protect tea plants by scavenging ROS (OH^- , O_2^-) and neutralizing active oxygen species (H_2O_2 , $^1\text{O}_2$). Their multiple phenolic groups enable successive oxidation, enhancing free radical scavenging efficiency.^{8,9} Previous studies indicate that exogenous polyphenol application enhances polyphenol accumulation in tea plants, aiding stress adaptation by regulating stomatal closure, energy metabolism, sphingolipids, arginine, glutathione, and the JA-ILE pathway. It also promotes compatible solutes like proline and glycine betaine for cell turgor maintenance while scavenging ROS and stabilizing thylakoid membranes to preserve photosynthetic efficiency.^{10–12} Polyphenols can transfer electrons to guaiacol-type peroxidases to neutralize or eliminate H_2O_2 .¹³ Catechin reacts with O_3 to form catechin-quinone, which can stimulate the expression of glutathione S-transferase (GST) and ascorbate peroxidase (APX) genes.¹⁴ Polyphenols can also modulate the levels of plant hormones like salicylic acid (SA), jasmonic acid (JA), and ethylene, which play key roles in the plant's defense responses.¹² In response to biotic stress, it inhibits bacterial, fungal, and viral growth by disrupting cell walls and membranes. It activates defense-related genes involved in phytoalexin synthesis and pathogenesis-related proteins. Additionally, it strengthens the plant cell wall by promoting lignin deposition and phenolic compound accumulation, enhancing resistance to pathogen invasion.^{15,16}

Tea production faces abiotic stresses like drought, heat, and waterlogging, reducing leaf yield, stunting growth, and inhibiting roots. These stresses lower water content, stomatal conductance, and photosynthetic rate, with oxidative damage being a key limiting factor.^{17,18} Biotic stresses from pathogens, pests, and herbivores further impact production and quality. Fungal, bacterial, and viral pathogens cause diseases like blister blight, gray mold, and root rot, while insects, nematodes, and herbivores reduce growth and yield.¹⁹ So, increasing polyphenol content before stress exposure would greatly benefit tea plants by enhancing their resilience.

It is widely recognized that the accumulation of polyphenols in plants is improved by the foliar administration of SA.²⁰ SA is derived from chorismite, which is an intermediate product of the shikimic acid (ShA) pathway via either the ICS or PAL pathways.²¹ During stress, SA signaling triggers NPR1 monomerization through redox modifications that reduce its disulfide bonds. The monomerized NPR1 translocates to the nucleus via its nuclear localization signal (NLS) and interacts with TGA transcription factors to activate defense-related genes, including PR1, strengthening plant defense.²² Studies have shown that SA treatment significantly increases phenolic compound levels in various plants, including *Thymus*, *Salvia*, *Carthamus*, *Ajuga*, *Zingiber*, *Capsicum*, and *Ginkgo biloba*. It upregulates diverse phenolic compounds, such as anthocyanins, rosmarinic acid, apigenin, rutin, chlorogenic acid, and caffeic acid.^{23–25} SA-induced enhancement of phenolic biosynthesis is consistently linked to increased total polyphenol content and antioxidant activity across plant species like Lamiaceae *Theobroma cacao* calli, spinach, yarrow, and grapes.^{26–30} However, the effectiveness of SA as an inducer in tea plants is not fully established, and the role of NPR1 signaling in polyphenol synthesis remains unclear. Understanding NPR1's involvement in regulating the phenylpropanoid pathway could offer new

insights into its mechanism. On the other hand, ShA is a key precursor for secondary metabolite biosynthesis, and a crucial intermediate in the ShA pathway.^{31,32} Research on ShA's efficacy as an inducer is limited, mainly due to its restricted availability.³³

After induction, evaluating the effect's durability is crucial for determining reapplication timing, as plants naturally return to homeostasis. In polyphenol synthesis, feedback mechanisms regulate the phenylpropanoid pathway. Trans-cinnamate (tCA), a PAL product, inhibits PAL activity by competing for phenylalanine binding and disrupting the catalytic cycle. Additionally, tCA induces Kelch-domain-containing F-box (KFB) proteins, which promote PAL ubiquitination and degradation, further modulating phenylpropanoid flux.³⁴ But research on the persistence of the effect is limited, particularly regarding the tea plant.

This study aimed to evaluate the effectiveness of SA and ShA as inducers of polyphenol synthesis and antioxidant activity in tea plants at two growth stages, nursery and plantation, through elicitation. It also examined the time-dependent return to homeostasis, the induction mechanism, and temporal dynamics. Additionally, polyphenol levels and antioxidant activity were compared across growth stages, and their correlation was analyzed. Molecular docking simulations were conducted to investigate NPR1 signaling in modulating the phenylpropanoid pathway via PAL regulation. These findings uncover valuable insights for sustainable tea cultivation and the role of polyphenols in plant defense and human health.

MATERIALS AND METHODS

Pot experiment

The pot experiment for the temporal analysis was designed based on previous studies on spinach leaves.²⁸ The experiment was conducted in the general laboratory under a rain-exclusion shelter at the Department of Biochemistry and Molecular Biology, Shahjalal University of Science and Technology, Sylhet, during the premonsoon season, with an average temperature of 25°C. Tea plant specimens were obtained from the Lakkatura Tea Estate, located in Sylhet, Bangladesh. The plants belonged to the Biclinal Tea Stock 1 (BTS1) clone, resulting from a cross between BT1 and TV1 varieties. Seedlings aged 4 months and mature plants aged 2 years were selected for experimentation to represent two distinct growth stages: the nursery and the plantation stages. As the ideal period for transferring plants from the nursery to the field is 10–18 months,³⁵ the 4-month-old and 2-year-old plants effectively represent the early and established stages of growth, respectively. The experiment was conducted using a completely randomized design (CRD)³⁶ with three replicates per treatment. Initially, a preparatory phase was conducted following the collection of the plants; plants were allowed a 2-week acclimatization period to adjust to the new environmental conditions and recuperate from any stress induced by transportation. On day 0, baseline data were collected through chemical assays to assess the initial conditions before the application of inducers. Inducers were then administered for three consecutive days (days 0, 1, and 2). Subsequent data collection occurred on days 4, 7, and 10 to evaluate the effectiveness and efficacy of the inducers.

Application of inducers

A concentration of 4 mM of SA was administered, based on findings from the existing literature.³⁷ A similar concentration of ShA was applied for comparative analysis. Both solutions were prepared in water. Additionally, 0.1% tween 20 was incorporated into both inducer solutions to serve as an adhesion molecule. Each plant received 15 mL of the inducer solution daily for three consecutive days.

Sample preparation

For the preparation of the methanolic extract utilized in all five chemical assays, several sequential steps were followed following established protocols.³⁸ Two leaves were collected from each plant and cut into pieces to increase surface area exposure for drying. The leaves were dried in a regulated dryer at 40°C for 12 h. Once dried, the leaves were powdered using a mortar and pestle. For each sample group, 100 mg of the resulting powder was weighed and soaked in 10 mL of methanol, creating an initial concentration of 10 mg/mL. The samples were then placed in an orbital shaker for 12 h to facilitate thorough polyphenol extraction. Following extraction, the samples were centrifuged at 4400 rpm for 15 min at room temperature. The supernatant was then filtered using Whatman filter paper to purify the extract.

Total-polyphenol-content

The total phenolic content (TPC) in the sample was assessed using a modified Folin–Ciocalteu (FC) method³⁹ based on literature adaptations.⁴⁰ Specifically, 100 µL of the plant extract was mixed with 400 µL of distilled water and vortexed. A blank was prepared using 500 µL of distilled water. Subsequently, 150 µL of a 1:30 diluted Folin–Ciocalteu reagent was added to each sample and vortexed, followed by a 10-min incubation. Then, 500 µL of a 7.5% Na₂CO₃ solution was added, and the mixture was vortexed again. After a 1-h incubation period in the dark, the absorbance was measured at 725 nm. A standard curve of gallic acid was made by following the same protocol. Then, the polyphenol concentration was calculated in GAE/gm (DW-mg/gm) utilizing that standard curve where $y = 0.9521x + 0.0124$.

Total-flavonoid-content

The total flavonoid content (TFC) in the plant sample was determined using the Aluminum Chloride Colorimetric method.⁴¹ In this procedure, 100 µL of the plant extract was mixed with 400 µL of methanol, and a blank was prepared with 500 µL of methanol. Next, 100 µL of a 10% AlCl₃ solution and 100 µL of 1 M CH₃COONa were added to the sample mixture. The resulting solution was incubated in darkness for

45 min. Absorbance was then measured at 415 nm. A standard curve of quercetin was made by following the same protocol. The flavonoid content was quantified in QE/gm (DW-mg/gm) using that standard curve where $y = 1.8459x + 0.0846$.

DPPH assay

The antioxidant activity of the plant samples was assessed using a modified DPPH (2,2'-diphenyl-1-picrylhydrazyl) assay.⁴² For this, 100 µL of the sample was taken and diluted with methanol to a total volume of 600 µL. The control consisted of 600 µL of methanol. Following this, 200 µL of a 0.004% DPPH solution was added to each diluted sample and mixed thoroughly. The mixtures were then incubated in the dark for 30 min to protect the light-sensitive DPPH. Absorbance was measured at 517 nm, with methanol serving as the blank. The percentage of radical scavenging activity was calculated using the formula:

$$\text{Radical scavenging activity (\%)} = (\text{absorbance of control} - \text{absorbance of sample}) / \text{absorbance of control} \times 100.$$

Antioxidant-reductive-potential assay

The antioxidant activity of the sample can be ensured by its reductive potential.⁴³ A 100 µL volume of the methanolic extract was placed in an Eppendorf tube, to which 250 µL of 0.2 M phosphate buffer (pH 6.6) and 250 µL of 1% potassium ferrocyanide solution, K₄Fe(CN)₆ were added. The mixture was thoroughly mixed and then incubated at 50°C for 20 min. After incubation, 250 µL of 10% trichloroacetic acid (C₂HCl₃O₂) was added, and the mixture was vortexed. Subsequently, 250 µL of this solution was transferred to a new tube and combined with 250 µL of distilled water. Finally, 100 µL of 0.1% ferric chloride (FeCl₃) solution was added, and the mixture was vortexed again. The absorbance of the resulting blue-colored complex was measured at 700 nm, using methanol as the blank.

Total monomeric anthocyanin content

Total monomeric anthocyanin content (TMAC) was determined using a modified version of the pH differential method,⁴⁴ adapted from the literature.⁴⁵ 0.6 mL of 5-fold diluted extract was mixed with 1.2 mL of buffers at pH 1.0 and pH 4.5, respectively, and allowed to equilibrate in the dark for 30 min. TMAC was expressed as mg cyanidin-3-O-glucoside equivalents per gram.

Molecular docking analysis

To validate the pathway, several dockings have been performed for the prediction of biological changes. The protein has been downloaded from Uniprot (<https://www.uniprot.org/>) and the RSCB PDB

database (<https://www.rcsb.org/>). Molecular dockings were performed to investigate the binding interactions between SA and key proteins involved in polyphenol biosynthesis, including WRKY transcription factors, TGA transcription factors, E3 ligase, NPR1, PAL, and NPR4. The primary aim of these simulations was to elucidate the molecular mechanisms underlying the induction of polyphenols and antioxidant activity in tea plants.^{46,47} By understanding these interactions, we gain deeper insights into how these compounds could influence gene regulation and metabolic pathways associated with polyphenol production.

Protein structures for NPR1, NPR4, WRKY1, TGA, PAL, and E3 ligase were retrieved from the UniProt Database. These structures were then preprocessed using PyMol 2.5. Missing residues in the protein structures were modeled to maintain structural integrity. Once the proteins were optimized, they were saved in PDB format, which is compatible with HDock (<http://hdock.phys.hust.edu.cn/>) for the subsequent docking process. Ligands, including SA, flavone, cinnamic acid, and other polyphenolic compounds, were prepared using Avogadro software for energy minimization. Each ligand underwent energy minimization to remove steric clashes and optimize its conformation. The ligands were then saved in the appropriate PDB format for compatibility with HDock, ensuring that the docking would be performed on well-prepared molecules.⁴⁸

The flexible docking protocol in HDock was used to refine the binding orientations and ensure a reliable prediction of the binding mode for each ligand-protein complex. The results of the docking simulations were analyzed by evaluating the docking scores, which are indicative of the binding affinity between the ligands and their corresponding proteins. Lower docking scores suggest stronger binding interactions, and these were used to identify the most stable complexes.^{48,49}

Data analysis

The data analysis was conducted primarily using Microsoft Excel. Paired *t*-tests were performed to assess significant differences between datasets collected on different days. This test was chosen due to the related nature of the data. Pearson correlation coefficients were calculated using R Studio to evaluate the relationships between variables.

RESULTS

Age-dependent phytochemical and antioxidant variations between 2-year-old and 4-month-old plants under control conditions

Baseline comparisons between 4-month-old and 2-year-old plants, before elicitor treatment, revealed significant age-dependent variations in phytochemical content and antioxidant activity, suggesting an increase with age (Figure 1). Two-year-old plants consistently

exhibited higher levels of all measured parameters compared with 4-month-old plants. TPC increased by 12.17% (62.13 vs. 55.39 mg/g) (Figure 1a), and TFC showed the most pronounced and statistically significant increase of 30.31% (23.76 vs. 18.23 mg/g) (Figure 1b). Furthermore, TMAC increased slightly by 18.31% (0.0252 vs. 0.0213 mg/g) (Figure 1c). Moreover, DPPH radical scavenging activity showed a marginally higher activity of 5.89% (35.07% vs. 33.12%) (Figure 1d). Lastly, antioxidant reductive potential showed an enhanced antioxidant capacity of 5.84% (0.647 OD vs. 0.6108 OD) (Figure 1e).

Foliar application of SA and ShA significantly boosts phytochemicals and antioxidant activity in 4-month-old plants

The exogenous application of SA and ShA in 4-month-old plants induced significant initial effects across all measured parameters by day 4 after foliar application. Among these inducers, SA performed better, but no significant change was observed in the control plants. TPC increased by 30.13% to 70.85 mg/g with SA, as compared to a 24.60% increase to 68.12 mg/g with ShA treatment (Figure 2a). Similarly, SA treatment resulted in a 26.62% increase in TFC to 25.1 mg/g, while ShA induced a 17.33% increase to 19.3 mg/g (Figure 2b). The most pronounced changes were observed in TMAC, where SA treatment resulted in a 71.43% increase to 0.038 mg/g, compared with a 62.5% increase to 0.042 mg/g under ShA treatment. Interestingly, a slight increase (12.5%) was also observed in the control plants (Figure 2c). Antioxidant properties also showed greater initial enhancement with SA treatment, with percent scavenging activity increasing by 33.48% compared to the increase of 27.12% with ShA (Figure 2d), and antioxidant reductive potential rising by 32.34% to 0.802 OD with SA versus 26.89% to 0.755 OD with ShA (Figure 2e).

Enhanced sustainability impact of ShA over SA in boosting plant phytochemicals and antioxidant properties in 4-month-old plants

Examining the sustainability effects of SA and ShA, the percentage of effects decreases from peak values observed at day 10 in the case of both treatments. In most cases, ShA showed better sustainability than SA. For TPC, SA-treated plants exhibited a 13.64% decrease by day 10, while ShA-treated plants showed a slightly lower 13.48% decrease (Figure 2a). In terms of TFC, SA treatment resulted in a 12.34% decrease by day 10, whereas ShA treatment showed only a 6.61% decrease (Figure 2b). The most notable difference in sustainability was observed in TMAC, with SA treatment leading to a 33.33% decrease by day 10, while ShA treatment resulted in a less pronounced 23.08% decrease (Figure 2c). For antioxidant activity, SA-treated plants showed a 12.78% decrease by day 10 compared to a 10.76% decrease in ShA-treated plants,

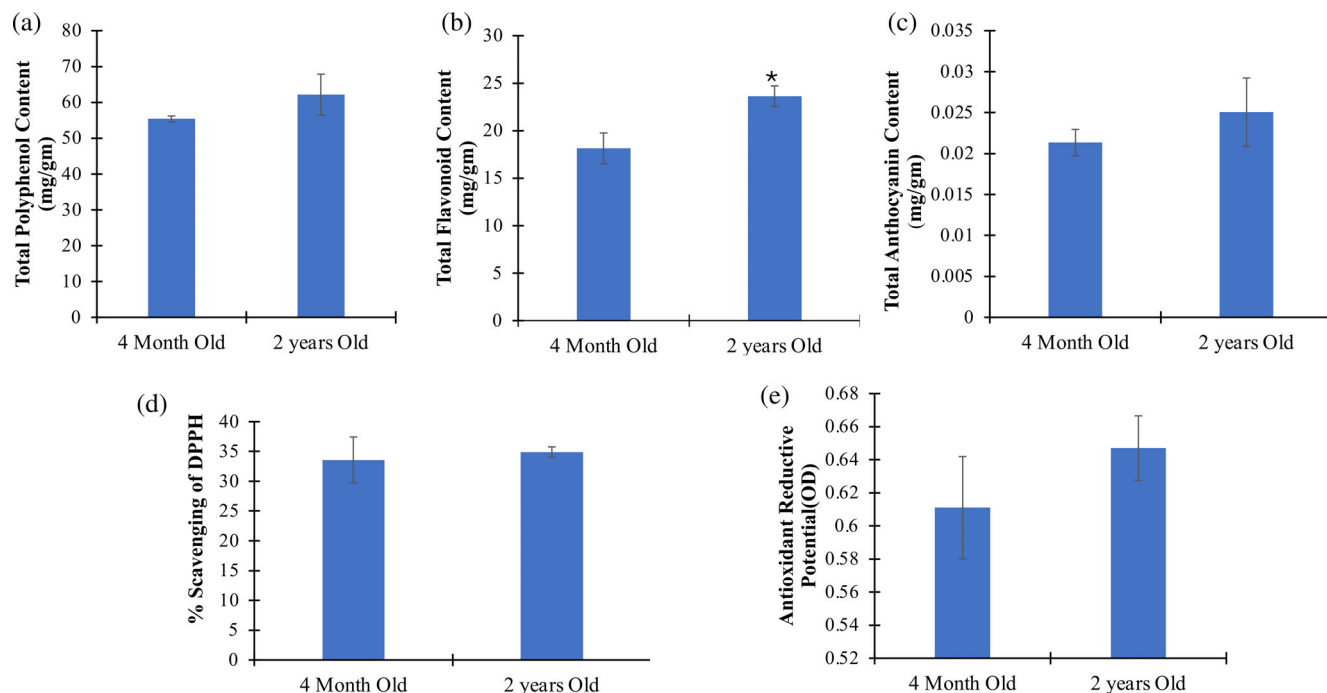


FIGURE 1 Comparison of the initial data between 4-month-old and 2-year-old plants. (a) Folin–Ciocalteu assay, (b) Aluminum chloride colorimetric assay, (c) total monomeric anthocyanin assay, (d) DPPH (2,2'-diphenyl-1-picrylhydrazyl) assay, and (e) antioxidant reductive potential assay. All values represent the mean of three replicates $\pm$ standard error of the mean ($n = 3$). The significance level of the independent Student's t -test is denoted by (*) with a p -value less than 0.05.

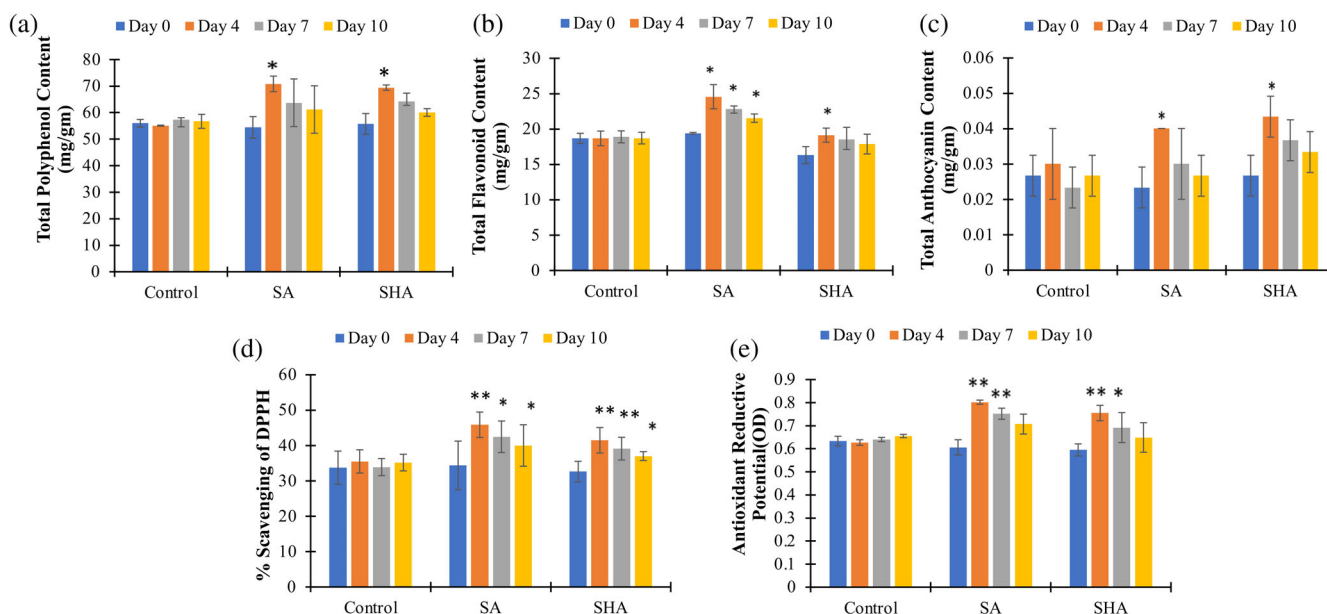


FIGURE 2 Effect of inducers on 4-month-old plants. (a) Folin–Ciocalteu assay, (b) Aluminum chloride colorimetric assay, (c) total monomeric anthocyanin assay, (d) DPPH (2,2'-diphenyl-1-picrylhydrazyl) assay, and (e) antioxidant reductive potential assay. All values represent the mean of three replicates $\pm$ standard error of the mean ($n = 3$). The significance level of the paired t -test is denoted by (*) and (**) with a p -value less than 0.05 and 0.01, respectively.

suggesting slightly better sustainability of antioxidant activity with ShA treatment (Figure 2d). Lastly, antioxidant reductive potential in SA-treated plants decreased by 11.85% by day 10, while ShA-

treated plants showed a 14.04% decrease, indicating slightly better maintenance with SA treatment, contrary to the trend in other parameters (Figure 2e).

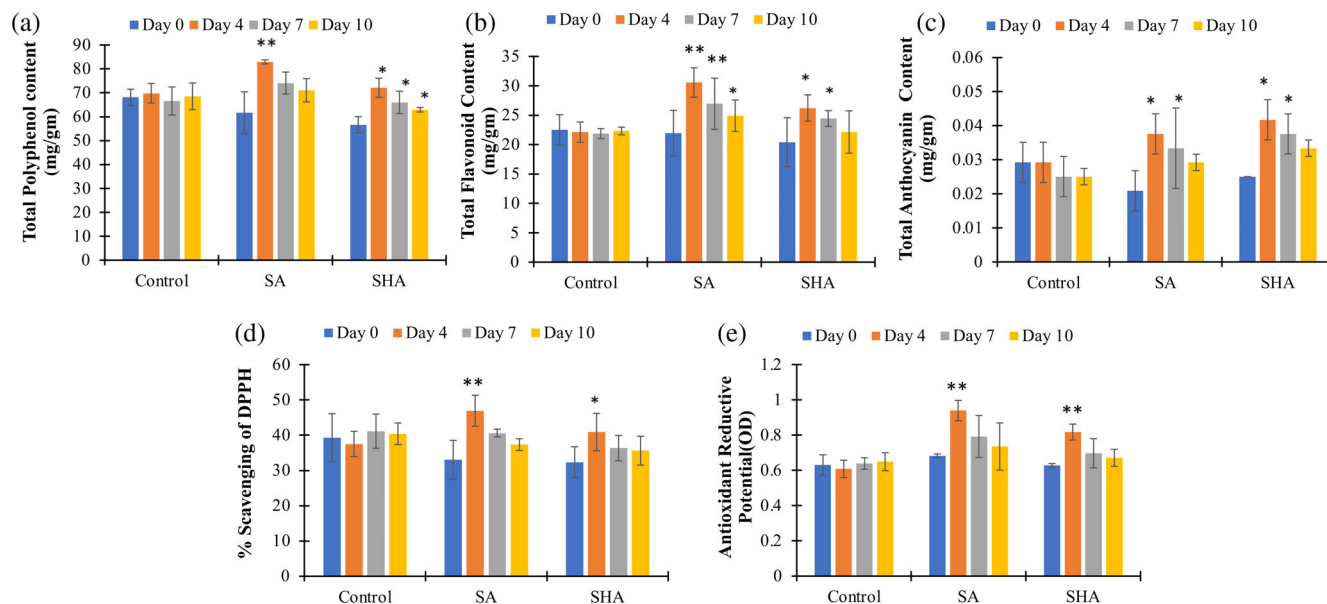


FIGURE 3 Effect of inducers on 2-year-old plants. (a) Folin–Ciocalteu assay, (b) Aluminum chloride colorimetric assay, (c) total monomeric anthocyanin assay, (d) DPPH (2,2'-diphenyl-1-picrylhydrazyl) assay, and (e) antioxidant reductive potential assay. All values represent the mean of three replicates $\pm$ standard error of the mean ($n = 3$). The significance level of the paired t -test is denoted by (*) and (**) with a p -value less than 0.05 and 0.01, respectively.

Foliar application of SA and ShA significantly boosts phytochemicals and antioxidant activity in 2-year-old plants

Like in 4-month-old plants, treatment with SA and ShA in 2-year-old plants also induced significant initial effects across all measured parameters by the fourth day of foliar application. Among these inducers, SA performed better, but no significant change was observed in the control plants. TPC increased by 34.55% to 83.5 mg/g with SA treatment, compared to a 27.26% increase to 71.9 mg/g with ShA (Figure 3a). SA treatment also resulted in a 39.18% increase in TFC to 31.2 mg/g, while ShA induced a 28.39% increase to 27.2 mg/g (Figure 3b). The most dramatic changes were observed in TMAC, where SA treatment led to an 80% increase to 0.039 mg/g, compared to a 66.67% increase to 0.042 mg/g with ShA (Figure 3c). Antioxidant properties similarly showed greater initial enhancement with SA treatment, with percent scavenging activity increasing by 42.13% compared to 27.57% with ShA (Figure 3d), and antioxidant reductive potential rising by 37.58% to 0.939 OD with SA versus 30.19% to 0.817 OD with ShA (Figure 3e).

Enhanced sustainability impact of ShA over SA in boosting plant phytochemicals and antioxidant properties in 2-year-old plants

Assessing the sustainability effects of SA and ShA treatments for all measured parameters at day 10 compared to their peaks at day 4, ShA sustained better inducer effects in all parameters, mirroring

trends observed in younger plants. We observed that SA-treated 2-year-old plants showed a 14.31% decrease in TPC, while ShA-treated plants exhibited a slightly lower 12.67% decrease (Figure 3a). For TFC, SA-treated plants experienced an 18.49% decrease, whereas ShA-treated plants showed a 15.55% decrease (Figure 3b). The sustainability of TMAC also followed a similar trend, with SA-treated plants showing a 22.22% decrease, and ShA-treated plants showing a 20% decrease (Figure 3c). Regarding antioxidant activity, SA-treated plants exhibited a 20.5% decrease compared to a 12.93% decrease in ShA-treated plants (Figure 3d). However, in contrast to younger plants, antioxidant reductive potential in SA-treated 2-year-old plants decreased by 21.73%, while ShA-treated plants showed a less pronounced 17.87% decrease, suggesting slightly better maintenance with ShA treatment in this parameter (Figure 3e).

Age-dependent variations of inducer effect: 2-year-old plants outperform 4-month-olds

A comparative analysis was conducted on control plants versus those induced with SA and ShA at two developmental stages: younger (4 months) and mature (2 years). The induction with both SA and ShA led to improved outcomes compared to the controls at both stages, with more pronounced effects observed in mature plants (Figure 4a,b). In SA-induced plants, the differences ranged from 5.371% to 21.007% (Figure 4b), while ShA-induced plants showed differences from 3.607% to 16.667% (Figure 4a). Notably, TPC exhibited the lowest values, while TMAC registered the highest values in both treatments (Figure 4a,b).

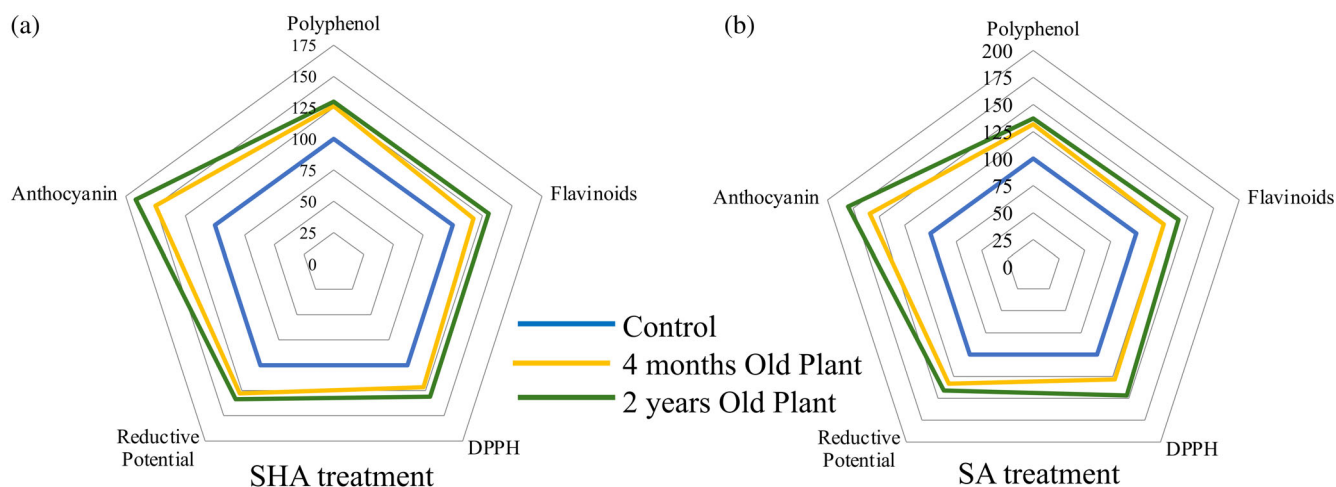


FIGURE 4 Comparison of inducers on two age groups. (a) Effect of Shikimic acid and (b) salicylic acid on 4-month-old (yellow) and 2-year-old (green) plants, compared to the control plants (blue). Here, a = shikimic acid (ShA) treatment, b = salicylic acid (SA) treatment.

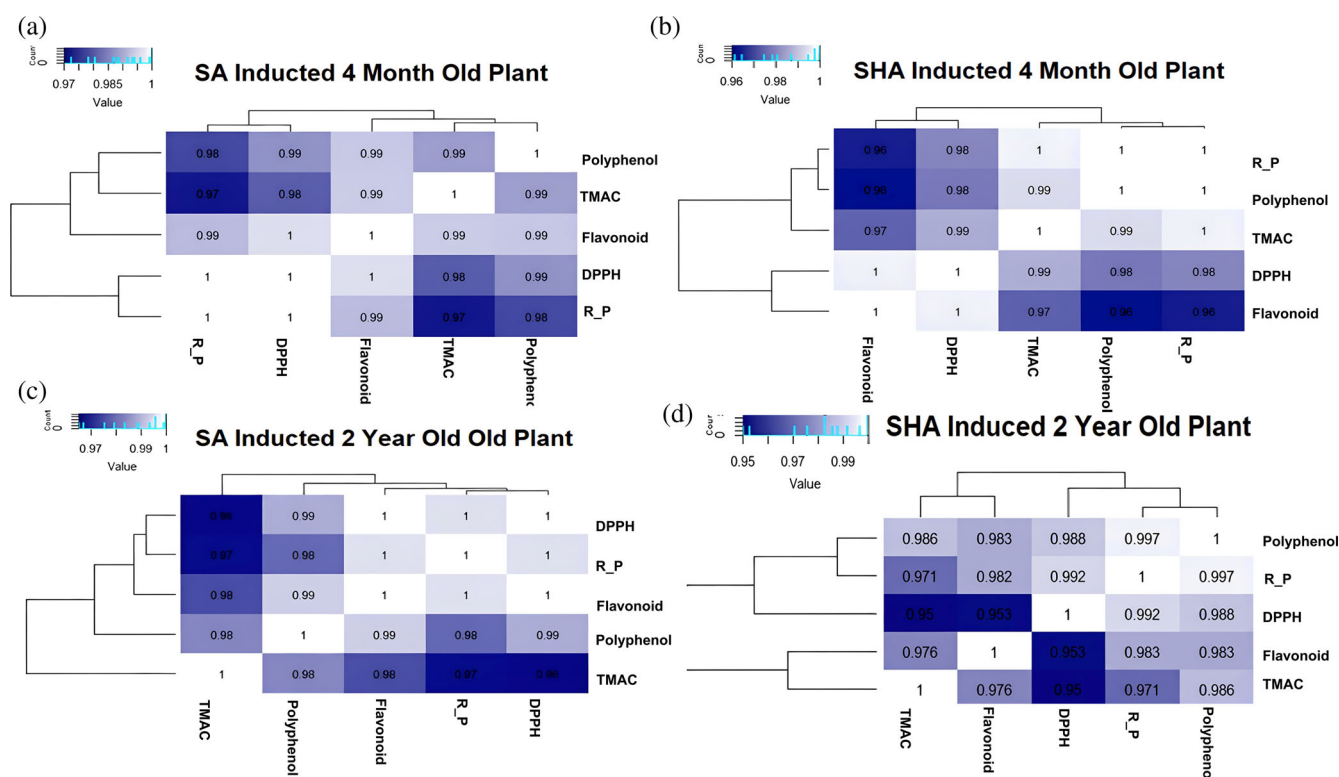


FIGURE 5 Interconnectivity between measured variables and treatments. Pearson correlation between all five chemical assays, ranging from 0.95 to 1, with colors transitioning from blue to white. (a) Salicylic acid (SA) induced 4-month-old-plant, (b) Shikimic acid (ShA) induced 4-month-old-plant, (c) SA induced 2-year-old-plant, and (d) ShA induced 2-year-old-plant.

Analysis also revealed significant differences in TFC, DPPH-free radical scavenging activity, and reductive potential between mature and younger plants treated with SA, with values of 14.295%, 18.502%, and 7.783%, respectively (Figure 4b). For the ShA-treated plants, the corresponding differences were 12.789%, 9.258%, and 5.749%, respectively (Figure 4a).

Interconnectivity between polyphenol levels and antioxidant activity

Correlation coefficients approaching +1 signify strong interconnectivity. Notably, all correlation coefficients across the four conditions exceed 0.95, demonstrating a high degree of interrelationship among

TABLE 1 Docking scores for NPR1 and salicylic acid (SA) interactions.

Protein	Ligand	Docking score
NPR1	SA	−92.33
NPR1 dimer	SA	−126.94
NPR1 tetramer	SA	−257.34

the assays. In the SA-induced 4-month-old plant, the correlation coefficients range from 0.97 to 1.00, indicating strong positive correlations between all variables (Figure 5a). Similar patterns are observed in the SHA-induced 4-month-old plant, with coefficients ranging from 0.96 to 1.00 (Figure 5b).

For older plants, an SA-induced 2-year-old plant shows correlation coefficients between 0.97 and 1.00 (Figure 5c), while an SHA-induced 2-year-old plant exhibits slightly lower but still strong correlations, ranging from 0.95 to 1.00 (Figure 5d). The consistently high correlation coefficients across all conditions suggest that the measured variables, such as polyphenol, TMAC, flavonoid, DPPH, and reductive potential, are highly interrelated, regardless of plant age or induction treatment.

Salicylic acid activates signaling via NPR1

Molecular docking analysis was conducted to understand the induction effect of SA,⁵⁰ which was targeted to investigate whether it can interact as effectively as in other plants to initiate the SA signaling pathway. As observed in other plants, SA showed a strong interaction with NPR1 in tea. The docking simulations revealed a clear trend in the binding affinity of SA with NPR1. Initially, NPR1 in its monomeric form showed a moderate binding affinity with SA, represented by a docking score of −92.33. This indicates that while SA binds to the monomer, the interaction is less stable. Upon dimerization, the binding affinity significantly improved, with the NPR1 dimer showing a docking score of −126.94, reflecting stronger protein-ligand interactions. The most notable improvement was observed when NPR1 formed a tetramer, where the binding affinity drastically increased, yielding a docking score of −257.34. This trend indicates that SA may promote the reduction-mediated monomerization of NPR1, a critical step for its nuclear translocation and transcriptional activity. As SA shows stronger binding affinity toward oligomeric forms of NPR1, its interaction with these complexes may contribute to NPR1 stabilization and could facilitate transitions between oligomeric and monomeric states, ultimately supporting NPR1 activation. The table below summarizes the docking scores for each form of NPR1 in complex with SA (Table 1).

Another docking analysis revealed a strong interaction between NPR1 and NPR4, with the initial complex (in the absence of SA) exhibiting a docking score of −279.17, indicating a high binding affinity. However, upon the introduction of SA, the binding affinity decreased significantly, as reflected by the NPR1_SA-NPR4 complex docking

TABLE 2 Docking scores of NPR1 with various ligands in the presence and absence of salicylic acid (SA).

Receptor	Ligand	Docking score
NPR1	NPR4	−279.17
NPR1_SA	NPR4	−229.86
NPR1-NPR4	E3 ligase	−276.10
NPR1-NPR4_SA	E3 ligase	−229.86

score of −229.86. This suggests that SA weakens the stability of the NPR1-NPR4 complex.

The next phase of the analysis focused on the interaction between the NPR1-NPR4 complex and the E3 ligase. In the absence of SA, the docking score for the NPR1-NPR4 complex bound to E3 ligase was −276.10, indicating a strong binding affinity. However, upon the introduction of SA, the docking score decreased to −239.25, suggesting a reduced interaction. This result implies that E3 ligase associates more effectively with the NPR1-NPR4 complex when SA is absent, potentially influencing NPR1 regulation. Stronger interaction may facilitate ubiquitination and proteasomal degradation, a crucial mechanism in immune signaling and transcriptional regulation (Table 2).

These findings suggest that SA plays a critical role in decreasing the binding of NPR1 to NPR4 and E3 ligase. Furthermore, the results highlight the functional relevance of NPR1-NPR4 interactions in the presence of SA, particularly in recruiting E3 ligase for regulation via ubiquitination and proteasomal degradation, which ultimately affects plant immune responses and polyphenol biosynthesis.

NPR1 mediates SA-induced PAL expression through transcription factor interaction

This analysis explores the structural details and binding affinities of the NPR1 monomer with TGA and WRKY, providing insights into NPR1's role in polyphenol production.

NPR1 is in complex with TGA transcription factors and the PAL promoter. This figure shows the structural arrangement of NPR1 (purple) and TGA (cyan) in close contact with the PAL promoter, emphasizing the key residues involved in the binding interaction that facilitates polyphenol biosynthesis. NPR1 is complex with WRKY transcription factors and the PAL promoter. Here, NPR1 (purple) assists WRKY (green) transcription factors in binding to the PAL promoter, further contributing to the regulation of polyphenol biosynthesis in plants (Figure 6).

The strongest binding affinity is observed in the NPR1-TGA-PAL complex with a docking score of −299.41, highlighting NPR1's crucial role in facilitating the binding of TGA to the PAL promoter. This interaction is likely the key driver of polyphenol biosynthesis activation. The NPR1-WRKY-PAL complex, with a docking score of −273.99, shows strong binding, though slightly weaker than NPR1-TGA-PAL. This suggests that WRKY transcription factors, while important, might have a secondary role in regulating polyphenol biosynthesis in comparison with TGA.

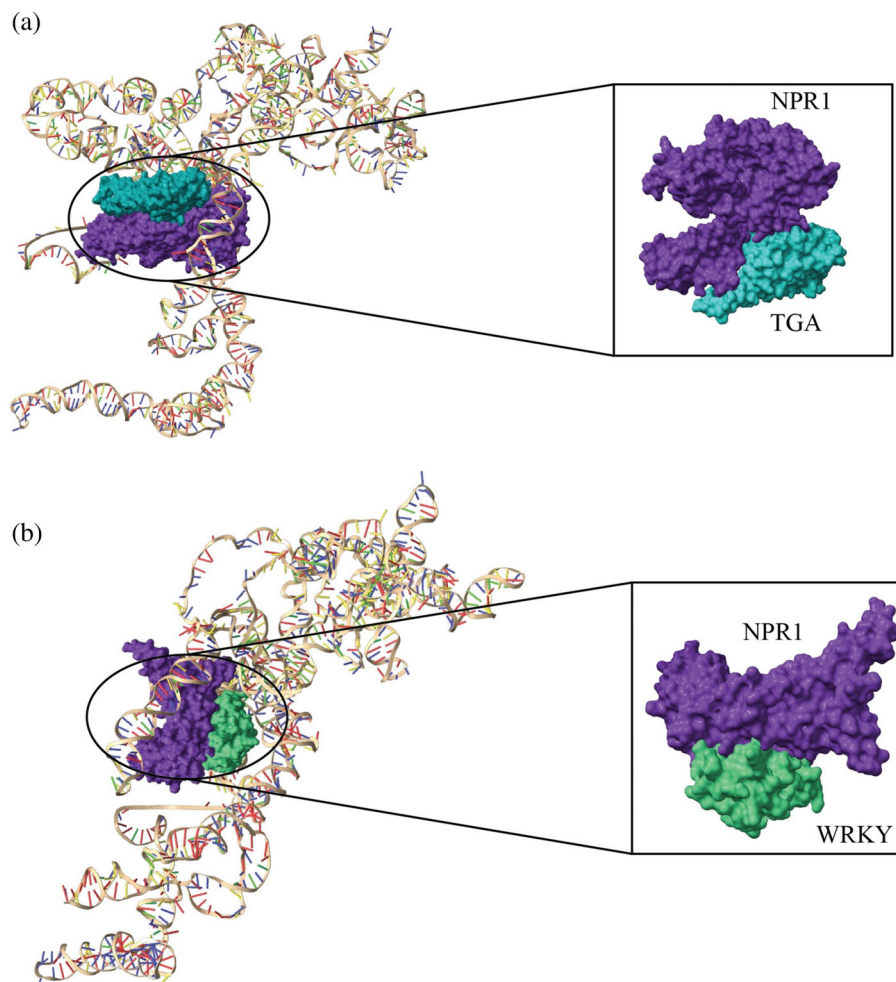


FIGURE 6 Docking of NPR1 monomer in complex with TGA and WRKY transcription factors, illustrating their interactions with the PAL promoter. (a) Docking of NPR1 monomer in complex with TGA transcription factors and the PAL promoter. The inset shows the close-up interaction between NPR1 (purple) and TGA (cyan), highlighting key binding sites involved in polyphenol biosynthesis regulation. (b) Docking of NPR1 monomer in complex with WRKY transcription factors and the PAL promoter. The inset displays the interaction between NPR1 (purple) and WRKY (green), showing how NPR1 facilitates the binding of WRKY to the PAL promoter, influencing polyphenol biosynthesis.

In the absence of the PAL promoter, the docking scores are lower (-257.03 for NPR1-TGA and -229.29 for NPR1-WRKY), indicating that the presence of the PAL promoter is crucial for stabilizing these complexes and facilitating efficient transcriptional regulation of polyphenol biosynthesis (Table 3). These docking results emphasize NPR1 as a central regulator in polyphenol biosynthesis. The NPR1-TGA-PAL complex, with the strongest binding affinity, likely represents the primary mechanism through which NPR1 activates the PAL gene for polyphenol biosynthesis. The NPR1-WRKY-PAL complex, though slightly weaker, still plays a significant role, suggesting that WRKY transcription factors support the overall process.

Feedback regulation of PAL by phenylpropanoid pathway products

The docking results for the interaction between the PAL protein and different ligands provide insights into the potential binding affinity of

each compound. The table below summarizes the binding scores for each ligand (Table 4).

Flavon exhibited the strongest binding affinity with a docking score of -151.45 , suggesting it has the most favorable interaction with the PAL protein, which could indicate a high potential for influencing polyphenol biosynthesis. Gallic Acid also showed a relatively strong binding affinity with a score of -125.23 , supporting its role as a significant modulator of the PAL enzyme activity.

Coumaric Acid and Caffeic Acid both had moderate binding scores (-116.78 and -108.45 , respectively), indicating that they also may have substantial roles in regulating the PAL gene and related biosynthetic pathways. Cinnamate had the weakest docking scores (-102.34), suggesting that while it might interact with the PAL protein, its binding is not as strong compared to others. The docking results emphasize the importance of flavonoids and phenolic acids, particularly Flavon, Gallic Acid, and Coumaric Acid, in the regulation of PAL, a key enzyme in polyphenol biosynthesis. These results support the hypothesis that certain ligands, especially flavonoids, may

TABLE 3 Docking scores and binding interactions of NPR1 with transcription factors and the PAL promoter.

Complex	Docking score	Interpretation
NPR1-TGA	−257.03	Strong binding between npr1 and tga transcription factors. Npr1 facilitates the interaction with the pal promoter, aiding polyphenol biosynthesis regulation.
NPR1-TGA-PAL	−299.41	Addition of the pal promoter further stabilizes the npr1-tga complex, enhancing the interaction and suggesting stronger transcriptional activation of polyphenol biosynthesis.
NPR1-WRKY	−229.29	Weaker binding affinity between npr1 and wrky compared to npr1-tga, though still significant. Indicates a role for wrky in polyphenol regulation.
NPR1-WRKY-PAL	−273.99	The pal promoter strengthens the interaction between npr1 and wrky, indicating that npr1 facilitates wrky's role in activating polyphenol biosynthesis, though slightly less potent than the npr1-tga-pal complex.

TABLE 4 Binding affinities of PAL protein with different polyphenolic compounds.

Protein	Ligand	Binding score
PAL	Cinnamate (5957728)	−102.34
PAL	Caffeic acid (689043)	−108.45
PAL	Gallic acid (370)	−125.23
PAL	Flavon (11349)	−151.45
PAL	Coumaric acid (637542)	−116.78

play a significant role in modulating polyphenol production by enhancing the binding affinity to the PAL enzyme.

The protein structure, as shown, highlights key features related to ligand binding. The hydrophobicity map reveals regions that are critical for the binding of various ligands, with the red areas indicating hydrophobic residues that likely interact strongly with nonpolar ligands. These hydrophobic regions are essential for maintaining the stability of the protein-ligand complex, as they facilitate better binding through nonpolar interactions.

The ligands, depicted in yellow, occupy the protein's binding pocket, indicating a favorable fit within the hydrophobic regions. This suggests that the ligand-binding process is driven by hydrophobic interactions, which are critical for stabilizing the complex.

When focusing on hydrophobicity and ligand-binding, it becomes evident that the protein's structure is optimized to accommodate such ligands. This could explain the docking scores observed in previous analyses, where ligands with strong hydrophobic characteristics tend to bind more effectively. Additionally, the hydrophobicity map can guide future experimental or computational approaches by identifying

residues that should be targeted for modification to improve binding affinity or specificity (Figure 7).

SA and ShA as key regulators of the phenylpropanoid pathway

A proposed pathway has been developed explaining how SA and ShA function as inducers (Figure 8). Foliar application of ShA triggers the shikimic acid pathway, enhancing phenylalanine synthesis. Phenylalanine enters the phenylpropanoid pathway, driving polyphenol and SA synthesis. Since SA is a component of the phenylpropanoid pathway, both foliar SA application and endogenous SA production activate SA signaling. At low SA levels, some NPR1 undergoes redox-mediated monomerization, allowing its nuclear translocation. In the nucleus, monomeric NPR1 interacts with NPR4, leading to NPR1 ubiquitination and proteasomal degradation, thus inhibiting downstream signaling. However, elevated SA levels promote NPR1 monomerization while reducing its affinity for NPR4, preventing degradation. This stabilization of NPR1 enables it to activate transcription factors like WRKY and TGA, which bind to the PAL promoter, inducing PAL gene expression and stimulating the phenylpropanoid pathway through increased PAL enzyme activity. As polyphenol levels rise, a feedback mechanism is triggered. Compounds like trans-cinnamic acid, coumaric acid, gallic acid, caffeic acid, and flavones inhibit PAL activity, gradually reducing the pathway's induction.

DISCUSSION

The main goal of this experiment was to establish SA and ShA as inducers for the induction of polyphenols and antioxidant activity in tea plants. Both SA and ShA effectively functioned as inducers, as evidenced by the significant changes observed from day 0 to day 4. The role of SA and ShA as inducers in tea plants is supported by previous studies.⁵¹ The induction of various polyphenols, such as flavonoids, specifically anthocyanins, has been supported by previous research on various plants, including *Salvia*, *Ginkgo biloba*, *Carthamus*, *Ajuga*, *Cap-sicum*, *Zingiber*, and *Thymus*.^{23–25} Moreover, the elevation of total phenolic content and antioxidant activity through these inductions aligns with findings from studies on *Lamiaceae*, *T. cacao* calli, spinach, tomato, and grapes.^{26–30} The induction effects observed with ShA align with previous research on tomato plants, which has discussed that ShA application enhances polyphenol production. This increase in polyphenols is associated with an elevated transpiration rate in plants.⁵²

However, a decreasing trend was observed after day 4, consistent with findings from previous research on spinach leaves.²⁸ This suggests that the plants return to their homeostatic condition after some time. This finding also provides insights into the timing of the inducers for optimal efficacy, indicating that the application of inducers should be carefully timed to ensure proper effectiveness.

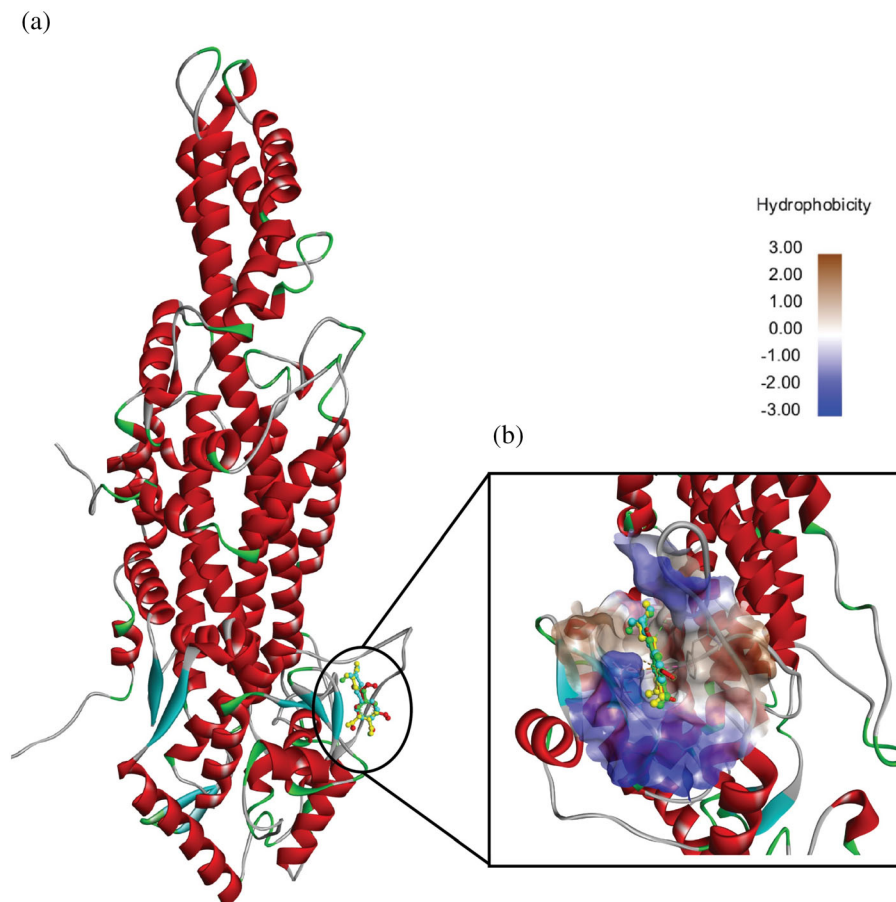


FIGURE 7 PAL with Feedback inhibition with a different ligand. (a) Ribbon representation of the protein structure with hydrophobicity levels indicated by the color gradient (from red for hydrophobic to blue for hydrophilic). The ligand (yellow) is bound within the protein's active site, showcasing its interaction with the hydrophobic regions. (b) A close-up view of the protein-ligand complex, displaying the surface representation of the protein with hydrophobicity coloring. The ligand (yellow) is positioned in the binding pocket, illustrating how the hydrophobic areas interact with the ligand.

Among the measured parameters, anthocyanin content exhibited the most pronounced increase. Interestingly, a slight elevation in anthocyanin was also observed in the control plants, although this increase was markedly lower than that observed in SA- and ShA-treated plants. This minor increase in controls can be attributed to two intrinsic plant mechanisms. First, anthocyanin biosynthesis is subject to dynamic metabolic flux within the flavonoid pathway, where flavonol synthase (FLS) and dihydroflavonol 4-reductase (DFR) continuously compete for common substrates such as dihydroflavonols.⁵³ A transient shift in substrate allocation favoring DFR may result in modest, spontaneous anthocyanin accumulation, even in the absence of exogenous elicitors. Second, basal photosynthetic and metabolic activity inherently produces low levels of ROS, which can function as signaling molecules to trigger mild, transient activation of anthocyanin biosynthetic genes.⁵⁴ Although this elevation is minimal, it is sufficient to cause a detectable increase in anthocyanin content without significantly impacting total polyphenol levels or overall antioxidant activity. Importantly, the plants maintain metabolic homeostasis over time, as evidenced in this study by the redistribution of anthocyanin levels

observed on day 7, and their return to near-baseline values by day 10, indicating tight endogenous regulation of flavonoid metabolism.

Based on the proposed pathway developed from molecular docking simulations, it is suggested that SA may induce a signaling cascade that ultimately upregulates PAL. Docking analysis indicates that SA shows higher binding affinity for NPR1 oligomers compared to monomeric or dimeric forms, which may support the role of SA in promoting redox-mediated conversion of NPR1 oligomers into monomers in plants such as *Arabidopsis thaliana*.⁵⁵ In the presence of SA, the affinity of NPR4 for NPR1 may decrease. Consistently, the relatively lower docking score of the SA-NPR4-NPR1-E3 ubiquitin ligase complex compared with the NPR4-NPR1-E3 ligase complex suggests that SA may induce conformational changes in NPR4 that weaken its interaction with NPR1. Similar SA-dependent modulation of NPR protein interactions has been reported in other plant systems, supporting this interpretation.⁵⁶ The strong predicted binding of the NPR1-TGA-PAL promoter and NPR1-WRKY-PAL promoter complexes suggests that NPR1 may participate in PAL promoter activation in a manner analogous to its established role in PR gene regulation, where NPR1

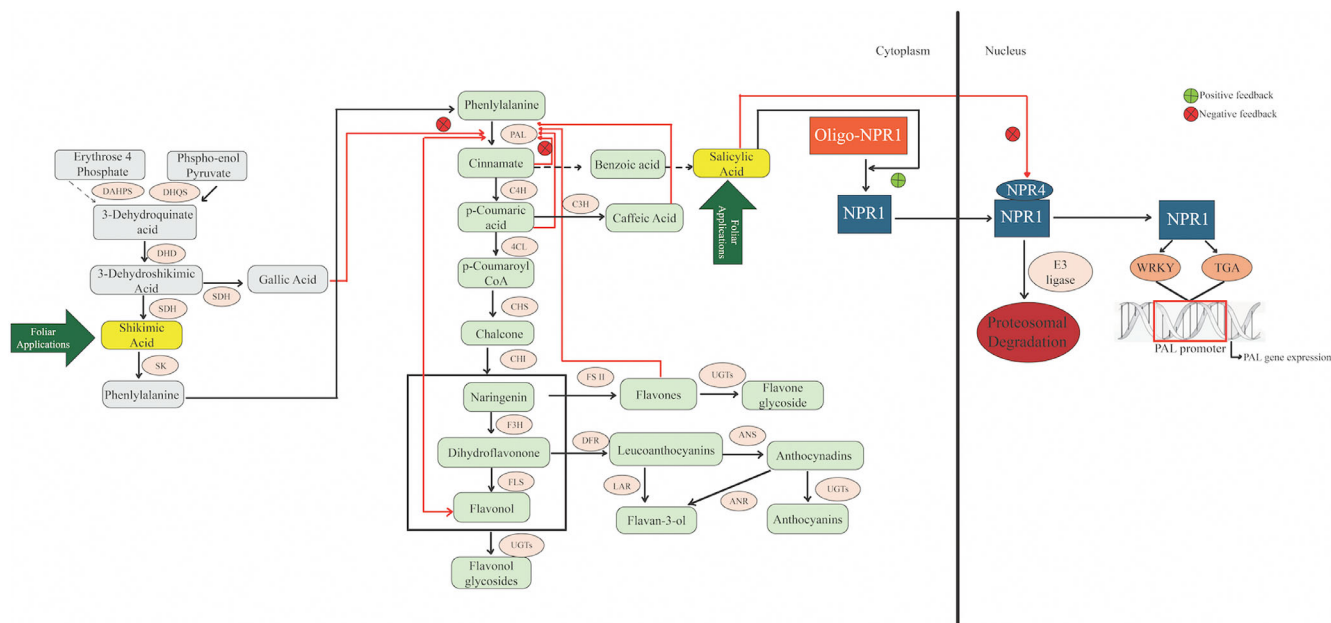


FIGURE 8 A proposed pathway on the effect of salicylic acid and shikimic acid on the phenylpropanoid pathway. Illustrating how NPR1 signaling induces PAL enzyme activity to promote the synthesis of polyphenols. These polyphenols then act in a feedback mechanism on PAL enzyme activity.

functions through transcription factors such as TGA.²² The higher binding scores of flavones, coumaric acid, caffeic acid, and gallic acid with PAL, compared to their product and proven inhibitor trans-cinnamic acid, clearly indicate feedback inhibition of PAL by these compounds. Since PAL is not directly related to them, any interaction beyond feedback is unlikely. This is supported by previous studies, such as the inhibition of PAL by coumaric acid in *Rhodobacter sphaeroides*,⁵⁷ caffeic acid in Glycine max,⁵⁸ gallic acid in Ipomoea batatas,⁵⁹ and flavonol in *Arabidopsis thaliana*.⁶⁰ Another reason these compounds may act in a feedback mechanism is that, like cinnamic acid, they also bind directly to the active site of PAL, suggesting competitive inhibition.

Overall, SA induced a more pronounced induction effect compared to ShA. However, sustainability, or the capacity to maintain the induced response over time, was notably better with ShA induction. This suggests that while SA may elicit a stronger immediate response, ShA induces a more enduring and sustainable effect. SA primarily functions as a signaling molecule, directly activating the NPR1-dependent pathway, which leads to a rapid transcriptional burst of phenylpropanoid-related genes,²² including PAL. This sharp increase in pathway activity results in a swift accumulation of downstream metabolites. However, the rapid rise in product concentration can quickly saturate enzyme-active sites,⁶¹ particularly at rate-limiting steps, thereby triggering feedback inhibition and regulatory attenuation of PAL activity. Consequently, although SA elicits a strong and immediate response, this induction is often transient due to faster engagement of feedback control mechanisms. In contrast, ShA acts as a metabolic precursor that is gradually assimilated into the phenylpropanoid pathway, where metabolic flux is distributed across multiple

branches, including the general phenylpropanoid pathway (hydroxycinnamic acids and lignin precursors), the flavonoid pathway (flavonols and flavones via CHS-CHI-FLS), and the anthocyanin branch (anthocyanidins and anthocyanins via DFR-ANS-UGT).^{31,32} This substrate-driven reinforcement may lead to a progressive and balanced increase in metabolite synthesis rather than a sharp surge. Because the flux is partitioned among several downstream products, enzyme saturation may occur more slowly, reducing the likelihood of rapid feedback inhibition. As a result, ShA may promote a more sustained and stable enhancement of secondary metabolite accumulation.⁶¹ The comparison between two different growth stages revealed that 2-year-old plants exhibit higher levels of polyphenols and antioxidant activity, along with better efficiency of inducers. This finding supports the practice of transferring plants from the nursery to the plantation at around 10–18 months,³⁵ as during this period, tea plants acquire a better ability to combat various stresses due to their enhanced polyphenol and antioxidant activity compared to nursery plants. Consequently, 2-year-old plants are already well-equipped to withstand stress and are, therefore, suitable for transplantation into the plantation, as they possess superior polyphenol and antioxidant activity compared to 4-month-old nursery plants.

The measured interconnectivity between all five assays demonstrates a strong correlation, aligned with the previous research on stone fruits.⁶² Anthocyanin is a subclass of flavonoids,⁶³ and flavonoids, in turn, are a subclass of polyphenols.⁶⁴ Hence, it is expected that these assays would be interconnected. Additionally, the interconnection between polyphenols and antioxidant activity arises from their shared characteristics.^{65,66} Catechins possess a strong hydrogen supply capacity on the B and C rings, and the presence of a

2,3-double bond and an unsaturated 4-oxo group in the C-ring promotes electron delocalization (free electron) of the ortho-dihydroxy catechol in the B-ring. These characteristics contribute to their antioxidant properties, which in turn correlate with the measured interconnectivity between polyphenol and antioxidant activity assays.¹²

These findings have the potential to significantly influence both plant and human well-being. The elevation of polyphenols can have a significant impact on human health, as tea polyphenols possess various beneficial properties, including anticancer, antidiabetic, anti-cardiovascular, anti-neurological, bacteriostatic, and anti-inflammatory effects.³ The most significant factor is the increase in anthocyanin levels, which have a direct effect on immunostimulation and anticancer activity. This is supported by previous research on purple tea, which is enriched with anthocyanins.⁶⁷

The elevation of polyphenols and antioxidants is equally important for the tea plant itself, as polyphenols can protect the plant from various abiotic stresses.¹⁶ Protection against abiotic stress is crucial for tea plants, particularly in the context of drought and heat stress, which are major concerns for the tea industry.⁶⁸ On the other hand, flash floods are a significant concern in the North East Bangladesh and India (NEBI) region due to extreme single-day rainfall events (>150 mm/day) occurring from May to October.⁶⁹ These flash floods can lead to severe waterlogging and damage tea plants.¹⁸ Another significant concern is heavy metal stress, resulting from the accumulation of heavy metals in the soil of tea gardens due to the use of different pesticides, chemical fertilizers, and industrialization.⁷⁰

The future aspects of this study include assessing the effectiveness of SA and ShA as inducers in real-world conditions. While this study has proposed that SA and ShA can function as inducers in a lab setup, it is necessary to evaluate their effectiveness in tea gardens to confirm their practical applicability and benefits in agricultural settings. Further research is needed on ShA to fully understand the mechanism by which it acts as an inducer.

CONCLUSION

This study observed that SA and ShA are effective inducers for enhancing polyphenol synthesis and antioxidant activity in tea plants, with 2-year-old plants showing greater responsiveness compared to 4-month-old plants. The strong correlations between the assays confirm the robustness of these findings. Novel insights into ShA's role were uncovered, as it had not been previously studied as an inducer. The temporal analysis showed that the application of these inducers should be timed strategically to prepare for or mitigate abiotic stress. Another novel insight is that the induction of PAL is mediated by the same mechanism as the induction of PR genes in NPR1 signaling.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors declare that all the data and plant materials will be available without restrictions. All the plant materials and the raw data used in the manuscript will be available to Dr. Ajit Ghosh (aghosh-bmb@sust.edu).

ETHICS STATEMENT

All experimental research on plants, including the collection of plant materials, has been conducted by following the relevant institutional, national, and international guidelines and legislation.

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