

Article

Investigating the Effect of Pipecolic Acid on Specialized Metabolites Involved in Tomato Plant Defense Mechanisms Against *Ralstonia solanacearum* Wilt Pathogens

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Abstract: The role of pipecolic acid (Pip) in plant immune responses, particularly against bacterial wilt pathogens, is significant. This research aimed to understand the interaction between plant defense-responsive enzymes and Pip by analyzing methanolic extracts from different treatments of tolerant (GAT5) and susceptible (GT2) tomato cultivars. LC-MS analysis demonstrated that the foliar application of Pip significantly influenced tomato metabolites, especially in bacterial wilt-infected plants, with a more pronounced effect in tolerant varieties. Principal component analysis (PCA) revealed that Pip-treated plants of tolerant varieties exhibited better coordinated metabolome profiles than those of susceptible varieties. Notable variations were observed in the levels of specialized metabolites, such as salicylic acid (SA), N-hydroxy pipecolic acid (NHP), and Pip, which are essential for producing defense compounds. Molecular docking studies further explored Pip's interactions with key plant enzymes involved in defense mechanisms and showed that Pip acts as an effective organic inducer of systemic acquired resistance (SAR). These findings highlight Pip's potential as a natural agent for enhancing plant tolerance to pathogens, offering promising implications for agricultural practices and improving crop resilience against diseases. This study enhances our understanding of Pip's role in plant defense and provides a foundation for developing Pip-based strategies for sustainable agriculture.

Keywords: LC-MS; docking studies; pipecolic acid; PCA; systemic acquired resistance; tomato plant



Academic Editor: Marcello Locatelli

Received: 4 December 2024

Revised: 4 January 2025

Accepted: 7 January 2025

Published: 9 January 2025

Citation: Sabharwal, U.; Rai, P.K.; Choure, K.; Subramanian, R.B.; Joo, J.C.; Pandey, A. Investigating the Effect of Pipecolic Acid on Specialized Metabolites Involved in Tomato Plant Defense Mechanisms Against *Ralstonia solanacearum* Wilt Pathogens. *Analytica* **2025**, *6*, 2. <https://doi.org/10.3390/analytica6010002>

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1. Introduction

Pathogen interactions with plants trigger a series of metabolic changes that play a critical role in plant protection. Bacterial pathogens can disrupt biochemical pathways by altering the biochemical composition of plants [1,2]. Plant responses to pathogens vary significantly depending on their acquired tolerance mechanisms, treatment methods, and genetic susceptibility. Some susceptible plants are unable to withstand pathogen attacks [3,4]. However, their tolerance can be enhanced through the foliar application of various chemicals or biofertilizers [5].

Piperidine-2-carboxylic acid (Pip), a non-protein amino acid, plays a crucial role in plant priming and defense [6]. Pip preconditions plants for the optimal production of phenolic defensive signals and salicylic acid (SA), enhancing systemic acquired resistance (SAR) through both SA-dependent and SA-independent signaling mechanisms, where the former plays a major role [7,8]. Previous studies have shown changes in xylem sap proteomes and metabolomes in response to abiotic and biotic stresses. Pathogen infections can increase phenolic compounds and salicylic acid derivatives in the xylem sap [9,10]. Plant secondary metabolites play vital roles in growth, development, defense mechanisms, and responses to various environmental stresses [6,11]. Metabolomics studies based on mass spectrometry have been extensively used to identify metabolites essential for plant–environment interactions, agriculture, nutrient assimilation, stress responses, and plant–microbe interactions, including pathogen-related interactions [8,12,13].

Tomato plants were selected for this study due to their economic importance and widespread cultivation [14]. However, they are susceptible to numerous pathogens, especially *Ralstonia* sp., one of the most devastating plant pathogens affecting over 200 plant species [15]. This study was conducted on two tomato cultivars: GT-2 (susceptible to pathogens) and GAT-5 (tolerant). The objective was to gain comparative insights into the pathogen defense mechanisms in plants by analyzing the distribution, function, biosynthesis, and transport of metabolites using LC-MS and molecular docking studies.

2. Materials and Methods

2.1. Pot Trails

GT-2 and GAT-5 seeds were planted in pots and maintained in a greenhouse at Anand Agriculture University, Gujarat. The soil was inoculated with 24-h-old *Ralstonia solanacearum* cultures (10^8 CFU/mL) at 28 °C to induce symptoms in the tomato plants. After six days, when symptoms had developed, 1 mM of Pip was sprayed on all treatments except the control group. Leaves were collected in triplicate from both cultivars' treatments 48 h after the Pip application.

The treatments were as follows, and more details are mentioned in Table 1:

T1: Control (no inoculation or Pip treatment).

T2: Inoculated with *Ralstonia solanacearum*.

T3: Inoculated with *Ralstonia solanacearum* and treated with Pip.

T4: Treated with Pip only.

Table 1. Treatments applied to susceptible (GT-2) and tolerant (GAT-5) varieties with corresponding experimental conditions.

S.No.	Treatments	Varieties	
		Susceptible (GT-2)	Tolerant (GAT-5)
1	Control	G2T1	G5T1
2	<i>Ralstonia solanacearum</i>	G2T2	G5T2
3	<i>Ralstonia solanacearum</i> + Pipecolic Acid	G2T3	G5T3
4	Only Pipecolic Acid	G2T4	G5T4

The seedlings were irrigated daily and maintained under greenhouse conditions of 25 ± 5 °C and $80 \pm 10\%$ relative humidity for 50 days. Finally, LC-MS analysis was performed to compare the levels of three metabolites—Pip, NHP, and SA—across the treatments to determine whether pathogen infection and elicitor treatment induced changes in the acquired tolerance of both cultivars.

2.2. Chemicals and Reagents

Acetonitrile (ACN) and methanol (MeOH) (LC-MS grade, Sigma Aldrich, St. Louis, MO, USA) were used for the LC-MS analysis. Ultra-pure water was obtained from a Millipore system (Milford, MA, USA).

2.3. Sample Preparation

Three leaflets from each triplicate sample were freeze-dried using liquid nitrogen and carefully crushed to ensure thorough mixing of different tissue types. Approximately 100 mg of the sample was homogenized in methanol and centrifuged at $10,000\times g$ for 20 min. The supernatant was collected, filtered through a $0.22\ \mu\text{m}$ Nylon PVDF syringe filter, and $1\ \mu\text{L}$ was injected into the LC instrument for LC-MS analysis.

2.4. Equipment and LC-MS Condition

Purified extracts were separated using a $5\ \mu\text{m}$ Hypersil Gold C18 LC column ($250\ \text{mm} \times 4.6\ \text{mm}$) (LC-MS/MS, USA) with an acetonitrile (ACN) gradient ranging from 2% to 90% over 22 min at a flow rate of $0.8\ \text{mL/min}$ and a column oven temperature of $40\ ^\circ\text{C}$. Measurements were conducted in both positive and negative ion modes, covering a mass range of $50\ m/z$ to $2000\ m/z$ [16]. MS data were acquired in targeted selective ion monitoring (t-SIM) mode using an electrospray ionization Orbitrap Q-Exactive system linked to an UltiMate 3000 RSLC [17,18].

The mass spectrometry conditions were as follows:

Polarity: Positive and negative ionization modes.

Spray voltage: 5 kV for positive mode; 2.0 kV for negative mode.

Sheath gas flow rate: 55 Units.

Auxiliary gas flow rate: 30 Units.

Sweep gas flow rate: 0 Units.

Heated capillary temperature: $300\ ^\circ\text{C}$.

Additional specifications included the following:

LCQ fleet mass range: 50–2000 Daltons.

TSQ Quantum Access mass range: 30–3000 Daltons.

Pump: Dual-piston delivery system with a built-in vacuum degasser.

Heated capillary temperature: $380\ ^\circ\text{C}$.

S-lens RF level: 50.

Auxiliary gas heated temperature: $350\ ^\circ\text{C}$ [19,20].

The resolution was set at 70,000, with an AGC (automatic gain control) target of 5×10^4 . The ion injection time was limited to 200 ms, and a $10\ m/z$ isolation window was employed [21,22].

2.5. LC-MS Data Analysis

Mass calibration and data detection for each metabolite were conducted using Data Analysis 4.1 Software (Brukers Daltonics, Bremen, Germany), with the following parameters for the “Find Molecular Features” function: a signal-to-noise ratio threshold of 5, a correlation coefficient of 0.7, a minimum compound length of 8, and a smoothing width of 2. Extracted Ion Chromatograms (EICs) were obtained from the calibrated data files using QuantAnalysis 2.1 Software, with an extraction window set to 0.3 Da. Peaks were integrated by aligning them within a retention time window of 0.5 min. The analyzed peak area data were saved as CSV files, reformatted in Microsoft Excel 2010, and subsequently utilized for graphical representation and statistical analyses using Origin 10.5.1.

For statistical analyses, a one-way analysis of variance (ANOVA) with a significance level of <0.01 was conducted to evaluate general statistical significance in multi-class

comparisons. Post hoc significance testing was performed using Tukey's test and the Least Significant Differences (LSDs) method to compare sample classes. These tests were performed using the SPSS IBM 22 software package. The Relative Standard Deviation (RSD) of metabolites was calculated from the peak regions of eight QC samples, including Pip ([M + H]⁺ 130.0, 70.0, 56.0), NHP ([M + H]⁺ 146.0, 128.0, 110.06, 100.07), and SA ([M + H][−] 137.025, 93.03).

Principal component analysis (PCA) was conducted using Origin software (version 8.5.1) to classify multivariate data and assess feature importance for separation based on their positions in the PCA loadings plots of the first three principal components. Multivariate ANOVA (MANOVA) was also performed on these components using Origin Lab. Raw data files were processed with Qual Browser software in Xcalibur 4.2.0.7. (Thermo Fisher Scientific, Waltham, MA, USA), and quantification was carried out using Quan Browser software in Xcalibur 4.2.0.7. Finally, comparative analyses were supported by Student's *t*-tests conducted in Microsoft Excel.

2.6. Molecular Docking Studies

To investigate the potential role of Pip, NHP, and SA in activating systemic acquired resistance (SAR) in plants, the involvement of key plant metabolites such as ALD1, SARD4, NPR1, and U76B1 was explored using docking studies. These metabolites play a crucial role in SAR, which had not been extensively studied in previous research. As the sequences of these tomato enzymes were unavailable in the database, homology modeling was performed using the sequences of *Arabidopsis thaliana*. The 3D structures of the target enzymes, which were also absent from the database, were generated using the AlphaFold2 server integrated with Chimera X [23,24]. Docking studies were conducted using AutoDock V1.2.0, and analysis was carried out using PyMOL (version 3.1.3) for visualization and interaction assessment. Binding affinity data (in kcal/mol) and the interaction patterns of the plant metabolites were determined, leveraging insights from previous studies [25].

3. Results

The plant metabolites Pip, NHP, and SA were analyzed across all treatments. These metabolites were detected in the target-selected ion monitoring mode by measuring the accurate mass of monoisotopic ions. The data analysis utilized *m/z* values, and retention times differed among the three metabolites. The objective was to investigate the relationship between these metabolites and tomato plants. To achieve this, eight treatment samples from two different plant cultivars were analyzed using LC-MS. The results revealed significant variations in the Pip, NHP, and SA peak areas between the two cultivars. Forty-eight hours after spraying, G5T3 plants exhibited the highest accumulation of Pip, with a maximum peak area of 31×10^5 mAU. Conversely, the lowest accumulation of Pip's precursor, NHP, was also observed in G5T3, with a peak area of 0.12×10^5 mAU. The salicylic acid levels were highest in G5T4, with a peak area of 7×10^5 mAU, while the lowest levels were recorded in G5T1 (control), with a peak area of approximately 4×10^4 mAU (Figure 1a–d).

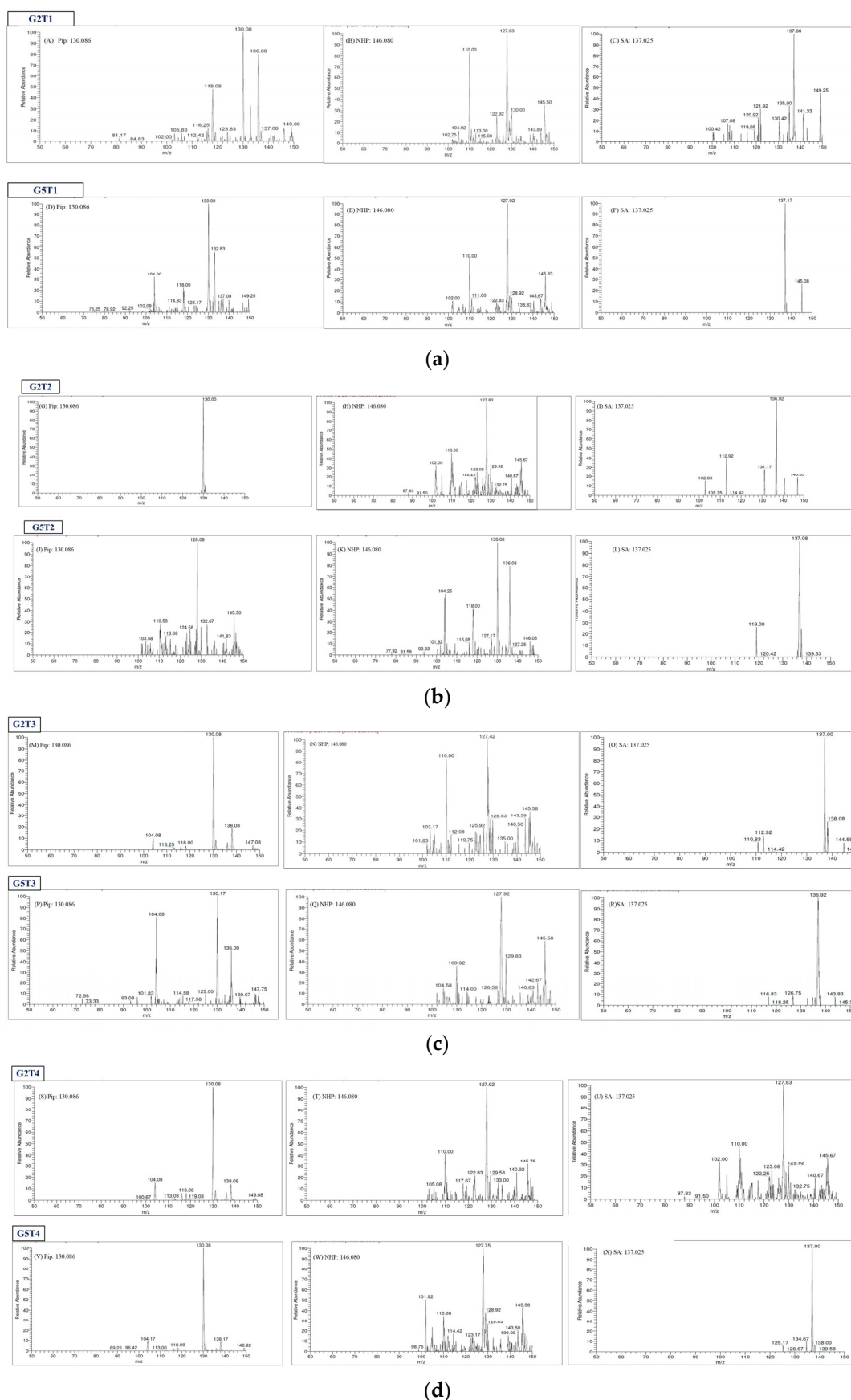


Figure 1. (a) LC-MS/MS chromatogram of control treatments G2T1: (A): pipecolic acid, (B): N-hydroxypipecolic acid, (C): salicylic Acid; G5T1, (D): pipecolic acid, (E): N-hydroxypipecolic acid, (F): salicylic acid. (b) LC-MS/MS chromatogram of treatments G2T2: (G): pipecolic acid, (H): N-hydroxypipecolic

acid, (I): salicylic Acid; G5T2, (J): pipelicolic acid, (K): N-hydroxypipelicolic acid, (L): salicylic acid. (c) LC-MS/MS chromatogram of treatments G2T3: (M): pipelicolic acid, (N): N-hydroxy pipelicolic acid, (O): salicylic Acid; G5T3, (P): pipelicolic acid, (Q): N-hydroxypipelicolic acid, (R): salicylic acid. (d) LC-MS/MS chromatogram of treatments G2T4: (S): pipelicolic acid, (T): N-hydroxypipelicolic acid, (U): salicylic Acid; G5T4, (V): pipelicolic acid, (W): N-hydroxypipelicolic acid, (X): salicylic acid.

3.1. Pip, NHP, and SA Production

Exogenous Pip significantly enhanced tolerance to bacterial wilt in both varieties. When 1 mM of Pip was applied as a foliar spray to individual plants, amino acid uptake occurred from the leaves and stems. Among all the treatments, Pip levels increased in the G5T3 treatment from the basal value observed in the control plants, whereas G5T4 accumulated a comparatively lower peak area than G5T3. In contrast, G2T4 plants accumulated a slightly higher concentration of Pip, approximately 9.7×10^5 mAU, which is higher than the peak area of G2T3, measured at 8.7×10^5 mAU, as shown in Figure 2.

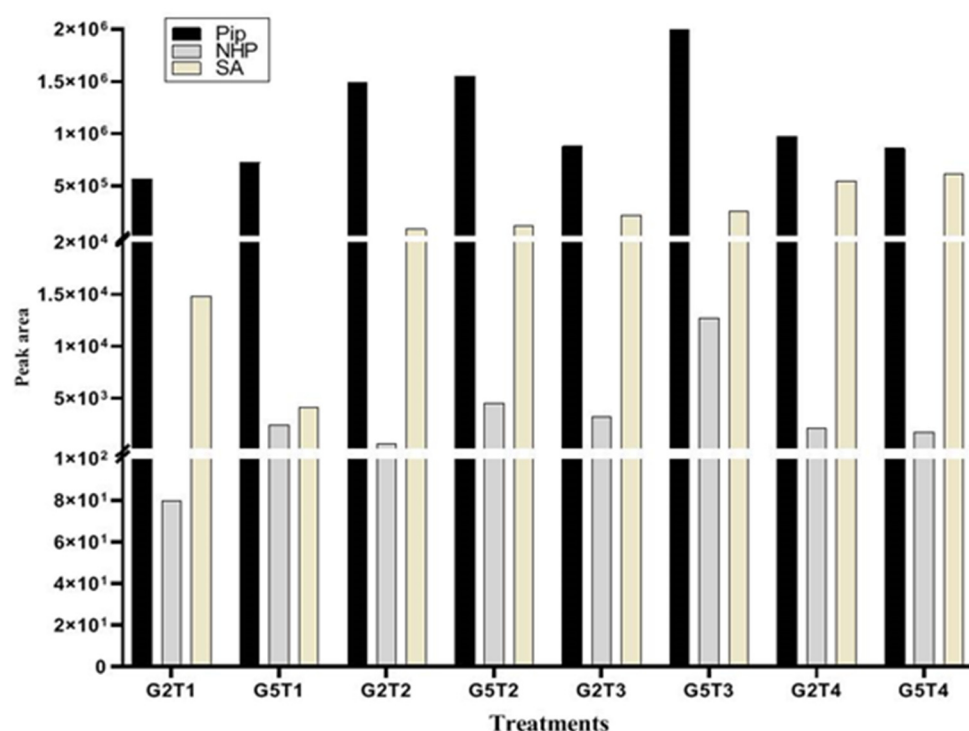


Figure 2. Peak area of treatments, tolerant (GT-2) and resistant (GAT-5) varieties, including control (G2T1, G5T1), *Ralstonia solanacearum* infection (G2T2, G5T2), *Ralstonia solanacearum* + pipelicolic acid (G2T3, G5T3), and only pipelicolic acid (G2T4, G5T4).

NHP was detected in all treatments of both susceptible and tolerant varieties. The lowest level of NHP was observed in the G2T1 treatment, but NHP levels increased progressively in the order G2T2 (79.95 mAU) < G5T4 (1.7×10^3 mAU) < G2T4 (2.1×10^3 mAU) < G2T3 (3.2×10^3 mAU) < G5T3 (1.2×10^4 mAU). The maximum accumulation of NHP was observed in G5T3 (1.2×10^4 mAU), which was significantly higher than in G2T3 (3.2×10^3 mAU). By contrast, NHP levels in *Ralstonia solanacearum*-treated G5T2 were higher than in G2T2. However, the NHP levels were nearly similar in G2T4 and G5T4, at 2.1×10^2 and 1.7×10^2 mAU, respectively.

The Pip-triggered elevation confers tolerance to bacterial pathogens, with the marked potentiation of SA production. SA accumulation was higher at basal levels compared to NHP. In G5T1 (4.1×10^2 mAU), SA levels were comparatively lower than in G2T1 (1.4×10^3 mAU). The highest SA accumulation was observed in G5T4 (6.1×10^5 mAU).

compared to G2T4 (5.4×10^5 mAU). However, SA levels were nearly similar in T3 treatments, where G2T3 accumulated 2.1×10^4 mAU and G5T3 accumulated 2.5×10^4 mAU, as shown in Figure 2. Enhanced SA production was evident in T2 and T3 treatments, particularly following pathogen infection (Tables 2 and 3).

Table 2. The details of the physicochemical properties of Pip, NHP, and SA ligands.

Name of Ligand	PubChem ID	Mol. Wt.	HBD *	HBA *	Rotatable Bonds	TPSA * ($\text{\AA}^2$)
Pip	849	129.16	2	3	1	49.3 $\text{\AA}^2$
NHP	92246037	145.16	2	4	1	60.8 $\text{\AA}^2$
SA	338	138.122	2	3	1	57.5 $\text{\AA}^2$

* HBD = hydrogen bond doner; HBA = hydrogen bond acceptor; TPSA = topological polar surface area.

Table 3. Molecular docking results show binding energy and several hydrogen bonds of ligand–protein interaction between plant SAR enzymes.

Sr. No.	Target	Ligand	Binding Energy (kcal/mol)	Total No. of Hydrogen Bonds	Hydrogen Bond and Length ($\text{\AA}^\circ$)	Interaction with Amino Acids
1	ALD1	SA	−6.1	One	Ala256: 2.3 $\text{\AA}^\circ$	Ala256
		Pip	−7.3	Two	Ala255: 2.3 $\text{\AA}^\circ$, Ala256: 2.9 $\text{\AA}^\circ$	Ala255, Ala256
		NHP	−7.72	Eight	Ala142: 2.2 $\text{\AA}^\circ$, Ser281: 2.2 $\text{\AA}^\circ$, Ser283: 2.5 $\text{\AA}^\circ$, 2.1 $\text{\AA}^\circ$, Arg292: 2.6 $\text{\AA}^\circ$, 2.5 $\text{\AA}^\circ$, 1.2 $\text{\AA}^\circ$, 2.1 $\text{\AA}^\circ$	Ala142, Ser281, Ser283, Arg292
2	NPR1	SA	−7.7	Two	Arg404: 1.7 $\text{\AA}^\circ$, Gln400: 1.8 $\text{\AA}^\circ$	Arg404, Gln400
		Pip	−7.4	Four	Lys340: 1.7 $\text{\AA}^\circ$, Arg161: 2.0 $\text{\AA}^\circ$, 1.7 $\text{\AA}^\circ$, 2.6 $\text{\AA}^\circ$	Lys340, Arg161
		NHP	−5.2	Two	Arg161: 3.0 $\text{\AA}^\circ$, Arg363: 3.2 $\text{\AA}^\circ$	Arg161, Arg363
3	SARD4	SA	−8.1	Seven	Arg165: 2.0 $\text{\AA}^\circ$, 1.8 $\text{\AA}^\circ$, Thr166: 1.9 $\text{\AA}^\circ$, Asn: 2.1 $\text{\AA}^\circ$, 2.0 $\text{\AA}^\circ$, Arg169: 2.4 $\text{\AA}^\circ$, 2.4 $\text{\AA}^\circ$	Arg165, Thr166, Asn, Arg169
		Pip	−8.9	Four	Arg114: 2.2 $\text{\AA}^\circ$, Lys71: 2.0 $\text{\AA}^\circ$, Arg42: 2.9 $\text{\AA}^\circ$, 2.4 $\text{\AA}^\circ$	Arg114, Lys71, Arg42
		NHP	−6.9	Four	Arg114: 2.6 $\text{\AA}^\circ$, 2.6 $\text{\AA}^\circ$, Lys71: 2.8 $\text{\AA}^\circ$, 2.2 $\text{\AA}^\circ$	Arg114, Lys71
4	U76B1	SA	−6.8	Three	Ala328: 2.3 $\text{\AA}^\circ$, Trp327: 2.5 $\text{\AA}^\circ$, His246: 2.2 $\text{\AA}^\circ$	Ala328, Trp327, His246
		Pip	−7.5	Six	Gln330: 2.4 $\text{\AA}^\circ$, His246: 2.0 $\text{\AA}^\circ$, Trp327: 2.1 $\text{\AA}^\circ$, Ser242: 2.7 $\text{\AA}^\circ$, Gln18: 2.3 $\text{\AA}^\circ$, Asn22: 2.6 $\text{\AA}^\circ$	Gln330, His246, Trp327, Ser242, Gln18, Asn22
		NHP	−8.8	Four	Gln330: 2.7 $\text{\AA}^\circ$, Glu353: 2.7 $\text{\AA}^\circ$, 2.3 $\text{\AA}^\circ$, Asn22: 2.4 $\text{\AA}^\circ$	Gln330, Glu353, Asn22

Based on LC-MS results, a PCA score plot (Figure 3) was generated to show that the clustering of plant metabolites varies among the treatments. Low levels of plant metabolites can be considered indicative of no infection and lower plant defense mechanisms. The PCA plot shows a clear variation between G2T1 and the cluster comprising G5T1, G5T2, and G2T2, while G2T3 and G5T3 are distinct from this cluster. However, G2T4 and G5T4 were clustered together. The proximity of T3 is noteworthy, as these treatments differ in variety. A second cluster of G2T4 and G5T4 was also observed.

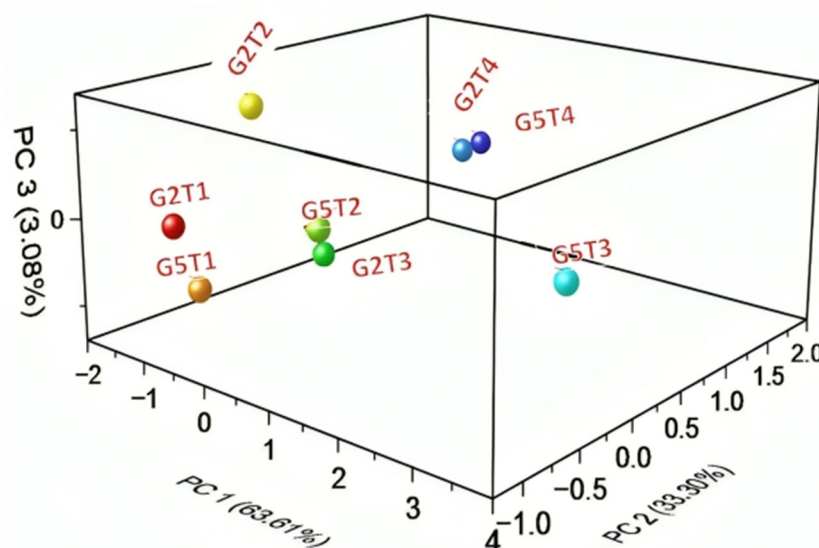


Figure 3. PCA scores plot created using peak areas of eight treatments in two different varieties, GT2 and GAT5.

Studies have shown that plant metabolites such as Pip, SA, JA, and azelaic acid (AzA) contribute to enhancing plant defense metabolism, even at low concentrations [26]. The PCA score plot (Figure 3), generated from the LC-MS results, displays the clustering of plant metabolites that vary among the treatments. Low levels of these metabolites indicate no infection and lower plant defense mechanisms. Additionally, research supports that plant metabolites like Pip, SA, JA, and AzA increase plant defense metabolism, even at low concentrations. The PCA plot clearly shows a variation in the G2T1 treatment.

3.2. Docking Results

To better understand plant defense mechanisms at the molecular level and determine why Pip application enhances SAR more effectively than SA and NHP, we conducted docking studies using AutoDock Vina and PyMOL. The decision to utilize molecular docking in our research stemmed from the need to elucidate the specific interactions between pipelicolic acid (Pip) and key enzymes involved in systemic acquired resistance (SAR) and defense priming [27]. By performing molecular docking, we predicted the binding affinities and interactions between Pip and these enzymes, providing valuable insights into their roles in plant immunity.

The docking study revealed that the best interaction between U76B1 (target) and the ligand (NHP) had an RMSD (Root Mean Square Deviation) value of 2.40 Å. The pose showed interactions with four hydrophilic amino acids and a binding energy (B.E.) of −8.8 kJ/mol. Additionally, U76B1 exhibited significant binding interactions with Pip, with a binding energy of −7.5 kJ/mol, involving six amino acids (both hydrophobic and hydrophilic).

The docking study of SARD4 (target) with ligands (Pip and SA) demonstrated the most significant binding energies of −8.9 kJ/mol and −8.1 kJ/mol, respectively. NPR1 showed the best binding affinities with SA and Pip, with binding energies of −7.7 kJ/mol and −7.4 kJ/mol, respectively. As referenced in Tables 2 and 3, ALD1 exhibited the highest binding affinities with NHP and Pip, with binding energies of −7.72 kJ/mol and −7.3 kJ/mol, respectively (Figure 4).

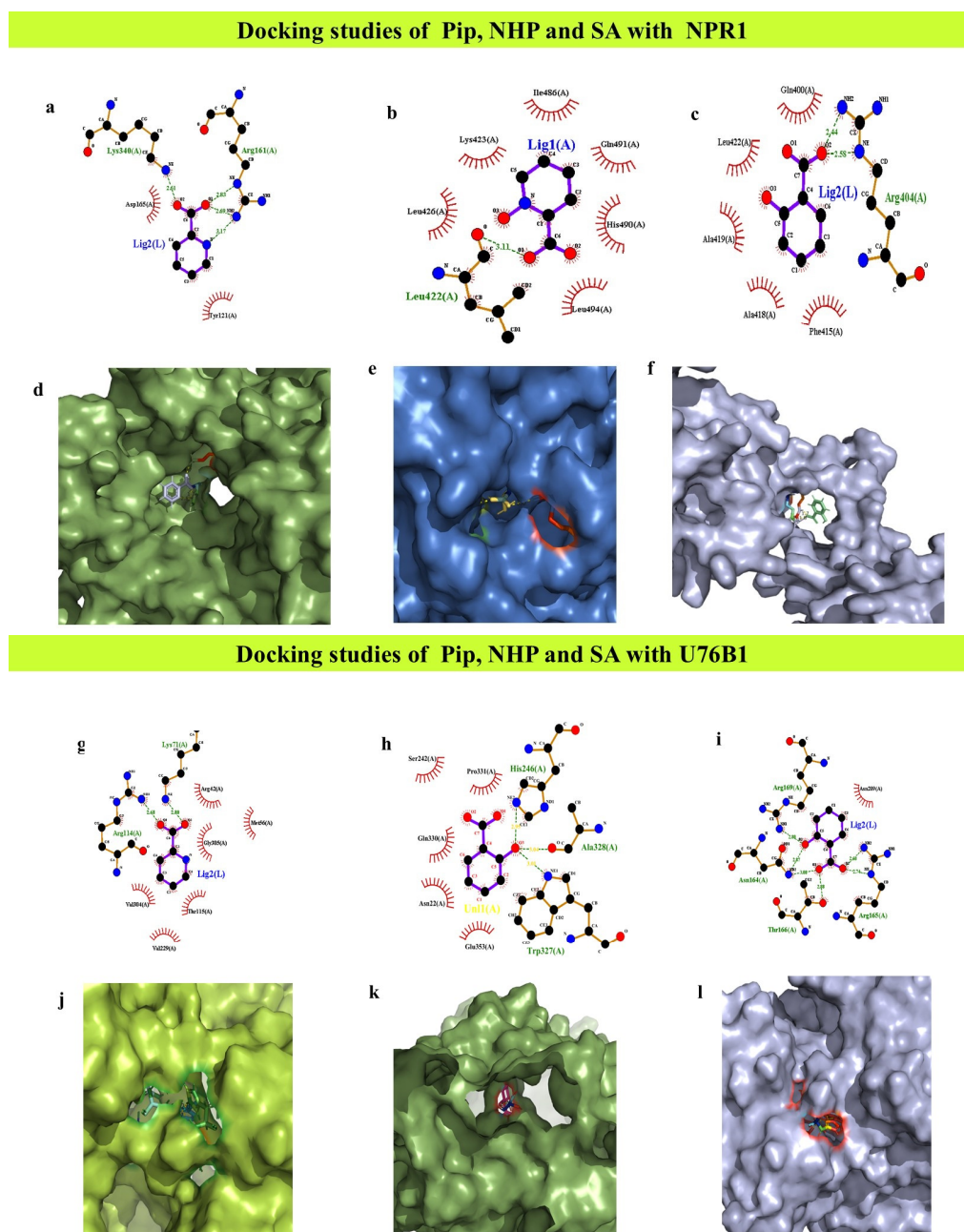


Figure 4. Three-dimensional models of the interactions between Pip, NHP, and SA with NPR1 (a–c) are presented, where Pip, NHP, and SA are shown as surfaces, while the docked NPR1 structures are displayed as sticks (d–f). Similarly, 3D models of the interactions between Pip, NHP, and SA with U76B1 (g–i) are shown, with Pip, NHP, and SA represented as surfaces and the docked U76B1 structures depicted as sticks.

4. Discussion

Bacterial wilt, caused by *Ralstonia solanacearum*, is a highly destructive disease that severely affects tomato plants grown in tropical and subtropical climates. These regions provide favorable environmental conditions for the pathogen's proliferation, leading to significant crop losses [21]. SA, NHP, and Pip are well-characterized plant hormones that function as signals integrated into the SAR pathway, enhancing plant resistance to pathogens. However, evidence suggests that although SA, NHP, and Pip activate defense responses, their direct application may not be effective for all tomato cultivars, as some varieties exhibit tolerance [22]. Several studies provide experimental evidence for the

beneficial effects of these metabolites on bacterial wilt resistance. For example, metabolomic studies using nuclear magnetic resonance (NMR) spectroscopy have identified major metabolites involved in resistance across various tomato varieties. Resistant strains show a higher accumulation of leucine and other metabolites associated with immune responses compared to susceptible ones [28].

The exogenous application of NHP and Pip can influence disease resistance development in crops, promising their utility in controlling bacterial wilt on farms. Gene expression analyses have shown that treatments with NHP and Pip lead to the increased expression of defense-related genes, such as AGD2-like defense response protein1 (ALD1) and flavin-dependent monooxygenase1 (FMO1), which are critical for effective pathogen response [29]. The enzyme UGT76B1 utilizes NHP and SA as endogenous substrates. Plants treated with foliar applications of Pip show increased SA levels, possibly because SA is not hydrolyzed into other compounds [30,31]. Bacterial wilt is a highly destructive plant disease prevalent in tropical and subtropical regions. Plant metabolites play a crucial role in balancing growth and defense mechanisms [13,21,32]. The possibility of a central player in SAR remains a topic of discussion, as new hypotheses emerge. The differential accumulation of SA, NHP, and Pip may result from the glycosylation of NHP by UGT76B. Our study indicates that Pip foliar application may alter defense priming in systemic tissues.

SA acts as a defense hormone that induces immunity against plant pathogens with biotrophic and hemi-biotrophic infection modes in Arabidopsis, tobacco, and other plants [33]. In this study, the results align with previous findings, showing that SA accumulates in T3 and T4 treatment plants (leaves), generating long-lasting and broad-spectrum SAR. Pip enhances SAR in plants (Figure 3). Pip levels in plants can be induced by pathogen infections such as *Pseudomonas syringae* pv. tomato DC3000 [33–35]. SA positively regulates Pip biosynthesis in the early stages of pathogen infection but may negatively modulate free, bioactive Pip levels at later stages by promoting its glucosylation. N-hydroxy pipecolic acid (NHP), a downstream metabolite of Pip, plays a crucial role in SAR through the direct induction of SAR gene expression, signal amplification, priming for enhanced defense activation, and the positive regulation of SA-dependent pathways. Exogenous Pip application can induce SAR and restore acquired tolerance defects in Pip-deficient mutants, underscoring Pip's central role in regulating both SA-dependent and SA-independent defense responses [36].

Our findings show that plant metabolites SA, Pip, and NHP exhibit varying affinities for active sites in key SAR pathway enzymes. Negative binding energy scores indicate significant interactions between these metabolites and their target enzymes. For instance, SA binds to ALD1, NPR1, SARD4, and U76B1 with binding energies ranging from -6.1 to -8.1 kcal/mol, forming one to seven hydrogen bonds. In comparison, Pip shows stronger binding to most targets, with energies between -7.3 and -8.9 kcal/mol, forming more hydrogen bonds, particularly with ALD1 and SARD4. Although NHP exhibits weaker binding energies, such as -5.2 kcal/mol with NPR1, it demonstrates notable interactions with U76B1 (-8.8 kcal/mol). This molecular modeling investigation suggests that Arg is a crucial residue for metabolite binding, except for SA and ALD1. L-arginine is an essential amino acid in plants, serving as a vital nitrogen reserve and a precursor for producing polyamines, nitric oxide, and other compounds. These molecules function as signaling agents in plant growth, development, and stress responses [37]. The foliar application of Pip can enhance plant defense against bacterial wilt by increasing SA and NHP accumulation in both susceptible and tolerant varieties [38]. Although the roles of SA, Pip, and NHP in bacterial wilt disease tolerance are evident, further research is needed to evaluate Pip's effects on plant morphology and fruit yield. Transcriptomics studies at different infection and treatment stages could provide insights into which metabolites offer protection in tomatoes.

5. Conclusions

The current study investigated the impact of pipelicolic acid (Pip) on enhancing the defense mechanisms of tomato plants against bacterial wilt. It was observed that Pip increases salicylic acid (SA) levels, particularly in specific treatments, by inhibiting SA hydrolysis. The interaction between Pip, SA, and N-hydroxy pipelicolic acid (NHP) with key enzymes in the systemic acquired resistance (SAR) pathway underscores their critical role in plant defense. Molecular docking studies revealed that Pip and SA share similar binding mechanisms, supporting their effectiveness in inducing SAR. While Pip's role in disease tolerance is well documented, its effects on plant morphology and fruit yield require further investigation. Future research, including transcriptomic analyses, will help uncover the underlying molecular mechanisms.

Author Contributions: Conceptualization, methodology, and investigations: U.S., R.B.S., and P.K.R.; software: R.B.S. and K.C.; formal analysis: P.K.R.; validation: U.S. and R.B.S.; resources: A.P. and J.C.J.; data curation: U.S.; writing—original draft preparation: U.S.; writing—review and editing: U.S., A.P., and P.K.R.; visualization: U.S. and P.K.R.; supervision and project administration: R.B.S.; funding acquisition: A.P. and J.C.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All the data are included in this manuscript.

Acknowledgments: The authors are grateful to Rajesh Acharya for providing seeds of the tomato plant variety of Main Vegetable Research Station, Anand Agricultural University. They would also like to thank the Parul Institute of Applied Sciences of Parul University for encouraging the author.

Conflicts of Interest: The authors declare no conflicts of interest.

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