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# Anti-TMV activity and mode of action of perillaldehyde in perilla essential oil

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**Abstract:** Perilla essential oil (PEO) is reported as an aromatic yellowish oily substance with a volatile odor extracted from perilla leaves. It exhibits various biological activities except anti-tobacco mosaic virus (TMV) activity. In this study, we investigated the main components and anti-TMV activity of PEO, identified its primary active components, and examined its mode of action. The results indicated that PEO exhibited anti-TMV activity (65.58%) at 800 μg/mL, with perillaldehyde identified as the main active component. The protective, curative, and inactivation activities of perillaldehyde at 800 μg/mL were 80.41%, 73.42%, and 34.93%, respectively. These values were significantly higher than those of the control drug (commercial chitosan oligosaccharide) and the protective and curative activities were superior to those of ningnanmycin. The results of the mode of action showed that perillaldehyde induced a hypersensitive response (HR) in tobacco. Transmission electron microscope (TEM) observation revealed that perillaldehyde had no direct effect on TMV particles. The treatment of *Nicotiana glutinosa* with perillaldehyde at 800 μg/mL indicated that perillaldehyde had significant induction activity (58.46%). The expression of three pathogenesis-related tobacco genes (*PR* genes), including nonexpressor of pathogenesis-related genes 1 (*NPR1*), pathogenesis-related protein 1 gene (*PR1*), and pathogenesis-related protein 5 gene (*PR5*), were induced and upregulated by perillaldehyde treatment. Perillaldehyde also induced the overexpression of the phenylalanine ammonia-lyase gene (*PAL*), respiratory burst oxidase homolog B gene (*RBOHB*), and protochlorophyllide oxidoreductase gene 1 (*POR1*). Furthermore, perillaldehyde increased the salicylic acid (SA) and $H_2O_2$ contents in tobacco leaves, and enhanced the activities of four defense enzymes: superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and phenylalanine ammonia-lyase (PAL). *N. glutinosa* was treated with perillaldehyde at 800 μg/mL for 24 h, and the results showed that the highest SA and $H_2O_2$ contents (1032.08 pmol/L and 23.40 μmol/g FW, respectively) were obtained in tobacco leaves. Defense enzyme activities also reached a maximum at 800 μg/mL, and the activities of CAT, PAL, POD, and SOD increased by 1.76, 1.95, 2.17, and 3.78 times, respectively, compared to the control. The results of the study showed that perillaldehyde may enhance resistance to pathogen infection by inducing systemic acquired resistance (SAR), which may contribute to the activation of SA signal transduction pathway. Therefore, perillaldehyde has the potential for application in agriculture as a novel antiviral agent and immune inducer.

**Keywords:** perilla essential oil; perillaldehyde; tobacco mosaic virus (TMV); induced resistance; systemic acquired resistance (SAR); plant immunity

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The authors declare that they have no competing interests.

# 紫苏精油中紫苏醛的抗 TMV 活性及作用方式

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**摘 要：**紫苏精油 (PEO) 是从紫苏叶中提取的一种具有挥发性芳香气味的淡黄色油性物质，具有多种生物活性，但关于其抗烟草花叶病毒 (TMV) 活性尚未见报道。本研究测定了紫苏精油及其主要成分的抗 TMV 活性，明确了其主要活性成分，并在此基础上进行了作用方式研究。结果表明：PEO 在 800 μg/mL 剂量下对 TMV 的活性高达 65.58%，其主要活性成分紫苏醛在该剂量下的保护、治疗和钝化活性分别为 80.41%、73.42% 和 34.93%，均显著高于对照药剂商品化药物壳寡糖，而其保护和治疗活性优于宁南霉素。作用方式研究结果表明：紫苏醛诱导了烟草的过敏反应 (HR)，透射电镜 (TEM) 观察显示紫苏醛对 TMV 粒子没有直接作用。紫苏醛在 800 μg/mL 剂量下处理心叶烟，具有显著的诱导抗病活性，为 58.46%。紫苏醛处理诱导了烟草病程相关基因非表达子 1 (*NPR1*)、病程相关蛋白 1 基因 (*PR1*) 和病程相关蛋白 5 基因 (*PR5*) 3 个致病相关基因 (*PR* 基因) 表达上调，也诱导了苯丙氨酸解氨酶基因 (*PAL*)、呼吸爆发氧化酶 B 基因 (*RBOHB*) 和原叶绿素酸酯氧化还原酶基因 1 (*POR1*) 过表达。此外，紫苏醛提高了烟草叶片中水杨酸 (SA) 和 $H_2O_2$ 含量，增强了超氧化物歧化酶 (SOD)、过氧化氢酶 (CAT)、过氧化物酶 (POD) 和苯丙氨酸解氨酶 (PAL) 4 种防御酶的活性。紫苏醛在 800 μg/mL 剂量下处理心叶烟 24 h, 结果表明：烟草叶片中的 SA 和 $H_2O_2$ 含量最高，分别为 1032.08 pmol/L 和 23.40 μmol/g FW，防御酶活性也达到最大值，CAT、PAL、POD 和 SOD 活性分别比对照提高了 1.76、1.95、2.17 和 3.78 倍。该研究结果表明，紫苏醛可能通过诱导系统性获得抗性 (SAR) 来增强植物对病原体感染的抵抗力，这可能是通过 SA 信号转导途径介导的。因此，紫苏醛作为一种新型抗病毒药物和免疫诱导剂在农业上具有广阔的应用前景。

**关键词:** 紫苏精油； 紫苏醛； 烟草花叶病毒 (TMV)； 诱导抗性； 系统性获得抗性 (SAR)； 植物免疫

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## 0 Introduction

Plant viruses, also known as "plant cancer"[1], can spread using the internal mechanisms of the host plants as well as through living organisms and abiotic factors, thus seriously threatening the growth and yield of crops and resulting in huge economic losses in agriculture[2]. The tobacco mosaic virus (TMV) belongs to a rod-shaped virus with a length of 300 nm and a diameter of 18 nm and seriously harms crops[3]. TMV can infect more than 885 plants among 65 families[4]. Many important cash crops such as tobacco, tomato, pepper, and potato can be infected by TMV, leading to a decreased quality and yield[5]. Traditional chemical pesticides are used to control viral diseases by reducing the number of vectors; however, this requires large amounts of pesticides and cannot eliminate the virus because it is not directly affected[6-7]. The long-term and large-scale use of traditional chemical pesticides has brought serious problems to humans and the environment including poisoning, pesticide residues, drug resistance, and water and soil pollution[8-11]. Considering the disadvantages of traditional chemical pesticides, biopesticides are gaining more attention because of their low-residue, high-specificity, and low-resistance characteristics[12-13]. Research on new substances to combat plant viruses has made great progress and various active ingredients have been discovered, including proteins, polysaccharides, alkaloids, flavonoids, phenols, and essential oils[4]。

Perilla is an annual herb native to India and China that belongs to the Lamiaceae/Labiatae family[14]. The seeds and leaves of perilla are edible. Perilla essential oil (PEO) is an oily, aromatic substance extracted from perilla leaves, and its composition is

complex and diverse, comprising 150-200 compounds[15]. The major compounds in PEO are perillaldehyde, perilla ketone, and *β*-caryophyllene[16]. PEO exhibits various biological activities, including insecticidal, antibacterial, anticancer, antioxidant, and anti-inflammatory effects[17]. Perillaldehyde is a monocyclic terpene compound with an aromatic smell[18-19]. Perillaldehyde exhibits various bioactivities such as lipid-lowering, anti-inflammatory, antioxidant, and anticancer effects[20-23], as well as antifungal and insecticidal activities. Perillaldehyde has been reported to induce the apoptosis of *Aspergillus flavus*[24], inhibit the hyphal growth of *Botrytis cinerea*[25], and exhibit insecticidal activities against *Sitophilus zeamais*, *Bactrocera dorsalis*, and *Lasioderma serricorne*[26-28]. Most previous studies focused on the antibacterial and insecticidal activity of perillaldehyde, but there were few studies on antiviral activity. In this study, we examined the anti-TMV activity of PEO, identified its main active components, and conducted a study on the mode of action on this basis. The discovery of this study contributes to the utilization of perillaldehyde in agriculture as a candidate compound or lead compound for the development of novel antiviral drugs and immune inducers.

## 1 Materials and methods

### 1.1 Tested materials

**1.1.1 Pathogens and Plants** Plant pathogens: tobacco mosaic virus (TMV), *Sclerotiia sclerotiorum*, *Rhizoctonia solani*, and *Colletotrichum lagenarium* were obtained from the College of Plant Protection, Northwest A&F University. TMV virion solution at 16 mg/mL was stored at −80 ℃ and diluted to 20 μg/mL and 10 μg/mL before use. Three fungal pathogens were stored at −80 ℃ in glycerol, then activated twice before being cultured on a PDA medium.

Plants: *Nicotiana glutinosa*, *Nicotiana benthamiana*, *Brassica napus*, *Vicia faba*, and *Cucumis sativus* were obtained from the College of Plant Protection, Northwest A&F University. The plants were cultivated in a greenhouse at 25 ℃ for 14 h of light and 20 ℃ for 10 h of darkness.

**1.1.2 Experimental instruments and reagents** Gas chromatograph-mass spectrometer (GC-MS, Agilent Technologies Co., Ltd., State of California, USA); Transmission electron microscope (TEM, Thermo Fisher Scientific, Massachusetts, USA); Microplate reader (Tecan Trading Co., Ltd., Shanghai, China); QuantStudioTM 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA).

Perillaldehyde (purity ⩾ 90%), *β*-caryophyllene (purity ⩾ 80%), farnesene (purity ⩾ 95%), caryophyllene oxide and chitosan oligosaccharide were obtained from Macklin® (Shanghai, China), and stored at 4 ℃; Ningnanmycin was obtained from Deqiang Biological Co., Ltd. (Heilongjiang, China), and stored at room temperature. The kits for detecting salicylic acid (SA) and $H_2O_2$ contents (Beijing Solaibao®, Beijing, China); The kits for detecting peroxidase (POD), phenylalanine ammonia-lyase (PAL), superoxide dismutase (SOD), and catalase (CAT) activities (Jiangsu Meimian Industrial Co., Ltd, Jiangsu, China); Quick RNA isolation kit (Tiangen Biotech Co., Ltd., Beijing, China); HiScript II 1st strand cDNA synthesis kit (Vazyme Biotech Co., Ltd, Nanjing, China); 2 × SYBR Green qPCR Mix (Coolaber, Beijing, China).

### 1.2 Preparation of PEO and GC-MS analysis

PEO was prepared by hydrodistillation as previously described[16]. The air-dried perilla leaves were ground into fine powder. Distilled water (500 mL) was added into a 1 L round-bottomed flask. Then, the powder of dried leaves (10 g) was accurately weighed into the flask. A Clevenger-type apparatus was used to obtain essential oil by hydrodistillation for 3 h. The PEO obtained was dried with $Na_2SO_4$ and stored at 4 ℃ for use.

The PEO components were analyzed by GC-MS and an Elite-5MS capillary column (30 mm × 0.25 mm, 0.25 μm) was used for the GC analysis. Chromatographic separation using temperature-programmed desorption method: the temperature of the column oven increased from 40 ℃ to 220 ℃ at a rate of 5 ℃ per minute, and was maintained for 20 min. The injector temperature was 280 ℃, and nitrogen was used as the carrier gas (1 mL/min). PEO (10 μL) was diluted in 1000 μL of hexane and the sample size was 1 μL. The split ratio was 1:20. For MS analysis, the transmission line temperature was 280 ℃, the acquisition mass range was 30-500 *m/z*, and the ionization energy was 70 eV. The quantitative analysis of PEO components was performed using the peak area normalization method. The content of each component was estimated according to the corresponding peak area percentage.

### 1.3 Anti-TMV activity analysis

*N. glutinosa* at the 5-leaf stage was used to evaluate the anti-TMV activity of PEO and its main components (perillaldehyde, farnesene, *β*-caryophyllene, and caryophyllene oxide) for treatment, protection, and inactivation. Tested solutions were prepared at concentrations of 50, 200, and 800 μg/mL with sterile water. The protective activity was evaluated by spraying 3 mL of the tested solutions on three leaves of *N. glutinosa*. After 24 h, 10 μg/mL of TMV was inoculated by rubbing (20 μL/leaf). The tested leaves showed typical necrosis symptoms 3-4 days after inoculation, and the local lesion numbers were recorded to calculate the inhibition rate. The curative activity was evaluated by inoculating 10 μg/mL of TMV by rubbing. After 24 h, the tested solutions were spryed on the three leaves of *N. glutinosa*. The local lesions were counted 3-4 days after inoculation to determine the inhibition rate. The inactivating effect was determined using the half-leaf method as previously reported[29]. The TMV solution was mixed with the same volume of tested solutions for 30 min to inhibit the virus. The mixed solution (20 μL) was then inoculated on the left side of the tobacco leaves, while the mixture of distilled water and virus was inoculated on the right side of the leaves as a control. The numbers of local lesions on the left and right sides of the leaves were recorded to calculate the inhibition rate 3-4 days after inoculation. For each experiment, three functional leaves of *N. glutinosa* were selected as the tested objects. The positive controls used were chitosan oligosaccharide and ningnanmycin. The blank control consisted of sterile water. The experiments were conducted in triplicate to ensure the reliability of the results. The inhibition rates were calculated using the formula (1):

$$I/\% = [(C - T)/C] \times 100 \quad (1)$$

Where $I$ is inhibition rate, $C$ is the average number of local lesions of the control and $T$ is the average number of local lesions of the treatment.

### 1.4 TEM observation

Morphological observation of TMV particles treated with perillaldehyde was performed under a TEM. TMV (20 μg/mL) was combined with the same volume of perillaldehyde solution (800 μg/mL) and left to stand at 25 ℃ for 1 h. TMV and distilled water were mixed to be used as the control. Negative staining of TMV was performed as follows: the copper mesh (230 mesh) was removed from the sample box with tweezers, placed on filter paper, and a small drop of the sample was absorbed by a pipette (10 μL) and poured on the copper mesh. A phosphotungstic acid solution with a concentration of 2.00% (*W/V*) was prepared. After the sample was dried, an appropriate amount of staining solution was added to the same position of the copper mesh. After 1-2 min, the excess staining solution was absorbed using filter paper, and the mesh was slowly rinsed with double-distilled water ($ddH_2O$) three times.

### 1.5 Induced hypersensitive response in *N. glutinosa* leaves

With sterile water as the control, perillaldehyde solutions were prepared at concentrations of 1600, 800, 400, 200, 100, 50, and 25 μg/mL as tested solutions. Typically, 50 μL of each tested solution was injected into the backside of a functional tobacco leave to cover an area of 1 $cm^2$, using a sterile syringe without a needle. After 2 days, local lesions were observed at the injection site.

### 1.6 Induction of resistance to TMV by perillaldehyde

*N. glutinosa* at the 5-6 leaf stage were selected as the experimental subjects to assess the induced resistance effect of perillaldehyde. Perillaldehyde solutions (800, 200, and 50 μg/mL) were smeared on the lower three functional leaves of experimental objects, and the upper three leaves were inoculated with 10 μg/mL of TMV by rubbing after 24 h (20 μL/piece). Three days after inoculation, the local lesion numbers at the inoculation site were recorded and the inhibition rates were calculated using the formula (1). Each treatment had three replicates. Chitosan oligosaccharide served as a positive control agent, and the blank control was sterile water.

### 1.7 Measurement of salicylic acid (SA) and $H_2O_2$ accumulation

*N. glutinosa* at the 3-leaf stage were selected as the experimental object. Perillaldehyde solutions (50, 200, 800 μg/mL) were evenly applied to the surface of the tobacco leaves (3 mL/plant), and samples were taken 24 h later. To measure SA content, a 0.1 g tobacco leaf sample was accurately weighed and added to 1 mL of phosphate buffer saline (PBS) for

ice bath homogenization. To measure $H_2O_2$ content, 0.1 g tobacco leaf sample, and 1 mL acetone were added into a pre-cooled mortar for ice bath homogenization. Then the homogenization was centrifuged at 8000 *g* for 10 min at 4 ℃, and the collected supernatant was stored at −80 ℃ for further experiments. The $H_2O_2$ and SA contents were determined according to the manufacturer's instructions for the respective determination kits. The absorbances (OD value) were measured at 415 nm and 450 nm, respectively, using a microplate reader. The experiments were conducted in triplicate to ensure the reliability of the results.

### 1.8 Analysis of defense enzyme activities

The experimental treatment and experimental objects were the same as in Section 1.7. Tobacco leaf tissues were collected 24 h post-spraying and analyzed for defense enzyme activities. Tissue samples (0.1 g) were accurately weighed into a pre-cooled mortar with 1 mL of extracting solution for ice bath homogenate and centrifuged at 8000 *g* for 10 min at 4 ℃. The homogenate was stratified after centrifugation, and the supernatant was stored at −80 ℃. PAL, POD, SOD, and CAT activities were determined by measuring their absorbances, following the manufacturer's instructions for the corresponding reagent kits.

### 1.9 RT-qPCR analysis

Gene expression in *N. benthamiana* was analyzed using RT-qPCR on a QuantStudio™ 6 Flex Real-Time PCR System. *N. benthamiana* at the 3-leaf stage were treated with perillaldehyde (50, 200, and 800 μg/mL) (a pot of tobacco plant was sprayed with 3 mL of perillaldehyde). Leaf samples were collected after 24 h and then ground with liquid nitrogen in a pre-cooled mortar. Total RNA was extracted from the tobacco leaves using a Quick RNA isolation kit. All RNA samples were processed to remove genomic DNA contaminants. cDNA was synthesized with a HiScript II 1st strand cDNA synthesis kit. An amount of 1 μg of total RNA, 2 μL of HiScript II Enzyme Mix, 1 μL of Oligo (dT) 23VN, 10 μL of 2 × RT Mix, and RNase-free $ddH_2O$ formed a total reaction system of 20 μL. The obtained cDNA was stored at −20 ℃. The total volume of RT-qPCR reaction was 20 μL: 10 μL of 2 × SYBR Green qPCR Mix, 1.6 μL of primers (the forward primer and reverse primer were 0.8 μL each, at 10 μmol/L), 1.5 μL of cDNA, and 6.9 μL of $ddH_2O$. RT-qPCR was performed by applying the three-step method. The program included a preheating step at 95 ℃ for 5 min, followed by 40 cycles at 95 ℃ for 10 s, 55 ℃ for 20 s, and 72 ℃ for 20 s. Melting curve analysis was performed after the execution of the RT-qPCR program. The comparative threshold cycle method $2^{-\Delta\Delta C_t}$ was used to calculate the relative expression levels[30]. Each gene assay included three biological replicates, and the specific primers are listed in Table 1.

**Table 1 Primer information of defense-related genes in *N. benthamiana***

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *NPR1* | 5′-ACATCAGCGGAAGCAGTAG-3′ | 5′-GTCGGCGAAGTAGTCAAAC-3′ |
| *PR1* | 5′-CCTCGTACATTCTCATGGTCAAT-3′ | 5′-CCATTGTTACACTGAACCCTAGC-3′ |
| *PR5* | 5′-CCGAGGTAATTGTGAGACTGGAG-3′ | 5′-CCTGATTGGGTTGATTAAGTGCA-3′ |
| *RBOHB* | 5′-TTTTCTCTGAGGTTTGCCAGCCACCA-3′ | 5′-GCCTTCATGTTGTTGACAATGTCTTT-3′ |
| *POR1* | 5′-GGACCCACAAGCTATTCCCA-3′ | 5′-AACCAGTGCAACAGTCTCCG-3′ |
| *PAL* | 5′-GTTATGCTCTTAGAACGTCGCCC-3′ | 5′-CCGTGTAATGCCTTGTTTCTTGA-3′ |
| *β-actin* | 5′-TATGGAAACATTGTGCTCAGTGG-3′ | 5′-CCAGATTCGTCATACTCTGCC-3′ |

### 1.10 Persistence of anti-TMV activity

A perillaldehyde solution at 800 μg/mL was the tested solution. *N. glutinosa* at the 5-6 leaf stage, were used. A volume of 3 mL of the tested solution was sprayed on the lower three leaves, while the upper three leaves were inoculated with 10 μg/mL of TMV by rubbing at different time intervals (20 μL/leaf), including 1, 3, 5, 7, 9, and 11 days. Tobacco leaves showed obvious necrotic spots 3-4 days after inoculation with the virus. The local lesion numbers on tested leaves were determined and inhibition rates were calculated using the formula (1). Three biological repetitions were performed for each treatment.

### 1.11 Induction of resistance to fungal pathogens

The antifungal activity induced by perillaldehyde was determined against *S. sclerotiorum*, *R. solani*, and *C.*

*lagenarium*. The perillaldehyde solution (50, 200, and 800 μg/mL) was applied on the lower three leaves of healthy plants, and the corresponding pathogens were inoculated on the upper untreated three leaves 24 h after pre-spraying. *B. napus* and *V. faba* were inoculated with *S. sclero*tiorum and *R. solani* agar discs (5 mm/leaf), respectively, and *C. sativus* was inoculated with a spore suspension of *C. lagenarium* (20 μL/spot). Five plants were used for each treatment. The positive control was chitosan oligosaccharide with 200 μg/mL, and the blank control was sterile water. The disease incidence was evaluated 5 days after inoculation. Disease grading standards (Table 2) were used to calculate disease indexes by formula (2) and efficacies were calculated according to the formula (3):

$$DI = \sum(S \times n)/(N \times S_{\max}) \times 100 \quad (2)$$

Where $DI$ is disease index, $S$ is score, $n$ is numer of disease leaves, $N$ is total number of leaves investigated, and $S_{\max}$ is maximum disease score.

$$E/\% = [1 - DI_T/DI_c] \times 100 \quad (3)$$

Where $E$ is efficacy, $DI_T$ is disease index of the treatment, $DI_C$ is disease index of the control.

**Table 2 Grading standard of leaf diseases[31]**

| Grade | Grading standard |
|---|---|
| 0 | Leaves without diseased spots |
| 1 | The area of leaf lesions accounted for less than 1/100 of the total leaf area |
| 2 | The area of leaf lesions accounted for 1/50-1/20 of the total leaf area |
| 3 | The area of leaf lesions accounted for 3/50-1/5 of the total leaf area |
| 4 | The area of leaf lesions accounted for 1/5-2/5 of the total leaf area |
| 5 | The area of leaf lesions accounted for more than 2/5 of the total leaf area |

### 1.12 Data analysis

The data were analyzed by SPSS 26.0. Mean values were compared using Fisher's least significant difference (LSD) test at a significance level of $\alpha$ = 0.05 via SPSS 26.

## 2 Results and analysis

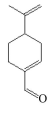
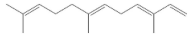
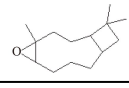
### 2.1 GC-MS analysis

GC-MS analysis revealed that the main components of PEO were perillaldehyde, *β*-caryophyllene, farnesene, and caryophyllene oxide. *β*-caryophyllene was the predominant compound (40.89%), followed by caryophyllene oxide, perillaldehyde, and farnesene, respectively (Table 3). Fig. 1 displays the GC-MS spectrum of PEO.

### 2.2 Anti-TMV activity analysis

The antiviral activity of PEO and its major compo-

**Table 3 The main volatile components (content greater than 5%) of PEO**

| No. | Retention time/min | Peak area percentage/% | Relative molecular mass | Compound name | Structure formula |
|---|---|---|---|---|---|
| 1 | 16.274 | 17.70 | 150 | Perillaldehyde |  |
| 2 | 20.009 | 40.89 | 204 | *β*-Caryophyllene |  |
| 3 | 21.759 | 15.53 | 204 | Farnesene |  |
| 4 | 23.982 | 19.78 | 220 | Caryophyllene oxide |  |

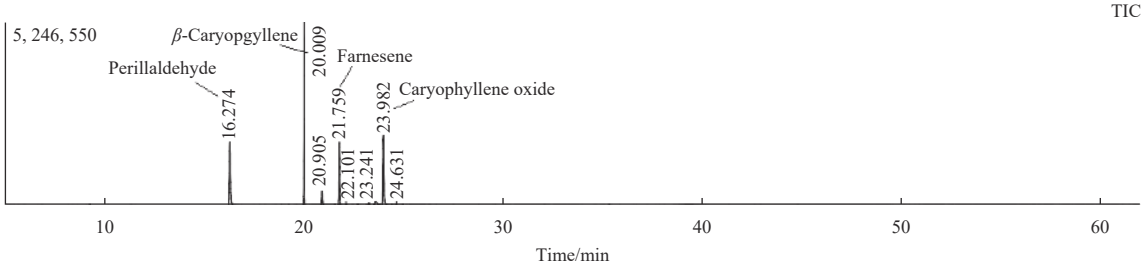



**Fig. 1 The GC-MS spectrum of PEO**

nents was evaluated in *N. glutinosa*. The protection, curative, and inactivation activities of PEO against TMV at 800 μg/mL were 65.58%, 56.33%, and 42.77%, respectively (Table 4). These results demonstrated that PEO had significant antiviral activity. Among the major components of PEO, perillaldehyde exhibited the highest anti-TMV activity. The protection, curative, and inactivation activities of perillaldehyde at 800 μg/mL were 80.41%, 73.42%, and 34.93%, respectively. The protective and curative effects of perillaldehyde were prominent and were significantly higher than those of the control agent at 200 μg/mL. The inactivating activity was similar to that of chitosan oligosaccharide, indicating that perillaldehyde was the main antiviral component of PEO.

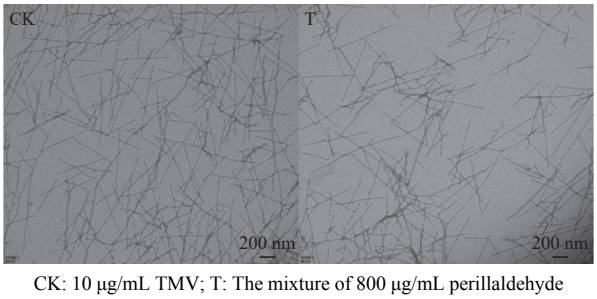
**Table 4 Anti-TMV activity of PEO and its main components**

| Treatment | Concentration/(μg/mL) | Control efficacy/% | | |
|---|---|---|---|---|
| | | Protection | Curative | Inactivation |
| PEO | 50 | 33.70 ± 2.45 gh | 35.40 ± 0.75 hj | 30.96 ± 1.81e fgh |
| | 200 | 47.05 ± 3.89 ef | 42.62 ± 3.52 ghi | 35.22 ± 1.74 def |
| | 800 | 65.58 ± 2.74 bc | 56.33 ± 3.24 cd | 42.77 ± 1.53 b |
| Perillaldehyde | 50 | 29.04 ± 2.25 h | 58.02 ± 0.77 c | 25.88 ± 1.16 h |
| | 200 | 62.62 ± 1.99 bc | 67.01 ± 1.72 b | 28.86 ± 2.69 gh |
| | 800 | 80.41 ± 3.87 a | 73.42 ± 1.22 a | 34.93 ± 2.42 defg |
| Farnesene | 50 | 29.19 ± 3.69 h | 19.80 ± 1.93 k | 14.21 ± 2.01 gi |
| | 200 | 40.25 ± 1.48 fg | 49.51 ± 0.80 defg | 29.57 ± 2.09 fgh |
| | 800 | 53.99 ± 1.53 de | 50.37 ± 4.96 def | 32.97 ± 2.86 defg |
| *β*-Caryophyllene | 50 | 46.42 ± 1.21 ef | 31.91 ± 2.54 j | 25.32 ± 0.52 h |
| | 200 | 58.25 ± 2.63 cd | 44.69 ± 1.15 fgh | 29.30 ± 1.70 fgh |
| | 800 | 68.49 ± 0.64 b | 55.08 ± 1.80 cde | 36.23 ± 2.06 cde |
| Caryophyllene oxide | 50 | 15.28 ± 3.69 i | 23.74 ± 1.76 k | 33.91 ± 1.49 defg |
| | 200 | 31.44 ± 2.81 h | 36.80 ± 2.74 ij | 38.36 ± 1.82 bcd |
| | 800 | 50.62 ± 1.03 de | 48.49 ± 1.91 efg | 41.23 ± 2.19 bc |
| Chitosan oligosaccharide | 200 | 53.62 ± 2.83 de | 23.38 ± 0.24 k | 32.78 ± 1.48 defg |
| Ningnanmycin | 200 | 47.63 ± 0.71 ef | 38.11 ± 1.24 hij | 57.47 ± 1.02 a |

Note: The data in the table were presented as mean ± standard error ($n$ = 3). The data followed by different lowercase letters were significantly different using the LSD method at the level of 0.05.

## 2.3 TEM observations

The morphology of TMV was observed by TEM to evaluate the effect of perillaldehyde on the virus. Fig. 2 shows that the morphology and structure of TMV particles treated with perillaldehyde remained intact. Compared with the control group, TMV particles showed no obvious morphological changes such as breakdown, aggregation, or distortion. These results indicated that perillaldehyde did not damage the morphology or structure of TMV particles.




CK: 10 μg/mL TMV; T: The mixture of 800 μg/mL perillaldehyde and 20 μg/mL TMV.

**Fig. 2 The morphology of TMV by TEM**

## 2.4 Induced hypersensitive response of *N. glutinosa* leaves

Perillaldehyde solutions were infiltrated into the mesophyll tissue of tobacco leaves for 2 days. As a result, no local lesions were observed on tobacco leaves at sites injected with 25, 50, 100, 200, and 400 μg/mL, respectively. However, local lesions were observed at sites injected with 800 and 1600 μg/mL, respectively (Fig. 3). Perillaldehyde at 800 and 1600 μg/mL, respectively induced a dose-dependent hypersensitive response (HR).

## 2.5 Induction of resistance to TMV by perillaldehyde

Perillaldehyde exhibited induced resistance activity against TMV, with effectiveness increasing as the

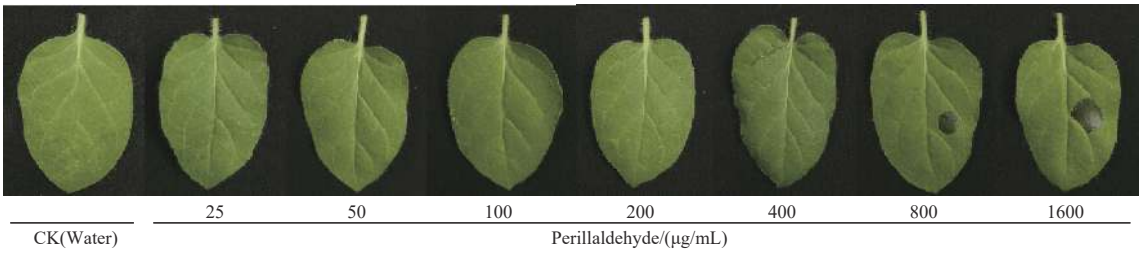
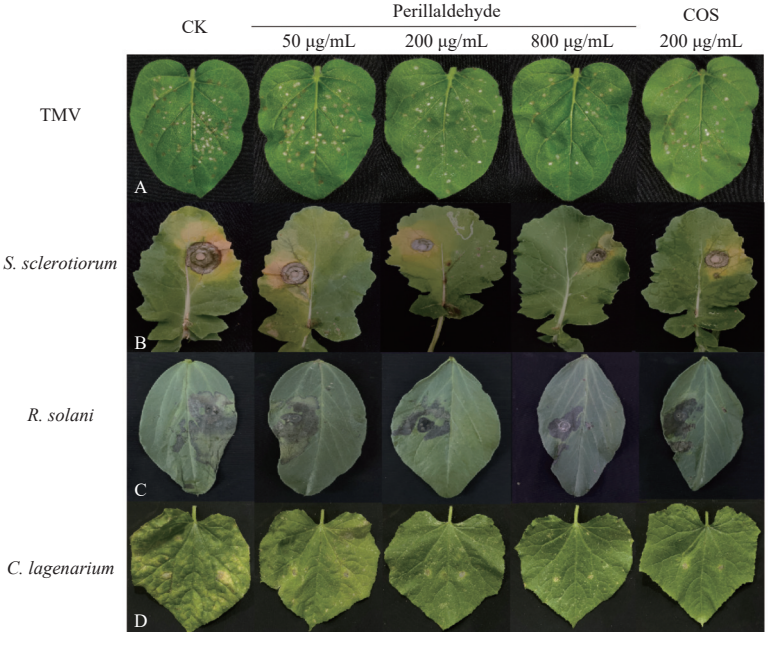



**Fig. 3 Hypersensitive response (HR) of *N. glutinosa* leaves by perillaldehyde**

concentration increased (Fig. 4A). The induced resistance activity of perillaldehyde was 58.46% at 800 μg/mL and 46.63% at 200 μg/mL, which was significantly higher than the activity of chitosan oligosaccharide (41.84%) (Table 5). These results demonstrate that perillaldehyde effectively induces disease resistance against TMV.




COS represents the chitosan oligosaccharide treatment. CK represents the sterile distilled water control.

**Fig. 4 Induced resistance of perillaldehyde to TMV and three kinds of fungal pathogens**

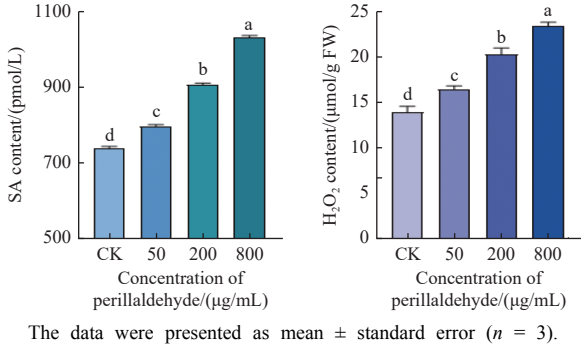
**Table 5 Induction of resistance to TMV by perillaldehyde**

| Treatment | Concentration/(μg/mL) | Control efficacy/% |
|---|---|---|
| Perillaldehyde | 50 | 34.12 ± 2.55 c |
| | 200 | 46.63 ± 1.52 b |
| | 800 | 58.46 ± 1.23 a |
| Chitosan oligosaccharide | 200 | 41.84 ± 2.72 b |

Note: The data in the table were presented as mean ± standard error ($n$ = 3). The data followed by different lowercase letters were significantly different using the LSD method at the level of 0.05.

## 2.6 Measurement of SA and $H_2O_2$ accumulation

Tobacco leaves showed enhanced SA and $H_2O_2$ accumulation after perillaldehyde treatment for 24 h (Fig. 5). The contents of SA and $H_2O_2$ increased in a dose-dependent manner, reaching their maximum value at 800 μg/mL. The highest concentrations were 1032.08 pmol/L for SA and 23.40 μmol/g FW for $H_2O_2$, respectively, both significantly higher than the control. Experimental results reveal that perillalde-



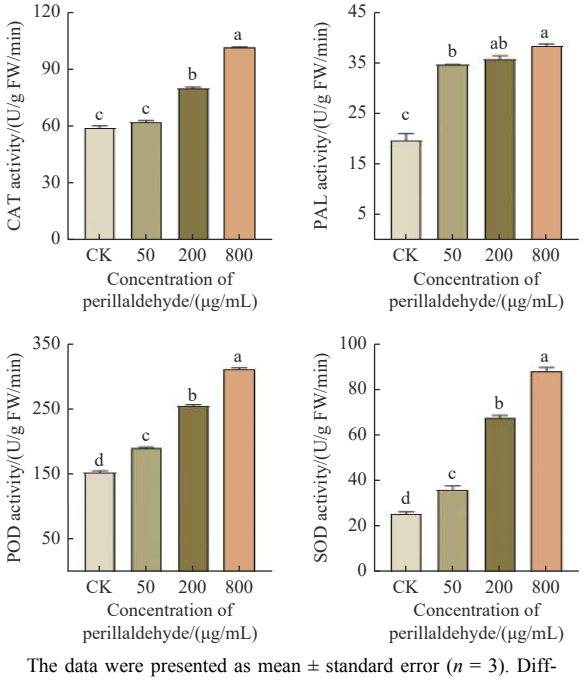

The data were presented as mean ± standard error ($n$ = 3). Different lowercase letters above the columns indicated significant differences in the data using the LSD method at the level of 0.05.

**Fig. 5 Effect of perillaldehyde on SA and $H_2O_2$ content in tobacco leaves**

hyde induced the accumulation of SA and $H_2O_2$ in tobacco leaves.

### 2.7 Analysis of defense enzyme activities

The influence of perillaldehyde on defense enzyme activities (CAT, PAL, POD, and SOD) in tobacco leaves were analyzed. Compared with the control, the activities of CAT, PAL, POD, and SOD were significantly enhanced by the perillaldehyde treatment, with maximum enhancement observed at 800 μg/mL (Fig. 6). Specifically, CAT activity increased by 1.76 times, PAL by 1.95 times, POD by 2.17 times, and SOD by 3.78 times. These results indicate that perillaldehyde enhances defense-related enzyme activities in tobacco leaves.

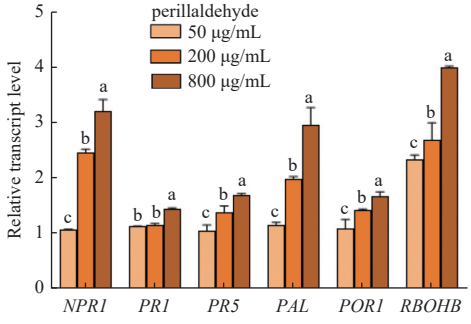



The data were presented as mean ± standard error ($n$ = 3). Different lowercase letters above the columns indicated significant differences in the data using the LSD method at the level of 0.05.

**Fig. 6 Effect of perillaldehyde on defense enzyme activities in tobacco leaves**

### 2.8 RT-qPCR analysis

To further study the effect of perillaldehyde on the relative expression of defense-related genes, gene expression was investigated in *N. benthamiana* with perillaldehyde treatment for 24 h, including *NPR1*, *PR1*, *PR5*, *PAL*, *RBOHB*, and *POR1*. The relative expressions of the six genes were increased in a dose-dependent manner. The expressions of *NPR1*, *PR1*, and *PR5* reached a maximum upregulation at 800 μg/mL and were increased by 3.19, 1.42, and 1.68 times, respectively, compared to the control (Fig. 7). Relative expression of the SA biosynthesis-related gene (*PAL*), chlorophyll biosynthesis key gene (*POR1*), and ROS outbreak key gene (*RBOHB*) also increased by 2.94, 1.75, and 3.92 times, respectively, at 800 μg/mL, compared to the control. These results indicate that perillaldehyde can upregulate defense-related genes to enhance plant disease resistance.




The data were presented as mean ± standard error ($n$ = 3). Different lowercase letters above the columns indicated significant differences in the data using the LSD method at the level of 0.05.

**Fig. 7 RT-qPCR analysis of defense-related genes in tobacco leaves with perillaldehyde treatment for 24 h**

### 2.9 Persistence of anti-TMV activity

To evaluate the persistence of perillaldehyde against TMV, the antiviral activity of perillaldehyde at 800 μg/mL was determined at different time intervals. Initially, the antiviral activity of perillaldehyde increased with a longer treatment time, reached the highest value (73.21%) on the 5$^{th}$ day, and then decreased. By the 11$^{th}$ day, the antiviral activity of perillaldehyde remained at 31.02% (Table 6). Compared with the positive control (chitosan oligosaccha-

**Table 6 Persistence of anti-TMV activity of perillaldehyde (800 μg/mL)**

| Time interval of inoculation/d | Control efficacy/% | |
|---|---|---|
| | Perillaldehyde | Chitosan oligosaccharide |
| 1 | 54.02 ± 2.53 bc | 56.80 ± 0.84 b |
| 3 | 53.39 ± 4.98 bc | 47.87 ± 1.43 cd |
| 5 | 73.21 ± 3.42 a | 35.76 ± 0.92 e |
| 7 | 49.86 ± 0.47 cd | 26.28 ± 1.31 g |
| 9 | 43.68 ± 2.87 d | 14.41 ± 1.18 h |
| 11 | 31.02 ± 1.89 ef | — |

Note: The data in the table were presented as mean ± standard error ($n$ = 3). Different lowercase letters following the data indicated the significant difference using analysis of variance (LSD method) at the level of 0.05.

ride) at the same dosage, perillaldehyde demonstrated long-lasting and effective induced resistance against TMV.

### 2.10 Induction of resistance to fungal pathogens

The perillaldehyde-induced resistance against three pathogenic fungi (*S. sclerotiorum*, *R. solani*, and *C. lagenarium*) was evaluated to assess its potential for broad-spectrum resistance. The perillaldehyde-induced resistance to *S. sclerotiorum* (Fig. 4B), *R. solani* (Fig. 4C), and *C. lagenarium* (Fig. 4D) at 800 μg/mL was 63.11%, 62.05%, and 49.12%, respectively (Table 7). These values were significantly higher than those of chitosan oligosaccharide at 200 μg/mL, especially for *S. sclerotiorum* and *R. solani*. These results demonstrate that perillaldehyde induces broad-spectrum resistance, not only against TMV but also against fungal pathogens in tobacco leaves.

**Table 7 Induction of resistance to fungal pathogens by perillaldehyde**

| Treatment | Concentration/(μg/mL) | *S. sclerotiorum* | | *R. solani* | | *C. lagenarium* | |
|---|---|---|---|---|---|---|---|
| | | Disease index | Efficacy/% | Disease index | Efficacy/% | Disease index | Efficacy/% |
| Perillaldehyde | 50 | 47.68 | 30.57±1.30 d | 46.34 | 35.92±1.77 c | 39.33 | 24.29±1.37 d |
| | 200 | 34.53 | 49.72±1.71 b | 35.28 | 51.22±1.84 b | 32.67 | 37.11±1.94 c |
| | 800 | 25.33 | 63.11±1.65 a | 27.45 | 62.05±2.09 a | 26.43 | 49.12±1.67 a |
| Chitosan oligosaccharide | 200 | 38.17 | 44.41±2.47 c | 37.93 | 47.56±1.92 d | 31.01 | 40.31±1.64 b |
| Blank control(water) | - | 68.67 | - | 72.33 | - | 51.95 | - |

Note: The data in the table were presented as mean ± standard error ($n$ = 3). Different lowercase letters following the data indicated the significant difference using analysis of variance (LSD method) at the level of 0.05.

## 3 Discussion

In this work, PEO was prepared by hydrodistillation and analyzed by GC-MS. The main components of PEO (content greater than 5%) were perillaldehyde, *β*-caryophyllene, farnesene, and caryophyllene oxide. The bioassay results demonstrated that PEO exhibited high antiviral activity. Among the four main components, perillaldehyde was the primary active ingredient of PEO which exhibited the most pronounced antiviral activity. The protective and curative activities of perillaldehyde were superior to its inactivating activity. The antiviral activity of perillaldehyde was higher than that of PEO. This difference can be attributed to the presence of other components in PEO, which dilute the concentration of perillaldehyde compared with the purified compound. As a result, the antiviral activity of PEO is lower than that of pure perillaldehyde at equivalent concentrations.

Natural products are an important source of antiviral drug discovery, providing novel structures and unique mechanisms for drug candidates[32]. In recent years, numerous natural products with antiviral activity have been discovered, showing potential for development into new antiviral drugs[33-35]. Small-molecule active compounds can directly act on the virion, disrupting its structure, inhibiting viral proliferation within the host, and inducing systemic resistance in the host. Thus, elucidating the antiviral mode of action of perillaldehyde is of interest. We found that perillaldehyde did not cause damage to the morphology or structure of the TMV particles. Thus, the high antiviral activity of perillaldehyde was not the result of direct effects on the virus. HR is a form of programmed cell death that activates defense responses by activating systemic signals in leaf vein endings, ultimately leading to SAR[36]. We found that perillaldehyde induced HR. Therefore, we hypothesized that the antiviral action of perillaldehyde may be attributed to its ability to induce systemic resistance in plants. The result of the pot experiment confirmed that perillaldehyde induced resistance against TMV, supporting the earlier speculation. This is further confirmed by perillaldehyde-induced upregulation of PR genes (*NPR1*, *PR1*, and *PR5*) and enhancement of defense enzyme activities, which activated the immune response of the plant[37].

SA is a crucial plant hormone that can act as a

signaling molecule in inducing local and systemic resistance responses to pathogen infection[38-39]. When a plant is attacked by a pathogen, it can use the two layers of its immune system to resist infection: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI)[40]. The defense response first produces a series of cellular responses at the infected site, including the generation of SA, which acts as a key signaling molecule to trigger systemic resistance to the same pathogen or other pathogens[41]. SA is an important elicitor of SAR, and the induction of SAR requires SA accumulation[42-43]. Perillaldehyde induced the upregulation of an SA synthesis-related gene (*PAL*) and the enhancement of PAL enzyme activity, and ultimately led to the accumulation of SA in tobacco. At the same time, perillaldehyde increased the relative expression of *NPR1*, which is a key regulator in SAR activation as an SA receptor gene[44]. The results suggest that perillaldehyde-induced resistance to pathogen infection may involve the induction of SA accumulation, which in turn activates SAR through NPR1 protein.

Reactive oxygen species (ROS) are derived from free and non-free oxygen radicals involved in the plant's immune response to pathogens. For instance, ROS may be involved in PTI and ETI as signaling molecules[45]. RBOHB (an NADPH oxidase) mediates ROS production, leading to ROS burst[46-47]. $H_2O_2$ is a stable active oxygen species that plays a crucial role in plant immune response to pathogen infection[48]. Perillaldehyde induced the overexpression of *RBOHB* and increased the $H_2O_2$ content, leading to a rise in ROS level. The activities of SOD, CAT, and POD were enhanced, which likely contributed to the removal of excessive ROS, avoiding damage to the plant cells, and enhancing plant resistance to the disease.

The induced resistance of 800 μg/mL perillaldehyde was maintained for more than 11 days. In addition, perillaldehyde can also induce resistance to fungal pathogens, including *R. solani*, *C. lagenarium*, and *S. sclerotiorum*. The results indicated that perillaldehyde-induced resistance was long-lasting and broad-spectrum. When elicitors induce plant resistance, they generally have a broad spectrum, with one elicitor capable of inducing resistance to a variety of diseases. For example, benzo-2,1,3-thiadiazole is a synthetic plant immune inducer that can induce plant resistance to a variety of diseases at the same time[49-50]. Citral can induce resistance to TMV, *Erysiphe cucurbitacearum*, *B. cinerea*, and *S. sclerotiorum*[31]. Perillaldehyde has been shown to induce plant resistance to a variety of diseases, demonstrating its broad-spectrum resistance characteristics. Therefore, this paper measured the induced resistances of perillaldehyde against three pathogenic fungi.

In addition, due to the rich source of perillaldehyde, it can be separated from PEO, and can also be synthesized by semi-synthetic or total synthesis, so the amount of resources is large and the acquisition cost is low. Perillaldehyde can be directly synthesized by chemical synthesis, the process route is short and the reaction process is easy to control. The chemical synthesis of perillaldehyde requires only two steps: the oxidation of $\alpha$-pinene to myrtenal, and then the isomerization of myrtenal to perillaldehyde[51]. In addition, perillaldehyde has a double bond at one end and an aldehyde group at the other end, both of which are good modification sites for chemical structural modification. It has been proved that a series of bioactive derivatives were produced by chemical modification of perillaldehyde as raw material[52-53]. Thus, perillaldehyde is expected to be a lead compound for the development of novel antiviral drugs and immune inducers. When using it for disease prevention, it is necessary to combine the rule of disease occurrence and apply it three times before or in the early stage of disease occurrence, with an interval of about 7 days. This study provides valuable data for the development and utilization of perillaldehyde in crop protection. The findings on the antiviral activity and mode of action of perillaldehyde provide a theoretical basis for its application in agriculture.

The disease resistance activity in this study was measured on living plants, results of pot experiments indicated that perillaldehyde, the main component of PEO, had strong disease resistance-inducing activity.

However, there is a lack of long-term plot experiments to verify these findings. Therefore, further study on complete and rigorous plot experiments is necessary to confirm the antiviral activity of perillaldehyde.

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