

# Limonene's Bacteriostatic Activity against *Ralstonia solanacearum* by Compromised Membrane Integrity: A Transcriptomic and Metabolomic Analysis

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## Abstract

*Ralstonia solanacearum*, the causative agent of bacterial wilt, is a soil-borne pathogen that poses a widespread threat to plants in the Solanaceae family. To elucidate the mechanism by which limonene exerts its effects on *R. solanacearum*, we first assessed the impact of limonene on the physiological indicators of the pathogen and subsequently analyzed its transcriptome and metabolome. Our findings indicate that limonene has a potent inhibitory effect on *R. solanacearum*, and it also suppresses the formation of the bacterial community biofilm. Limonene primarily regulates the terpene biosynthesis pathway in *R. solanacearum*, thereby potentially affecting signal transduction in the pathogen and disrupting its normal growth and development. These results significantly enhance our understanding of limonene's response to the induction of bacterial wilt and provide a reference for further prevention and control of *R. solanacearum*.

## Keywords

Limonene, Transcriptome, Metabolome, *Ralstonia solanacearum*, Terpenoid Backbone Biosynthesis

## 1. Introduction

Plant essential oils are aromatic oily liquids extracted from plants, known for their fragrant scents [1]. They contain a variety of chemical components, such as

monoterpenes, sesquiterpenes, aldehydes, phenols, and other compounds [2]. Essential oils are widely used for their medicinal properties, including antimicrobial, antiviral, antifungal, antioxidant, anti-inflammatory, anticancer, antihistamine, and hypoglycemic activities, and they also serve in food preservation [3]. To date, researchers worldwide have conducted extensive studies on essential oils due to their diverse bioactivities and have developed various commercial pesticide products. As a result, essential oils have been extensively applied in insecticides, herbicides, acaricides, and nematocides [4].

Limonene, a monocyclic monoterpene, is widely present in plants of the Rutaceae family and can be extracted from essential oils such as lemon, mandarin, sweet orange, and frankincense [5]. It is a compound widely used in cosmetics and household products [6], and due to its diverse biological properties, such as antioxidant, anti-inflammatory, anticancer, analgesic, and gastroprotective characteristics [7], as well as its agricultural properties, including insecticidal [8], antimicrobial [9], and herbicidal activities [10], it can also be utilized in medicine and agriculture. In China, limonene has been registered as a pesticide, bactericide, and pesticide adjuvant. Given its broad sources, easy extraction, extensive applications, and significant practical and research value, research on limonene is increasingly growing, expanding in depth and breadth, and its applications are gradually extending to fields such as food, medicine, and agriculture.

*Ralstonia solanacearum* (*R. solanacearum*), a polyphagous (hemi-) biotrophic plant pathogen, causes the destructive bacterial wilt disease and can persist in about 400 host and non-host reservoir species across more than 50 plant families [11]-[13]. Besides impacting bananas, eggplants, peanuts, peppers, tobacco, and tomatoes, the most severely affected major crop globally is the potato (*Solanum tuberosum* L.) [14]. The pathogen causes an estimated global economic loss of around one billion US dollars annually. In potato crops, regional yield losses range from 10% to 80% [15]-[17]. Owing to its economic and scientific significance, *R. solanacearum* ranks second among the top ten plant pathogenic bacteria worldwide [18]. This high ranking is due to its lethality in causing wilt in host plants and the difficulty in its eradication because of its long-term survival and high persistence in the environment [19] [20].

In this study, we experimentally verified the phenotypic effects of limonene on *R. solanacearum* and then analyzed the transcriptome and metabolome of limonene-treated *R. solanacearum* cultures. The objectives were to: 1) determine the impact of limonene on the pathogen's activity, chemotaxis, and biofilm formation capabilities; 2) explore the distribution of gene and metabolite expression regulated by limonene in *R. solanacearum*; 3) explain the metabolic pathways affected by limonene that influence *R. solanacearum*.

## 2. Materials and Methods

### 2.1. Experimental Materials and Treatments

Limonene was purchased from Aladdin (CAS: 7705-14-8). The bacterial wilt

pathogen, *Ralstonia solanacearum*, is the same strain used in the patent “Active components from marigold roots and their application in the control of *Ralstonia solanacearum*” (CN202010774068.0), which was applied for by the Hubei Provincial Tobacco Science Research Institute. The strain was acquired from Shanghai Mailier Chemical Technology Co., Ltd.

NA Medium: The composition of the NA medium includes peptone 3 g, yeast extract 1 g, proteose peptone 5 g, and glucose 10 g, with distilled water added to a final volume of 1000 mL. *Ralstonia solanacearum* was routinely cultured on Nutrient Agar (NA), Nutrient Broth (NB), and semi-solid medium containing 0.35% agar.

## 2.2. Experimental Methods and Procedures

### 2.2.1. Bacterial Suspension Preparation

The lyophilized *Ralstonia solanacearum* stored in our laboratory was inoculated into the NB liquid medium for revival. A sterile inoculation loop was used to transfer the revived bacterial suspension and streak it onto a solid NB agar plate. The plate was incubated overnight at 28 °C; single colonies grown on the plate were then inoculated into Polyamide bottles containing liquid NB medium. After cultivation at 28 °C with shaking at 180 r/min for 12 hours, the bacterial suspension was adjusted to a concentration of  $1 \times 10^6$  to  $1 \times 10^9$  CFU/mL to facilitate subsequent experiments.

### 2.2.2. In Vitro Antibacterial Assay

Sterile 96-well plates were placed on a laminar flow bench. In the sample test groups, 100  $\mu$ L of bacterial suspension and 100  $\mu$ L of *R. solanacearum* bacterial inhibitory liquid of different concentrations were added to each well. In the positive control group, 100  $\mu$ L of bacterial suspension and 100  $\mu$ L of a 20% ethanol solution were added to each well. The blank control group was supplemented with 200  $\mu$ L of NB liquid medium. The 96-well plates were sealed with a film, and incubated under conditions of 28 °C for 24 hours. A microplate reader was set to measure the absorbance values at 600 nm in each well at different time intervals. The inhibition rate (IR%) was calculated according to Equation (1).

$$IR\% = \frac{r_0 - r}{r_0 - 2.5} \quad (1)$$

In the formula,  $r_0$  represents the colony radius in the bacterial inhibitory liquid group, and  $r$  represents the colony radius in the control group.

### 2.2.3. Assessment of Biofilm Formation

The biofilm formation by *R. solanacearum* was assessed using a modified crystal violet staining method. Bacterial suspensions at  $1 \times 10^8$  CFU/mL were inoculated into NB medium with varying concentrations of the inhibitory liquid. After 24 hours of incubation at 37 °C, the wells were washed with PBS, air-dried, fixed with ethanol, and stained with 1% crystal violet for 30 minutes. Excess stain was

removed, and the plates were dried. The biofilms were then solubilized with 33% glacial acetic acid, and the OD<sub>490</sub> was measured using a microplate reader. The experiment was conducted in triplicate to ensure reliability.

### 2.3. Transcriptome Profiling

Total RNA was extracted from each sample (weighing  $120 \pm 10$  mg) using the Trizol reagent kit (Thermo Fisher Scientific, USA), and the absorbance at 260 and 280 nm was measured with the NanoDrop 2000 (Thermo Scientific, USA). The integrity of each RNA sample was assessed by 1% agarose gel electrophoresis and the Agilent 2200 Tape Station (Agilent Technologies, Germany). Eukaryotic mRNA from each qualified sample was enriched using magnetic oligo-dT beads and randomly fragmented using RNA fragmentation buffer. First-strand cDNA was synthesized using random hexamers, and double-stranded cDNA was purified with AMPure XP beads (Beckman Coulter, USA). RNA sequencing was conducted at the Shanghai Applied Protein Technology Co., Ltd. using the Illumina NovaSeq 6000 sequencing system, following the manufacturer's instructions (Illumina, USA).

### 2.4. Metabolome Profiling

Each sample (weighing  $310 \pm 10$  mg) was frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. Sixty milligrams of the powder was mixed with 1 mL of a methanol/acetonitrile/water solution (volume ratio 2:2:1) to remove proteins. The mixture was centrifuged at 14,000 g for 15 minutes at 4°C. The supernatant was then dried using a vacuum centrifuge. For liquid chromatography-mass spectrometry (LC-MS) analysis, the samples were reconstituted in 100 µL of acetonitrile/water (1:1, v/v) solvent.

To monitor the stability and reproducibility of the instrumental analysis, a quality control sample was prepared by pooling 10 µL from each sample and was analyzed alongside the experimental samples. The quality control samples were randomly interspersed and analyzed. The analysis was performed at the Shanghai Applied Protein Technology Co., Ltd. using an Agilent 1290 Infinity 2D-LC system (Agilent Technologies, Germany) coupled with a Triple TOF 6600 hybrid triple-quadrupole/time-of-flight mass spectrometer (AB Sciex, USA).

### 2.5. Data Statistics and Analysis

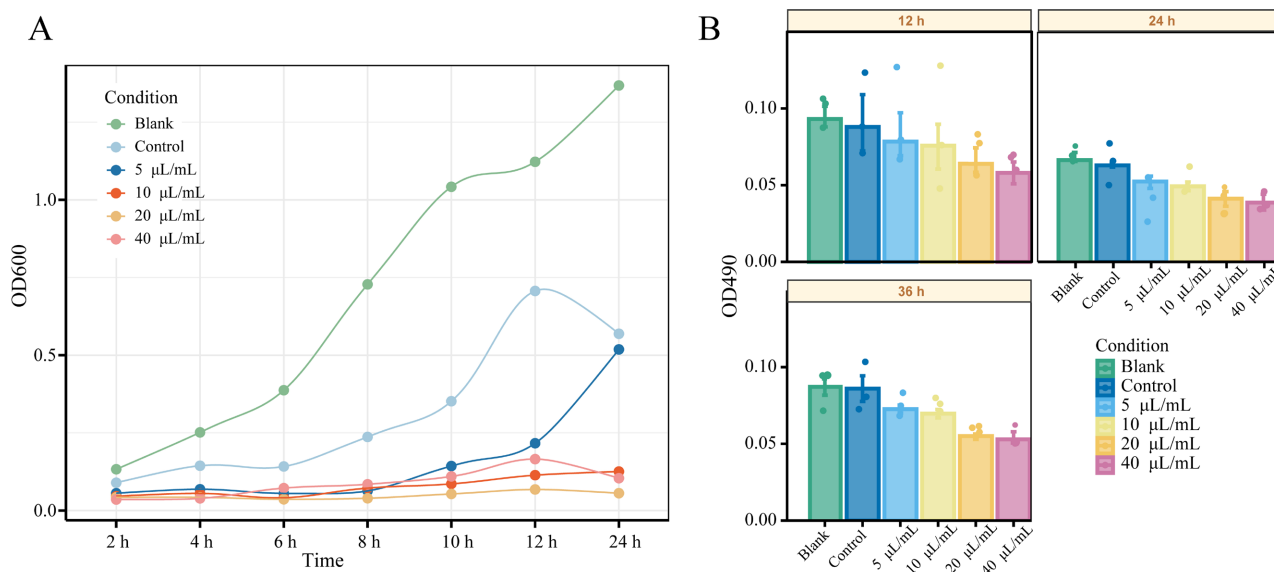
Measurement data were analyzed using one-way analysis of variance (ANOVA) in SPSS 27.0 software (SPSS, USA), followed by post-hoc comparisons using the least significant difference (LSD) and Duncan's methods. Results were visualized with Origin 2023 software (Origin Lab, USA). Statistical analysis was conducted at a significance level of  $p < 0.05$ , indicating significant differences. Values are presented as mean  $\pm$  standard deviation. Differentially expressed genes (DEGs) and differentially accumulated metabolites (DAMs) were identified and analyzed

using DEGseq software, and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotation analysis as well as KEGG enrichment analysis were performed on the DEGs and DAMs.

### 3. Results

#### 3.1. Phenotypic Effects of Limonene Treatment on *R. solanacearum*

As shown in **Figure 1**, limonene exhibits a strong inhibitory effect on *R. solanacearum*. When the concentration of limonene exceeds 10  $\mu\text{L}/\text{mL}$ , it significantly suppresses the growth of the bacteria, and this inhibitory capacity increases with concentration. There was no significant difference in the OD values between the negative control group (20% ethanol) and the blank control group ( $p > 0.05$ ), indicating that the growth states of the bacteria in both groups were essentially the same. Thus, the impact of the solvent for limonene (20% ethanol) on the growth of *R. solanacearum* can be neglected. The minimum inhibitory concentration (MIC) of limonene against *R. solanacearum* was determined to be 10  $\mu\text{L}/\text{mL}$ .



**Figure 1.** The impact of limonene on the physiological indices of *R. solanacearum*. (A) Growth curves of *R. solanacearum* under treatment with various concentrations of limonene; (B) Biofilm formation by *R. solanacearum* under treatment with various concentrations of limonene.

The minimum bactericidal concentration (MBC) test revealed that at a limonene concentration of 20  $\mu\text{L}/\text{mL}$ , no growth of *R. solanacearum* was observed on the agar plates, whereas at a concentration of 10  $\mu\text{L}/\text{mL}$ , the bacteria grew well. According to the guidelines in “Chemical Analysis of Antibacterial Drug Residues in Food,” the lowest drug concentration with no bacterial growth is defined as the MBC. Therefore, the MBC of limonene against *R. solanacearum* is 20  $\mu\text{L}/\text{mL}$  (**Table 1**).

The growth curve measurements showed that when the concentration of limonene was greater than 5  $\mu\text{L}/\text{mL}$ , it significantly inhibited the growth of *R. solanacearum* ( $p < 0.05$ ). Compared to the control group, the growth rate of the limonene-treated *R. solanacearum* was markedly delayed, with almost no increase observed. At a concentration of 20  $\mu\text{L}/\text{mL}$ , limonene severely inhibited bacterial growth, and there was hardly any growth of *R. solanacearum* after 6 hours of cultivation (**Figure 1(A)**).

Based on the OD490 values measured by the microplate reader, the comparative analysis of the biofilm formation quantity of the strain indicated that the biomass of all treatment groups decreased from 12 hours to 24 hours and then increased from 24 hours to 36 hours. The control group exhibited a significantly higher level of biofilm formation compared to other treatment groups with varying concentrations. The inhibitory effect of limonene on biofilm formation intensified progressively with the increase in concentration, particularly showing a strong inhibitory effect when the limonene concentration was at 5 and 20  $\mu\text{L}/\text{mL}$  ( $p < 0.05$ ) (**Figure 1(B)**).

**Table 1.** Determination of the minimum bactericidal concentration of Limonene Against *R. solanacearum*.

Bacteria	Control		Limonene Concentration ( $\mu\text{L}/\text{ML}$ )							
	Blank	Negative	0.625	1.25	2.5	5	10	20	40	
<i>R. solanacearum</i>	+++	+++	+++	+++	++	++	++	-	-	

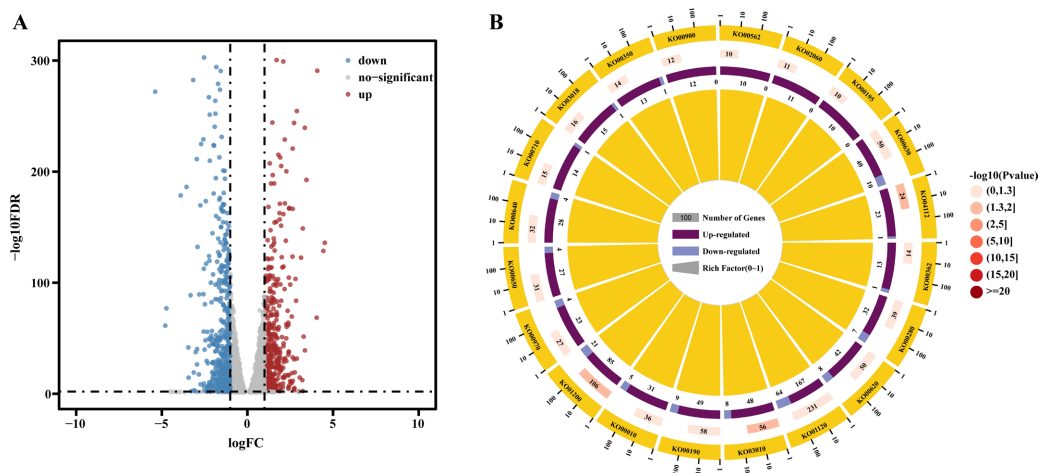
Note: “-” indicates no bacterial growth; “+” indicates few colonies; “++” indicates moderate colonies; “+++” indicates a large number of colonies.

### 3.2. Limonene Treatment Transcriptome Response in *Ralstonia solanacearum*

Using the Illumina high-throughput sequencing technology, a total of 2,722 genes were identified. The DESeq2 package was utilized to analyze differentially expressed genes (DEGs). The results indicated that the Limonene treatment significantly impacted gene expression in the leaves. After Limonene treatment, 465 genes were upregulated, and 536 genes were downregulated ( $p < 0.05$  and  $|\text{Log}_2(\text{Fold Change})| \geq 1$ ). After applying the Benjamini-Hochberg correction, these numbers were adjusted to 461 upregulated and 523 downregulated genes (**Figure 2(A)**).

As shown in **Figure 2(B)**, the DEGs were mapped to the KEGG database to analyze the metabolic pathways of the differential genes. In the comparison between Limonene and Control, 88 pathways were significantly enriched ( $p < 0.05$ ). After adjusting the  $p$ -values to false discovery rate.

(FDR) values using the Benjamini-Hochberg procedure, 26 metabolic pathways remained significantly enriched. In the comparison between Limonene and Control, the top three pathways were Terpenoid backbone biosynthesis, Phosphotransferase system, and Photosynthesis (**Figure 1(B)**).



**Figure 2.** Transcriptome Analysis of *R. solanacearum* Treated with Limonene. (A) Distribution of differentially expressed genes (DEGs); (B) KEGG enrichment analysis of DEGs.

### 3.3. Limonene Treatment-Induced Metabolic Response in *Ralstonia solanacearum*

Through the application of ultra-high-performance liquid chromatography coupled with a quadrupole-time-of-flight mass spectrometer (UHPLC-Q-TOF MS), a total of 5887 signals were acquired from the samples under different ionization modes, with 1167 signals successfully annotated against databases (BMDB/Decode/HMDB/KEGG/Masslist/mzCloud). Quality control samples demonstrated minimal variation across the experimental process, as evidenced by the overlay comparison of total ion current (TIC) profiles, where the response intensity and retention time of each chromatographic peak were largely overlapping, indicating negligible instrumental error. A Principal Component Analysis (PCA) model was derived from the amplified ion peaks extracted from all experimental samples (with six replicates each for blank control, negative control, and Limonene treatment groups) and quality control samples (four replicates per group) (**Figure 2(A)**). As shown in **Figure 3(A)**, the PCA results confirmed the stability of the analytical system used in this study, and the robustness and reliability of the collected data. The differences in metabolic profiles reflect the biological variations among the samples.

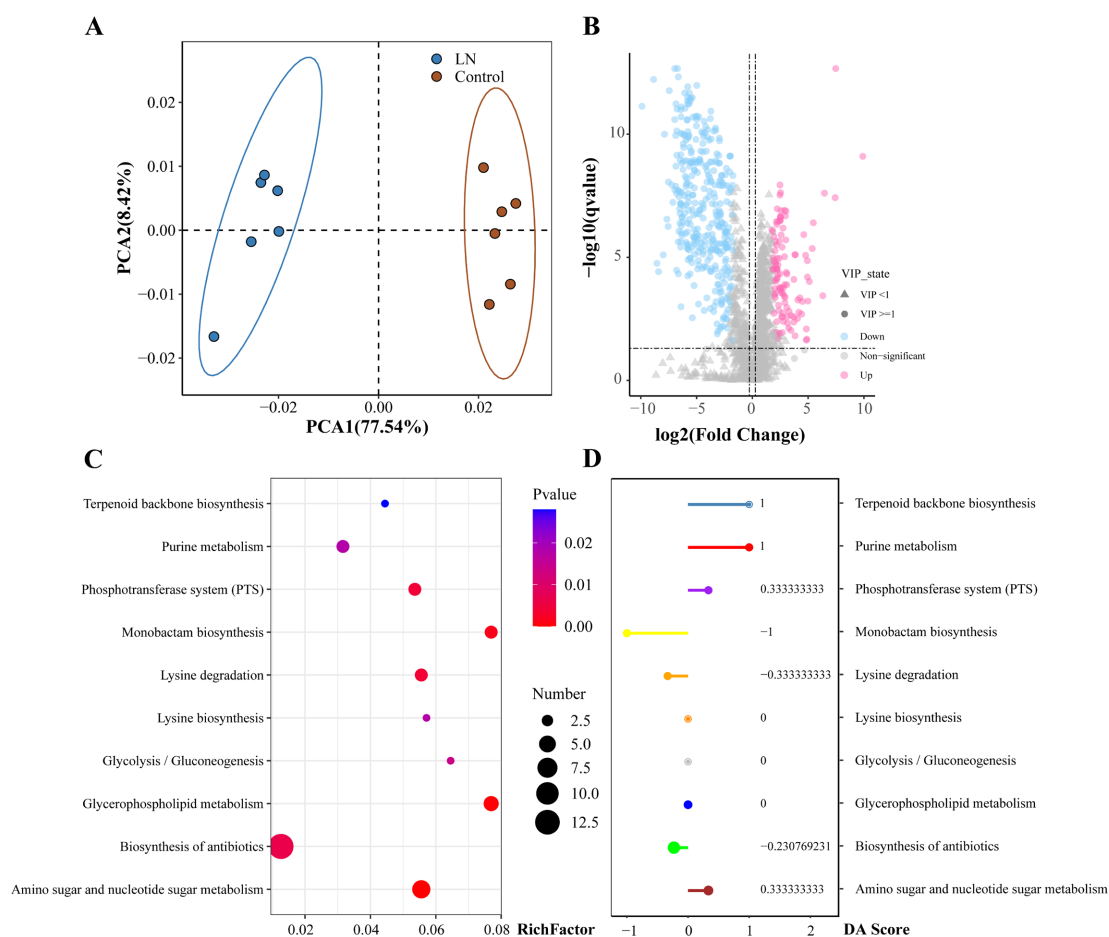
In the comparison between the Limonene treatment and the Control, 485 differentially accumulated metabolites (DAMs) were identified, with 117 upregulated and 368 downregulated (**Figure 3(B)**). KEGG pathway enrichment revealed that 56 metabolic pathways were enriched in the Limonene treatment compared to the Control, among which 16 pathways exhibited significant changes ( $p < 0.05$ ). The top five pathways were Amino sugar and nucleotide sugar metabolism, Glycerophospholipid metabolism, Metabolic pathways, Monobactam biosynthesis, and Lysine degradation (**Figure 3(C)**).

Ultimately, integrating the Differential Amplification scores, we identified three metabolic pathways in *R. solanacearum* that were significantly overexpressed following the Limonene treatment: Amino sugar and nucleotide sugar

metabolism (Differential Amplification = 0.33), Purine metabolism (Differential Amplification = 1), and Terpenoid backbone biosynthesis (Differential Amplification = 1) (**Figure 3(D)**).

### 3.4. Combined analysis of Transcriptome and Metabolome (DEGs/DAMs) Responding to Limonene Treatment in *Ralstonia solanacearum*

By assigning both differentially expressed genes (DEGs) and differentially accumulated metabolites (DAMs) to KEGG pathways ( $p < 0.05$ ), we aimed to understand the impact of Limonene treatment on *R. solanacearum*. The results indicate that DEGs and DAMs are concurrently involved in several metabolic pathways, including: Terpenoid backbone biosynthesis, Glycolysis/Gluconeogenesis, Phosphotransferase system (PTS), Inositol phosphate metabolism. These findings demonstrate the multifaceted influence of limonene on the metabolic processes of *R. solanacearum*, affecting both gene expression and metabolite accumulation. The overlap between DEGs and DAMs in these pathways suggests a coordinated response at the genetic and metabolic levels to limonene treatment.



**Figure 3.** Metabolomic analysis of *R. solanacearum* after limonene treatment. (A) PCA analysis; (B) Volcano plot of differential metabolites; (C) Rich factor values of enriched pathways; (D) DA values of enriched pathways.

## 4. Discussion

This study pioneers the integration of transcriptomics and metabolomics to analyze the response of *Ralstonia solanacearum* to limonene, deepening our understanding of how this bacterium reacts to secondary metabolite stress and revealing the regulatory effects of limonene at the genetic and metabolic levels.

Limonene, registered as an insecticide and bactericide in China, has been recognized for its potent properties. In 2018, Oro Agri International Limited registered D-limonene as an active insecticide (with a total active ingredient content of 92%, PD20184009) and a soluble bactericide/insecticide (with a total active ingredient content of 5%, PD20184008) for the control of various pests and pathogens, such as the citrus red mite (*Panonychus citri* McGregor), whitefly (*Bemisia tabaci* Gennadius), anthracnose (*Colletotrichum gloeosporioides* Penz.), lettuce and chicory downy mildew (*Erysiphe cichoracearum*), and grape powdery mildew (*Sphaerotheca fuliginea*). In 2021, Qingdao Riseon Crop Nutrition Co., Ltd. registered D-limonene as an insecticidal active ingredient (with a total active ingredient content of 93%, PD20211690), and in 2022, as a soluble insecticide (with a total active ingredient content of 18%, PD20220205), for the control of whiteflies. Limonene's strong penetrating and expanding properties enhance the permeability and absorption of traditional pesticides, making it a popular additive in China. Our study's findings of significant anti-*Ralstonia* activity provide a theoretical basis for the development and utilization of limonene in agricultural defense.

Our research indicates that limonene acts as a powerful antimicrobial agent, inhibiting the growth of *R. solanacearum*, disrupting biofilm formation, and affecting bacterial activity. Post-treatment with limonene, there were 461 upregulated and 523 downregulated genes, demonstrating limonene's antagonistic effect and its influence on the transcriptional and metabolic profiles of *R. solanacearum*.

Based on KEGG enrichment analysis, the pathway with the most accumulated metabolites was Amino sugar and nucleotide sugar metabolism. Amino sugars [21] [22] and nucleotide sugars [23] are common biomarkers in bacterial necrosis. The enrichment of this pathway further confirms that limonene treatment severely impacts the synthesis and repair of the bacterial cell membrane, disrupting its structure and promoting necrosis. Terpene synthesis is closely linked to bacterial growth [24] and serves as precursors for many signaling molecules [25]. Our multi-omics analysis confirms that limonene primarily affects the Terpenoid backbone biosynthesis, regulating the growth and development of *R. solanacearum*.

Biofilms play a crucial role in bacterial growth and quorum sensing [26] [27], and pathogens form biofilms to enhance resistance to antimicrobial agents [28]. The enzymatic degradation of biofilms is an efficient and environmentally friendly approach [29]. Our study suggests that limonene may affect the synthesis of terpenoids in *R. solanacearum*, increasing their accumulation in the bio-

film and reducing membrane permeability [30], leading to the dispersion of the biofilm community [31], providing strong evidence for the use of limonene in the control of *R. solanacearum*.

In this study, we demonstrate the functional role of limonene as an effective natural metabolite against *R. solanacearum*, suggesting its potential use in reducing the incidence of bacterial wilt. Limonene not only inhibits the growth activity of *R. solanacearum* but also affects its group movement and the integrity of biofilms, which are key factors for colonization at plant wound sites, indicating a strong antagonistic characteristic of limonene. Furthermore, limonene regulates the gene and metabolic expression network of *R. solanacearum*, disrupting its normal physiological activities. Finally, limonene modulates the Terpenoid backbone biosynthesis pathway in *R. solanacearum*, a molecular mechanism that corroborates the effects of limonene on chemotaxis and biofilm formation, further validating our conclusions. This research provides solid evidence for the development of limonene as a novel plant immune inducer.

## 5. Conclusions

In summary, this study reports for the first time the anti-*Ralstonia solanacearum* activity of limonene. With a minimum inhibitory concentration (MIC) of only 10  $\mu\text{L/mL}$  and a bactericidal concentration (MBC) of 20  $\mu\text{L/mL}$ , limonene demonstrates potent antibacterial properties. Transcriptomic and metabolomic analyses suggest that limonene's activity may be attributed to its ability to alter metabolic pathways such as Amino sugar and nucleotide sugar metabolism and Terpenoid backbone biosynthesis in *R. solanacearum*, disrupting membrane integrity and modifying biofilm permeability, thereby exerting a strong bacteriostatic effect.

As a natural active substance, limonene is cost-effective and readily available, which implies a high value for development and utilization. Beyond its use as an insecticide, limonene holds promise as a novel inhibitor for the plant soil-borne disease, bacterial wilt. This research not only enriches our understanding of the mechanisms by which natural products combat bacterial pathogens but also opens up new avenues for the application of limonene in agriculture, particularly in the management of diseases caused by *R. solanacearum*.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

## References

- [1] Archana, T., Kamalakannan, A., Gopalakrishnan, C., Johnson, I., Rajendran, L., Varanavasiappan, S., *et al.* (2024) Detection of Indian Isolates of Soil and Tuber Borne *Ralstonia solanacearum* (Smith) Infecting Potato (*Solanum tuberosum* L.) through a Colorimetric LAMP Assay. <https://doi.org/10.1007/s11540-024-09699-z>
- [2] Buddenhagen, I. and Kelman, A. (1964) Biological and Physiological Aspects of Bacterial Wilt Caused by *Pseudomonas solanacearum*. *Annual Review of Phytopathology*, **2**, 203-230. <https://doi.org/10.1146/annurev.py.02.090164.001223>
- [3] Chen, D., Kamran, M., Chen, S., Xing, J., Qu, Z., Liu, C., *et al.* (2023) Two Nucleotide Sugar Transporters Are Important for Cell Wall Integrity and Full Virulence of *Magnaporthe oryzae*. *Molecular Plant Pathology*, **24**, 374-390. <https://doi.org/10.1111/mpp.13304>
- [4] Ciofu, O., Moser, C., Jensen, P.Ø. and Høiby, N. (2022) Tolerance and Resistance of Microbial Biofilms. *Nature Reviews Microbiology*, **20**, 621-635. <https://doi.org/10.1038/s41579-022-00682-4>
- [5] de Groot, A. (2019) Limonene Hydroperoxides. *Dermatitis*, **30**, 331-335. <https://doi.org/10.1097/der.0000000000000465>
- [6] de Souza, M.A., da Silva, L., Macêdo, M.J.F., Lacerda-Neto, L.J., dos Santos, M.A.C., Coutinho, H.D.M., *et al.* (2019) Adulticide and Repellent Activity of Essential Oils against *Aedes aegypti* (Diptera: Culicidae)—A Review. *South African Journal of Botany*, **124**, 160-165. <https://doi.org/10.1016/j.sajb.2019.05.007>
- [7] Dias, A.L.B., Sousa, W.C., Batista, H.R.F., Alves, C.C.F., Souchie, E.L., Silva, F.G., *et al.* (2020) Chemical Composition and *in Vitro* Inhibitory Effects of Essential Oils from Fruit Peel of Three Citrus Species and Limonene on Mycelial Growth of *Sclerotinia sclerotiorum*. *Brazilian Journal of Biology*, **80**, 460-464. <https://doi.org/10.1590/1519-6984.216848>
- [8] Eddin, L.B., Jha, N.K., Meeran, M.F.N., Kesari, K.K., Beiram, R. and Ojha, S. (2021) Neuroprotective Potential of Limonene and Limonene Containing Natural Products. *Molecules*, **26**, Article 4535. <https://doi.org/10.3390/molecules26154535>
- [9] Fierascu, R.C., Fierascu, I.C., Dinu-Pirvu, C.E., Fierascu, I. and Paunescu, A. (2019) The Application of Essential Oils as a Next-Generation of Pesticides: Recent Developments and Future Perspectives. *Zeitschrift für Naturforschung C*, **75**, 183-204. <https://doi.org/10.1515/znc-2019-0160>
- [10] García, R.O., Kerns, J.P. and Thiessen, L. (2019) *Ralstonia solanacearum* Species Complex: A Quick Diagnostic Guide. *Plant Health Progress*, **20**, 7-13. <https://doi.org/10.1094/php-04-18-0015-dg>
- [11] Gettys, L.A., Thayer, K.L. and Sigmon, J.W. (2021) Evaluating the Effects of Acetic Acid and D-Limonene on Four Aquatic Plants. *HortTechnology*, **31**, 225-233. <https://doi.org/10.21273/horttech04769-20>

- [12] Han, L., Zhang, G., Miao, G., Zhang, X. and Feng, J. (2015) *Streptomyces kanasensis* Sp. Nov., an Antiviral Glycoprotein Producing Actinomycete Isolated from Forest Soil around Kanas Lake of China. *Current Microbiology*, **71**, 627-631. <https://doi.org/10.1007/s00284-015-0900-0>
- [13] Hayward, A.C. (1991) Biology and Epidemiology of Bacterial Wilt Caused by *Pseudomonas solanacearum*. *Annual Review of Phytopathology*, **29**, 65-87. <https://doi.org/10.1146/annurev.py.29.090191.000433>
- [14] Jian, R., Lin, Y., Li, Y., Wu, W., Ren, X., Liang, Z., *et al.* (2022) Larvicidal Activity of Two *Rutaceae* Plant Essential Oils and Their Constituents against *Aedes albopictus* (Diptera: Culicidae) in Multiple Formulations. *Journal of Medical Entomology*, **59**, 1669-1677. <https://doi.org/10.1093/jme/tjac083>
- [15] Joergensen, R.G. (2018) Amino Sugars as Specific Indices for Fungal and Bacterial Residues in Soil. *Biology and Fertility of Soils*, **54**, 559-568. <https://doi.org/10.1007/s00374-018-1288-3>
- [16] Karim, Z., Hossain, M. and Begum, M. (2018) *Ralstonia solanacearum*: A Threat to Potato Production in Bangladesh. *Fundamental and Applied Agriculture*, **3**, 407-421. <https://doi.org/10.5455/faa.280361>
- [17] Kong, H.G., Bae, J.Y., Lee, H.J., Joo, H.J., Jung, E.J., Chung, E., *et al.* (2014) Induction of the Viable but Nonculturable State of *Ralstonia solanacearum* by Low Temperature in the Soil Microcosm and Its Resuscitation by Catalase. *PLOS ONE*, **9**, e109792. <https://doi.org/10.1371/journal.pone.0109792>
- [18] Koo, H., Allan, R.N., Howlin, R.P., Stoodley, P. and Hall-Stoodley, L. (2017) Targeting Microbial Biofilms: Current and Prospective Therapeutic Strategies. *Nature Reviews Microbiology*, **15**, 740-755. <https://doi.org/10.1038/nrmicro.2017.99>
- [19] Kurabachew, H. and Ayana, G. (2017) Bacterial Wilt Caused by *Ralstonia solanacearum* in Ethiopia: Status and Management Approaches: A Review. *International Journal of Phytopathology*, **5**, 107-119. <https://doi.org/10.33687/phytopath.005.03.1829>
- [20] Liang, C., Lv, H., Liu, W., Wang, Q., Yao, X., Li, X., *et al.* (2024) Mechanism of the Adverse Outcome of *Chlorella Vulgaris* Exposure to Diethyl Phthalate: Water Environmental Health Reflected by Primary Producer Toxicity. *Science of the Total Environment*, **912**, Article 168876. <https://doi.org/10.1016/j.scitotenv.2023.168876>
- [21] Liaqat, I., Riaz, N., Saleem, Q., Tahir, H.M., Arshad, M. and Arshad, N. (2018) Toxicological Evaluation of Essential Oils from Some Plants of Rutaceae Family. *Evidence-Based Complementary and Alternative Medicine*, **2018**, Article ID: 4394687. <https://doi.org/10.1155/2018/4394687>
- [22] Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., *et al.* (2012) Top 10 Plant Pathogenic Bacteria in Molecular Plant Pathology. *Molecular Plant Pathology*, **13**, 614-629. <https://doi.org/10.1111/j.1364-3703.2012.00804.x>
- [23] Mendoza-Poudereux, I., Kutzner, E., Huber, C., Segura, J., Eisenreich, W. and Arrillaga, I. (2015) Metabolic Cross-Talk between Pathways of Terpenoid Backbone Biosynthesis in Spike Lavender. *Plant Physiology and Biochemistry*, **95**, 113-120. <https://doi.org/10.1016/j.plaphy.2015.07.029>
- [24] Parsons, J.W. (1981) Chemistry and Distribution of Amino Sugars in Soils and Soil Organisms. CRC Press.
- [25] Patil, V.U., Gopal, J. and Singh, B.P. (2012) Improvement for Bacterial Wilt Resistance in Potato by Conventional and Biotechnological Approaches. *Agricultural Research*, **1**, 299-316. <https://doi.org/10.1007/s40003-012-0034-6>
- [26] Rinaldi, M.A., Ferraz, C.A. and Scrutton, N.S. (2022) Alternative Metabolic Path-

- ways and Strategies to High-Titre Terpenoid Production in *Escherichia coli*. *Natural Product Reports*, **39**, 90-118. <https://doi.org/10.1039/d1np00025j>
- [27] Tsumori, C., Matsuo, S., Murai, Y. and Kai, K. (2023) Quorum Sensing-Dependent Invasion of *Ralstonia solanacearum* into *Fusarium oxysporum* Chlamydospores. *Microbiology Spectrum*, **11**, e00036-23. <https://doi.org/10.1128/spectrum.00036-23>
- [28] van Elsas, J.D., Kastelein, P., van Bekkum, P., van der Wolf, J.M., de Vries, P.M. and van Overbeek, L.S. (2000) Survival of *Ralstonia solanacearum* Biovar 2, the Causative Agent of Potato Brown Rot, in Field and Microcosm Soils in Temperate Climates. *Phytopathology*, **90**, 1358-1366. <https://doi.org/10.1094/phyto.2000.90.12.1358>
- [29] Wang, D., Qiu, D., Shi, L., Pan, H., Li, Y., Sun, J., *et al.* (2015) Identification of Insecticidal Constituents of the Essential Oils of *Dahlia pinnata* Cav. against *Sitophilus zeamais* and *Sitophilus oryzae*. *Natural Product Research*, **29**, 1748-1751. <https://doi.org/10.1080/14786419.2014.998218>
- [30] Yang, L., Wang, R., Lin, W., Li, B., Jin, T., Weng, Q., *et al.* (2024) Efficacy of 2,4-Di-*tert*-Butylphenol in Reducing *Ralstonia solanacearum* Virulence: Insights into the Underlying Mechanisms. *ACS Omega*, **9**, 4647-4655. <https://doi.org/10.1021/acsomega.3c07887>
- [31] Yuan, X., Xiong, J., Wu, X., Ta, N., Liu, S., Li, Z., *et al.* (2024) Ultrasmall Ce-Based Metal-Organic Frameworks Nanozyme with Hydrolytic Activity for Boosting Antibiofilm Therapy. *Chemical Engineering Journal*, **480**, Article 148246. <https://doi.org/10.1016/j.cej.2023.148246>