# Volatiles from the endophytic bacteria Bacillus sp. T6 confer Verticillium resistance in cotton

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# Abstract

[Aims] To obtain the endophytic bacteria with *Verticillium* wilt-resistance and explore the interactions between soil and plants. [Methods] Active tracking screening experiments were used to isolate the target strains. Morphological examination and 16s rRNA sequence analysis were performed to identify the strain. GC-mass spectrometry was used to explore the inhibition factor. Transcriptome analysis was carried out to explore the potential genes. Molecular docking and qRT PCR were used to validate the gene function. [Results] An endophytic bacterium *Bacillus* sp. T6 was obtained from the *Verticillium* wilt-resistant cotton *Gossypium barbadense* 'Xinhai15'. The strain possessed strong antagonistic abilities that inhibited *V. dahliae* spore germination and mycelial growth without contact, and thus, it was speculated that the active factor of the bacteria might be volatile compounds. A total of 46 volatile substances were detected. The styrene produced by the T6 strain was the main virulence factor. Following styrene induction, 247 genes in *V. dahliae*, including genes of four hydrolase, eight dehydrogenase, 11 reductase, and 17 genes related to transport and transfer were upregulated. Additionally, 72 genes, including two chitinase, two protease, five transport-related, and 33 hypothetical protein genes, were downregulated. The expression of the four genes VDAG_02838, VDAG_09554, VDAG_045572, and VDAG_08251 was increased by 3.18, 78.83, 2.71, and 2.92 times, respectively, compared with the uninduced control group. [Conclusions] The research provides a new reference for the development and application of the volatile compounds of endophytic bacteria as new biocontrol agents for the control of *Verticillium* wilt and as biological preservatives for agricultural products.


# Introduction

Vascular wilts are the most serious fungal diseases worldwide, and their pathogenic fungi are mainly the members of the genus *Verticillium* (Deketelaere et al. 2017). Plant pathogenic *Verticillium* species infect many kinds of hosts including important agricultural and cash crops (Inderbitzin and Subbarao 2014), among which *V. dahliae* is one of the most destructive soil-borne pathogens in crop plants. The fungus *V. dahliae* is distributed all over the world and can infect more than 660 species of plants, which causes serious yield losses in crop production. The consequences of *V. dahliae* infection can be far-reaching and global, and there is no effective method to control the diseases caused by it (Deketelaere et al. 2017). In particular, it has caused significant economic losses to cotton. The average yield reduction of cotton caused by *Verticillium* wilt is about 10–35% (Song et al. 2020). *V. dahliae* first infects the plant root through the soil, then the mycelium penetrates the plant root surface and colonizes the vascular bundle. Finally, the fungi cause the plant to die (Zhao et al. 2019).

The main pathogenic mechanism of *V. dahliae* is blocking blood vessels in the xylem vessel and producing toxins (Song et al. 2020). The process of *V. dahliae* infecting cotton is as follows: first, it penetrates roots to infect cotton; then, it spreads all over the root cortex and invades the xylem vessels, where it forms conidia. The mycelium then blocks vessels in the xylem, affecting the transport of water and nutrients in the plant (Zhao et al. 2019). According to one toxicity theory, the main cause of plant wilting after *V. dahliae* infection is the acid protein-lipopolysaccharide complex. The toxin seriously

destroys plant metabolism, immobilizes $CO_2$, breaks down $H_3PO_4$, and eventually kills the plant (Luo et al. 2014).

Plant pathogens are more difficult to control once they reach vascular tissue, and fungicides seem to be ineffective (Zhao et al. 2019). Eco-friendly alternatives such as biocontrol have become promising strategies for managing soil-borne *Verticillium* wilt in a variety of crops with growing concerns about the environment and human health.

Recently, several studies on the antifungal activity of microbial volatile organic compounds (VOC) have been carried out in a variety of plant pathogens, such as *Botrytis cinerea*, *Fusarium oxysporum*, *Magnaporthe oryzae*, and *V. dahliae* (Zhao et al. 2019). The cumulative data show that volatile substances play a more important role in microbial interactions than non-volatile compounds (Kanchiswamy et al. 2015). In addition, it has been shown that the exchange of aerial signals, such as VOCs, between microorganisms can cause changes in the metabolism of recipients (Rybakova et al. 2017). This reaction can enhance or reduce the production of certain soluble metabolites to ensure the survival of the recipients in the environment (Mulero-Aparicio et al. 2019). VOCs have the advantage of long-distance diffusion compared with larger molecules, which makes them a potential biological control agent. Therefore, it is very important to understand the specific mode of action of VOC to pathogenic fungi to develop VOCs as new biological fungicides.

The aim of the study was to explore the endophytes that utilize VOCs to resist cotton *Verticillium* wilt in the roots of the cotton *Gossypium barbadense* 'Xinhai15'. It was hypothesized that the VOCs produced by the endophytes in verticillium wilt-resistant cotton mediate the interactions between the plant, soil, and pathogens. To test the hypothesis, an endophytic bacterium *Bacillus* sp. T6 with antagonistic activity against *V. dahliae* was extracted from the *Verticillium* wilt-resistant cotton 'Xinhai 15'. The VOCs produced by T6 showed antifungal activity in *V. dahlia*. The antagonistic effect of *Bacillus* sp. T6 was mainly due to the production of bioactive compounds such as styrene. Furthermore, we determined the molecular mechanism of inhibition of the styrene against the fungus. The results of this study provide new insights into the mode of action of a potential VOC biocontrol agent, which is relevant for controlling *Verticillium* wilt using an ecologically friendly approach.

# Materials And Methods

### Microbial strains and materials

The fungus *V. dahliae* V991 and *Verticillium* wilt-resistant cotton 'Xinhai 15' were preserved in the State Key Laboratory of Cotton Biology, Henan University. The antagonistic strain *Bacillus* sp. T6 was screened and obtained from the roots of *Verticillium* wilt-resistant cotton 'Xinhai 9' samples, and the strain was deposited in the China Center for Type Culture Collection (CCTCC M2019618). The fungi were grown on Potato Dextrose Agar (PDA) plates (20.0% potato, 2.0% glucose, and 1.5% agar). The bacterial strains were incubated on Luria-Bertani culture media (1.0% tryptone, 0.5% yeast extract, and 0.5% NaCl)

containing the appropriate antibiotics. All of the chemical reagents and antibiotics used in this study were purchased from Sigma (St. Louis, MO).

### Screening and identification of the biocontrol strains against *V. dahlia*

The experiments on the screening and identification of the biocontrol strains against *V. dahlia* were performed based on a previous article (Lin Zhang 2020). Briefly, nine samples of the roots of 'Xinhai 15' were collected, surface disinfected, frozen in liquid nitrogen, and ground for 15 min with a 1-mL micro-dismembrator (Wheaton). After serially diluting with sterile water, approximately 50 μL of the suspension was plated onto LB and incubated at 28°C for 3 ds to culture the endophytic bacteria.

The antifungal activities of the obtained endophytic bacteria were tested via a dual culture method (Yu et al. 2018). The fungus *V. dahliae* V991 was grown for 7 ds at 25°C on PDA medium. Two hundred microliters of the cultured liquid bacterial suspensions were spread on LB agar plates and incubated for 12 h at 37°C. A 1-cm-diameter fungal block was fixed at the center of the PDA plate. An equivalent amount of tested bacterial strains and blank LB block was placed on both sides of the same plate. All of the isolates were incubated for 3–5 ds at 25°C, and the growth of the fungal mycelia was observed. Finally, the inhibition rate (IR) was calculated as follows: (control colony diameter-processing colony diameter) × 100% / (control colony diameter-fungus cake diameter). Each test was repeated three times in triplicate.

To further test the antagonistic activities of the bacterial strains, a pot efficiency test was conducted using our previously reported method. First, the spores of *V. dahliae* V991 were poured into the soil (loess/black soil/vermiculite 1:1:1) and mixed to obtain $1 \times 10^6$ spores/g of spore soil. Then, the tested bacterial liquid was cultured and adjusted to $OD_{600nm}$ = 1.0 using sterile water. Finally, the spore soil was poured in 10 mL of tested bacterial suspension ($OD_{600nm}$ = 1.0) + 90 mL of sterile water as the test group, while 100 mL of sterile water constituted the blank group. The cottonseeds were planted in the groups of soil and cultured at 25°C in a light incubator with a light/dark 16/8 h photoperiod. After 30 ds of incubation, the disease indices (DIs) were statistically counted every 5 ds.

Pure isolate strains of the endophytic bacteria with the strongest antagonistic against *V. dahlia* were identified through morphological examination and 16s rRNA gene sequence analysis.

### Exploration of the inhibition factor and gas chromatography (GC)-mass spectrometry

The thermostability of the antifungal metabolites of the bacteria was tested after boiling for 10 min. First, 40 μL of $1.0 \times 10^6$ CFU/mL *V. dahliae* spore suspension was spread onto a PDA plate. Then, 200 μL of boiled sterile fermentation filtrate and the untreated control were separately placed into holes at equal distances from the center of the plate. The suspension was then incubated at 25°C for 5 d. The germination of the fungal spores and the diameter of the inhibition zone were measured. The test was repeated three times.

The ‘upside-down plate’ method was used to measure the antifungal activities of the VOCs produced by the endophytic bacteria. First, *V. dahliae* was cultured in PDA medium for 14 ds, and a 1-cm-diameter hole punch of the cultured fungi clump was obtained and placed in the center of the PDA medium for incubation at 25°C for 3 ds. Then, 100 μL of the screened bacterial liquid was spread evenly on fresh LB solid medium and incubated at 37°C for 12 h. Finally, the pre-raised *V. dahliae* plate was inverted and placed inside the bacteria dish. The group of *V. dahliae* was inverted on the blank LB solid medium and used as a negative control. The reversed plate was incubated at 25°C for 15 ds, and the IR was calculated as follows: (control colony diameter-processing colony diameter) Í 100% / control colony diameter. Each test was repeated three times in triplicate.

The chemical components of the VOCs of the bacteria T6 were analyzed using gas chromatography-mass spectrometry (GC-MS). The VOCs in the cultured fermentation broth of the strain were collected using the headspace solid-phase microextraction technique (HS-SPME) (Wan MG 2008). The VOC components were identified using a GC-MS machine (6890N-5975B, Agilent Technologies Inc., CA, USA) following the procedures recommended by the manufacturer. Seven synthetic chemicals (1-dodecene, styrene, tetradecane, hexadecane, ethyl acetate, decane, caprolactam, 1-tetradecene) were selected based on the VOC profile of T6, as only these compounds were available from the chemical companies. The commercial pure products were purchased from Sigma-Aldrich Company. They were individually tested for the inhibition of mycelial growth and conidial germination of *V. dahliae* using the dual culture method as above. The concentration value for 50% inhibition of mycelial growth and conidial germination ($IC_{50}$), expressed as microliters per liter (μL $L^{-1}$), was inferred from the data on the inhibition percentages and the corresponding VOC doses applied in the double-dishes assay (Toral et al. 2021). Moreover, the morphological characteristics of the hyphae of *V. dahliae* treated with the pure VOC were observed under a scanning electron microscope to evaluate the suppression mechanisms of the VOCs.

### Transcriptome analysis of *V. dahliae* in response to styrene

Through antagonistic growth assay, it was found that the styrene produced by T6 showed the maximum antifungal activity. The *V. dahliae* was cultured in liquid medium for 3 ds, and then 60 mL of the cultured fungal liquid was extracted and evenly distributed into three 150 mL conical flasks. The sterilized 10 mL Eppendorf tube was placed into a conical flask with the mouth facing upward. One hundred microliters of $10^{-6}$ styrene were added into the Eppendorf tube. The fungi with styrene were cultured in a shaker at 200 rtm and 25°C for 2 h, 2.5 h, and 3 h. Fungi without any styrene added were used as a negative control. The four groups of samples were collected quickly and placed in liquid nitrogen to be frozen.

Total RNA was isolated from each fungal sample using a Fungal RNA Kit (OMEGA, Beijing, China). The RNA quality was measured using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Kit (Agilent Technologies, Beijing, China). Ribosomal RNA was removed using the Ribo-Zero AQ8. Equal amounts of RNA from each sample were used to construct the cDNA library using the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA), according to the manufacturer’s instructions. The cDNA fragments were purified with a QiaQuick PCR extraction kit and end-repaired. Following the addition

of poly (A), the fragments were ligated to Illumina sequencing adapters. The ligation products were size-selected by agarose gel electrophoresis, PCR amplified, and sequenced using the Illumina HiSeqTM 4000 System with the 2Í150 bp paired-end read module by Gene Denovo Biotechnology Co. (Guangzhou, China). Transcriptome assembly, transcriptome characterization, and differential gene expression analysis were carried out according to standard procedures.

Differential gene expression analysis was performed using the edgeR package (version: 3.10.2) with default parameters (Niu et al. 2012). Transcripts with a fold change ≥ 2 and a false discovery rate (FDR) < 0.05 were considered significant differentially expressed genes (DEGs). The DEGs for each sample were analyzed for Gene Ontology enrichment categories using BLAST2GO (version: 2.3.5), and the terms were deemed significant at FDR < 0.05. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database annotation of the unigenes, the DEGs for each comparison were analyzed for pathway enrichment, and pathways with FDR < 0.05 were considered significantly enriched pathways.

## Molecular docking of styrene

According to the transcriptome analysis results, proteins with significant differences in the expression levels of genes following treatment with styrene were selected for docking simulations. Their three-dimensional structures were downloaded from the Protein Data Bank (PDB) and optimized using Discovery Studio. After removing bound water, hydrogen atoms, and other irrelevant molecules, polar hydrogen atoms were added after processing. The file for the styrene was generated by Visualizer Studio 3.1 molecular docking to investigate the interactions between styrene and proteins using AutoDock 4.2 (Autodock Molecular Graphics Laboratory, the Scripps Research Institute, La Jolla, CA, USA) (Morris et al. 2009). Parameters, including a Lamarckian genetic algorithm (LGA) with a population size of 100 individuals, a maximum of 2,500,000 energy evaluations, and a maximum of 27,000 generations, were employed during the molecular docking, and all other parameters were set as default.

## Quantitative real-time (qRT) PCR validation of differential gene expression

Combined with gene differential expression analysis and functional annotation, the eight key genes in response to the growth inhibition of the fungus *V. dahliae* were selected and verified by qRT-PCR. An RNAclean Kit (BioTeck, China) was employed to purify the total RNA following total RNA isolation. The RNA concentration was determined by measuring the absorbance at 260 nm using a UV spectrophotometer. After the random-primed cDNAs were generated, qRT-PCR analysis was performed with a SYBR Green JumpStart Taq Ready Mix for qPCR kit (Sigma-Aldrich Co) following the manufacturer's instructions. The partial sequence of 18S rRNA amplified by primers N1 (ACGAGATCAGGACGGGCTT) and N2 (CGGCGTCTTCTGGAACATTTC) was used for internal control. The primers of the eight tested genes were designed, and their sequences are shown in Fig. 1. The PCR amplification consisted of 40 cycles of 94°C for 30 s, 60°C for 31 s, and 72°C for 40 s on an ABI PRISM 7000 Real-Time PCR machine.

# Results

### Isolation and identification of strain T6 with antagonistic against V. dahlia using VOCs

A total of 39 endophytic bacteria were isolated from the roots of the *Verticillium* wilt-resistant cotton 'Xinhai 15'. Among them, Proteobacteria (72%) and Firmicutes (23.6%) were the predominant bacterial groups in the 'Xinhai 15' root endophytes, particularly the genera *Bacillus* (40.35%), *Enterobacter* (22.97%), and *Pseudomonas* (11.06%). Six of the bacterial strains showed relatively strong fungistatic activities against the plant pathogenic fungus *V. dahlia* (Fig. 1), which accounted for approximately 15% of the population of endophytic bacteria in the plants. The inhibition rates of T6 and T4 against *V. dahlia* were 63.79% and 46.08%, respectively, which suggests that among the six endophytic bacteria, the two strains possessed significant inhibitory activity toward the *Verticillium* wilt pathogenic fungus *V. dahlia*.

The six strains were identified according to their morphological properties and the 16S rRNA sequences analysis. These six strains contained three *Bacillus*, one *Enterobacter*, one *Pseudomonas*, and one *Microbacterium* (Table 1), which showed that the genus *Bacillus* is the dominant endophyte in the rhizosphere and plays an important role in maintaining the health of cotton plants.

Table 1
Isolation, identification and their VOC inhibition activities of Endophytic Bacteria from XinHai15 roots

| Strain | Accessible number | Closest species in 16S rRNA gene sequence database | Similarity (%) | VOC inhibition activity |
|---|---|---|---|---|
| T4 | CP017184 | *Enterobacter roggenkampii* EN-117$^T$ | 100 | 30.05% |
| T6 | AMXN01000021 | *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429$^T$ | 99.93 | 95.66% |
| D2 | AB021406 | *Pseudomonas beteli* ATCC 19861$^T$ | 99.44 | 5.69% |
| R4 | LPVF01000003 | *Bacillus halotolerans* ATCC 25096$^T$ | 99.73 | 29.85% |
| S1 | Y17227 | *Bacillus siamensis* KCTC 13613$^T$ | 99.86 | 17.59% |
| R8 | AJVF01000043 | *Microbacterium oxydans* DSM 20578$^T$ | 99.86 | 11.02% |

Table 2
Volatile organic compounds produced by *Bacillus* sp. T6 in 3-day-old cultures detected by GC/MS analysis

| RT (min) | RA (%) | Possible compound | Matching degree (%) |
|---|---|---|---|
| 2.116 | 0.29 | Octane | 86 |
| 2.966 | 15.45 | Ethyl Acetate | 91 |
| 4.028 | 4.56 | Heptane | 86 |
| 4.202 | 0.63 | Nonane, 3-methyl- | 90 |
| 5.03 | 4.47 | Decane | 95 |
| 6.283 | 0.79 | Toluene | 90 |
| 8.953 | 1.07 | P-Xylene | 88 |
| 9.543 | 4.99 | Decane, 3,8-dimethyl- | 90 |
| 10.323 | 18.60 | Dodecane | 96 |
| 10.579 | 0.88 | 3-Undecene, 7-methyl-, (E)- | 90 |
| 11.134 | 1.53 | Undecane, 3-methylene- | 91 |
| 11.203 | 0.32 | 3-Dodecene, (E)- | 91 |
| 11.290 | 2.43 | 1-Dodecene | 96 |
| 11.671 | 1.02 | Styrene | 98 |
| 11.845 | 0.34 | 2-Dodecene, (Z)- | 96 |
| 13.70 | 4.14 | 3,5-Dimethyldodecane | 88 |
| 14.299 | 11.41 | Tetradecane | 98 |
| 14.485 | 0.78 | Cycloundecane, 1,1,2-trimethyl- | 92 |
| 14.966 | 1.16 | Tridecane, 3-methylene- | 91 |
| 15.101 | 1.25 | 1-Tetradecene | 99 |
| 15.564 | 0.90 | 2-Tetradecene, (E)- | 89 |
| 15.942 | 0.16 | 7-Tetradecene | 89 |
| 16.345 | 0.57 | Methoxyacetic acid, 2-tetradecyl ester | 87 |
| 16.605 | 0.31 | Benzaldehyde | 89 |

RT Retention time, RA Relative peak area (%), the values in that column are the average of two repeats; The VOCs of *Bacillus* sp. T6 with the relative peak areas less than 0.2% and the matching degree less than 85% are not included in this table.

| RT (min) | RA (%) | Possible compound | Matching degree (%) |
|---|---|---|---|
| 17.606 | 1.95 | Hexadecane | 96 |
| 18.330 | 0.53 | 5-Octadecene, (E)- | 91 |
| 21.053 | 1.42 | Benzene, 1-methoxy-4-(1-propenyl)- | 98 |
| 25.648 | 3.05 | Caprolactam | 99 |

RT Retention time, RA Relative peak area (%), the values in that column are the average of two repeats; The VOCs of *Bacillus* sp. T6 with the relative peak areas less than 0.2% and the matching degree less than 85% are not included in this table.

The effects of the VOCs of the six strains on antifungal activity were investigated. As shown in Fig. 2, the volatile substances produced by the six tested bacteria showed some antibacterial activity. Among them, the inhibition rate of the volatile substances produced by strain T6 reached up to 95.66%, representing the highest inhibition rate. The inhibition rates of the volatile substances produced by the other five bacterial strains were 30.05% (T4), 29.85% (R4), 17.59% (S1), 11.02% (R8), and 5.69% (D2) (Table 1).

The *V. dahliae* did not grow in the soil in which the T6 and R4 strains had been added. However, the fungi did grow well in the soil containing the other four strains, which is similar to that of the control group (Fig. 3), indicating that the T6 and R4 strains could inhibit the growth of *V. dahliae* not only on the Petri dish but also in the soil. Taken together, the T6 strain showed the most significant antifungal activity as a result of its VOCs. Therefore, the T6 strain was selected as the follow-up experimental object.

The results of the pot experiment carried out in the light incubator are shown in Fig. 4. The cotton group infected only with the pathogenic fungus suffered from the disease, and the leaves began yellowing and exhibited some defoliation. The infected plant also grew more slowly than the cotton in the blank control group with no disease symptoms. However, the cotton irrigated with T6 solution exhibited strong growth and was more resistant to *Verticillium* wilt than the control plants. The infection rates under the T6 treatment decreased to 13–19% compared to the blank control group only infected with the fungus, which had a 100% disease incidence. The average control effect of the T6 strain reached up to 92.55% after repeated experiments.

### GC-MS identification of the VOCs of Bacillus sp. T6

Based on the HS-SPME-GCMS spectral properties, we identified 28 VOCs in 3-day-old cultures after eliminating the compounds with a matching degree of less than 85% (Table 1). These compounds were classified as alkanes, alkenes, esters, benzenes, acids, and aldehydes. Alkanes and alkenes occupied the vast majority, accounting for 42.9% (12/28) and 35.8% (10/28), respectively.

# Antifungal Activity Of The Selected Commercial Vocs

A total of 10 compounds in the VOC profile of *Bacillus* sp. T6 with a relative content greater than 1% and matching degree greater than 90% were selected for the subsequent experiment. The 10 commercially pure compounds were tested for their antifungal activities. Five of the compounds (styrene, 1-tetradecene, 1-dodecene, ethyl acetate, and caprolactam) exhibited inhibitory activity against *V. dahlia* (Table 3). Among the 10 tested pure compounds, styrene showed the highest antifungal activity with the lowest 50% inhibition concentration values (IC50) for growth inhibition (12.8 μL $L^{-1}$) and for conidial germination (7.7 μL $L^{-1}$). Alkanes including decane, dodecane, tetradecane, tridecane, 3-methylene-, and hexadecane did not show any detectable inhibitory activity against *V. dahliae*.

Table 3
Fifty percent inhibition concentrations (IC50) of ten compounds on the mycelial growth and conidial germination of *V. dahliae*

| Compound | CAS number | IC 50, μL $L^{-1}$ | |
|---|---|---|---|
| | | Mycelial growth | Conidial germination |
| Ethyl Acetate | 000141-78-6 | 3699.9 | 97.2 |
| Decane | 000124-18-5 | -* | - |
| Dodecane | 000112-40-3 | - | - |
| 1-Dodecene | 000112-41-4 | 2863.5 | 96.3 |
| Styrene | 000100-42-5 | 12.8 | 7.7 |
| Tetradecane | 000629-59-4 | - | - |
| Tridecane, 3-methylene- | 019780-34-8 | - | - |
| 1-Tetradecene | 001120-36-1 | 2199.6 | 68.5 |
| Hexadecane | 000544-76-3 | - | - |
| Caprolactam | 000105-60-2 | 5533.2 | 49.4 |

*The symbol "-" indicates no inhibitory effect detected.

The bioassay experiment results showed that compared with the control group, the spores of *V. dahliae* hardly germinated when treated with 30 μL of pure styrene for 5 h, and the number of hyphae was significantly reduced (Fig. 5A&C). The inverted plate tests indicated that part of the hyphae of *V. dahliae* was dissolved (Fig. 5B). Moreover, the spore structure appeared incompact and irregular compared with the normal hyphae, and some holes appeared on the surfaces of the spores (Fig. 5D). The hyphae of *V. dahliae* were completely lysed as the treatment duration with styrene was extended to 5 ds.

**Transcriptome analysis of** V. dahlia **in the early response to styrene stress**

A total of six samples were designed for transcriptome analysis, including three samples of styrene treatment groups at three different time points (2 h, 4 h, and 6 h) and the corresponding control groups. After filtering the raw reads, a high rate of clean reads from each sample was achieved. A total of 46.39 Gb of clean data was obtained, and the clean data of each sample exceeded 7.2 Gb. Overall, more than 93.92% of the sequences could be mapped to the reference, and the GC content of all samples was stable with a distribution ranging from 52.70–55.32%. The Q20 and QC30 values of all samples were 98.29–98.54% and 94.82–95.38%, respectively. The results implied successful library construction, and thus the data could be used for subsequent bioinformatics analysis.

An FC > 2 or FC < 0.5, and a q-value ≤ 0.05 were used as thresholds to determine the DEGs. A total of 4818 DEGs (3092 upregulated and 1726 downregulated) were identified between the styrene-treated and control groups. There were 1370 DEGs (952 upregulated and 418 downregulated), 1820 DEGs (909 upregulated and 911 downregulated), and 1628 DEGs (1231 upregulated and 397 downregulated) at the three time points of styrene induction for 2 h, 4 h, and 6 h, respectively. The Venn diagram showed that there were 319 DEGs including 247 upregulated and 72 downregulated genes that were common among the three groups. The DEGs were further analyzed using GO enrichment and KEGG analyses. In detail, the upregulated genes in the styrene treatment versus control were significantly enriched in the biological process, molecular function, and cellular component categories, which were associated with metabolic enzymes, stress-stimulated response proteins, regulation factors, and membrane component proteins. The downregulated genes in the styrene treatment versus the control were mostly involved in the transport and catabolism, cell growth, and biosynthesis categories, specifically peptidase, lipase, proteases, chitinases, and methionyl-tRNA synthetase.

# Expression Levels Of Genes Related To Growth And Apoptosis

Transcriptome sequencing technology can provide a large amount of information regarding the DEGs that are involved in specific biological responses. The eight genes related to metabolic process and response to stimulus were screened from the DEGs based on the differential expression levels. The expression levels of genes involved in growth and stress (VDAG02212, VDAG06215, VDAG06215, VDAG09554) were upregulated, while VDAG04573, VDAG08882, VDAG09248, and VDAG09854 were downregulated in the styrene-treated group compared to the untreated control fungi. In addition, the qRT-PCR results showed that the expression patterns of the eight genes were identical to those detected by transcriptome sequencing (Fig. 6). The relative expression of genes related to lysozyme, epoxide hydrolase, retrograde regulation protein, and carbapenem antibiotics biosynthesis protein was upregulated by 9.78, 3.18, 2.92, and 2.71 times, respectively, compared with the control group. The relative expression of genes related to DNA polymerase lambda, meiotic coiled-coil protein, cellulose-growth-specific protein, and histone was downregulated by 7.15, 5.11, 4.20, and 3.55 times compared with the control group, respectively. The results confirmed the reliability of the RNA-seq data and further demonstrated that styrene is the virulence factor of the T6 strain that inhibits the growth of *V. dahliae*.

# Discussion

It has become particularly important to study the interactions between microbiota and their host plants. The plant root-associated microbiome represents a huge biodiversity reservoir of tens of thousands of species (Mulero-Aparicio et al. 2019). Plants depend upon beneficial interactions between roots and microbes to absorb nutrients and increase tolerance to various stresses and pathogen resistance (Kanchiswamy et al. 2015; Mohamad et al. 2018; Mulero-Aparicio et al. 2019; Rybakova et al. 2017; Xie et al. 2019). Microbial composition has been investigated in a variety of plant species, such as *Arabidopsis* (Duran et al. 2018; Schlaeppi et al. 2014; Zhao et al. 2014), *Populus* (Beckers et al. 2017; Gottel et al. 2011), maize (Edwards et al. 2015), rice (Edwards et al. 2015; Zhang et al. 2019), and cotton (Wei et al. 2019).

Cotton (*Gossypium hirsutum* L.) is an important cash crop grown worldwide. *Verticillium* wilt caused by soil-borne fungal pathogens, *V. dahliae*, is the main disease of cotton. *Verticillium* wilt resistance is mediated by quantitative trait loci, and these quantitative traits may be significantly influenced by other factors, including environmental conditions and plant-associated microbiota. The elementary inocula of *V. dahliae* are microsclerotia-fungal static structures in dead plant tissues and the soil. Microsclerotia may survive in the soil for more than 10 years in the absence of a host (Wei et al. 2019). Various cultivars differ in their susceptibility to *V. dahliae* (Wei et al. 2015). Wei et al. (2015) demonstrated that specific rhizosphere and endosphere microbes may be greatly helpful to the resistance of cotton against *V. dahliae*. They also demonstrated that several well-known taxonomic groups with resistant activities against *Verticillium* wilt contained profitable microbes, such as *Bacillales*, *Pseudomonadales*, *Rhizobiales*, and *Trichoderma*, with higher relative abundances related to resistant cultivars. Greenhouse data supported the function of beneficial rhizosphere microbes in reducing the development of *Verticillium* wilt (Wei et al. 2019). The tolerance on the *Verticillium* wilt has been reported to be closely associated with well-known beneficial bacteria, including *Bacillus* (Egamberdieva 2017), *Lysobacter* (Sullivan et al. 2003), *Streptomyces* (Niu et al. 2016), *Rhizobiales* (Wei et al. 2019), and *Pseudomonas* (Prieto et al. 2009). The relative abundance in wilt-susceptible cultivars, including the fungal endophytes of *Alternaria solani*, *Aspergillus aculeatus*, *V. longisporum*, and *Choanephora* and the rhizosphere fungi of *Alternaria*, *Magnaporthe grisea*, *Thielaviopsis basicola*, *Ceratobasidiaceae*, and *Ustilaginaceae*, was increased in many fungal groups (Wei et al. 2019; Wheeler et al. 2019).

*Bacillus* spp., in addition to possessing a multiplicity of effect mechanisms of cooperation that can act synergistically against phytopathogens (Fujimoto et al. 2022; Fujimoto and Kupper 2016), have the capacity to form endospores to ensure their long-term maintenance and survival in different environments. It is very advantageous to use these VOCs in disease control, and VOCs are ideal informational chemicals because they can function over long distances through the air and diffuse through the pores of the soil (Wheatley, 2002). The VOCs of the bacteria *Bacillus* genus exhibit potential antagonistic behavior against several phytopathogens. It is reported that the VOCs produced by the bacteria *B. subtilis* and *B. amyloliquefaciens* show up to 87% inhibition against the fungus *Rhizoctonia solani*. These compounds caused the deformation and death of the hyphae, which is confirmed by

scanning electron microscopy observation (Minerdi et al. 2009). Other authors have also identified deformations in the hyphae and spores of the phytopathogenic fungus *Macrophomina phaseolina* caused by volatile compounds from *B. subtilis* and *B. amyloliquefaciens* (Gotor-Vila et al. 2017; Torres et al. 2016). Moreover, volatile compounds from *Bacillus* spp. ACB-65 and *Bacillus* spp. ACB-73 provided 86% inhibition in the control of *Phyllosticta citricarpa* (Solanki et al. 2015). In addition, Gotor-Vila et al. (2017) verified the use of *B. amyloliquefaciens* VOCs in the control of *Monilinia laxa*, *Monilinia fructicola*, and *B. cinerea* in post-harvest sweet cherry fruits, thereby reducing the incidence of disease and sporulation of pathogens. However, little is known about the application of *Bacillus* VOCs in plant disease control, as well as the chemical composition of *Bacillus* VOCs (Zhao et al. 2019).

In this study, we investigated the *Bacillus* sp. T6 strain from the endophytic bacteria in *Verticillium* wilt-resistant cotton roots, which showed strong inhibitory activities against *V. dahliae* via its VOCs. The GC-MS results and pure product verification experiments showed that the styrene produced by the T6 strain represented an important antagonistic virulence factor. In the fungi treated with styrene treatment, the hyphae were obviously reduced and fell off, and the morphology of the spores was distorted and deformed. Furthermore, the antifungal mechanism of styrene at the molecular level was explored via transcriptome and qRT-PCR analysis. The results confirmed that the expression levels of the genes related to transportation and cell lysis in *V. dahliae* were upregulated, while the expression of growth-related genes was downregulated after styrene induction, thus inhibiting the growth of the fungus *V. dahliae*. The present work demonstrated that the volatiles produced by the bacterial isolates could be used for the development of new bioproducts, though more in-depth studies are needed. The results provide a promising approach for managing cotton *Verticillium* wilt and enriching the resources of VOC-producing antagonists.

# Declarations

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**Author contributions** QHN and LZ conceived and designed the experiments. YW and SWL performed the isolation and transcriptome analysis. HXZ and ZYL assayed the VOC and inhibition activities. YW performed qPCR analysis. SWL analyzed the pot experimental data. YW and SWL drafted the manuscript. JWY helped revise and polish the manuscript. All authors read and approved the final manuscript.

# References

1. Beckers B, Op De Beeck M, Weyens N, Boerjan W, Vangronsveld J (2017) Structural variability and niche differentiation in the rhizosphere and endosphere bacterial microbiome of field-grown poplar trees. Microbiome 5:25. doi: 10.1186/s40168-017-0241-2

2. Egamberdieva D (2017) "*Bacillus* spp.: a potential plant growth stimulator and biocontrol agent under hostile environmental conditions". Bacilli and Agrobiotechnology. Springer, Cham, pp 91–111
3. Deketelaere S, Tyvaert L, Franca SC, Hofte M (2017) Desirable traits of a good biocontrol agent against *Verticillium* wilt. Front Microbio 8:1186. doi: 10.3389/fmicb.2017.01186
4. Duran P, Thiergart T, Garrido-Oter R, Agler M, Kemen E, Schulze-Lefert P, Hacquard S (2018) Microbial interkingdom interactions in roots promote *Arabidopsis* survival. Cell 175: 973–983 e914. doi: 10.1016/j.cell.2018.10.020
5. Edwards J, Johnson C, Santos-Medellin C, Lurie E, Podishetty NK, Bhatnagar S, Eisen JA, Sundaresan V (2015) Structure, variation, and assembly of the root-associated microbiomes of rice. P Natl Acad Sci USA 112:E911–920. doi: 10.1073/pnas.1414592112
6. Fujimoto A, Augusto F, Fill TP, Moretto RK, Kupper KC (2022) Biocontrol of *Phyllosticta citricarpa* by *Bacillus* spp.: biological and chemical aspects of the microbial interaction. World J Microb Biot 38:53. doi: 10.1007/s11274-021-03214-z
7. Fujimoto A, Kupper KC (2016) Production of antifungal compounds and hydrolytic enzymes by *Bacillus* spp. as mechanisms of action against *Phyllosticta citricarpa*. IOSR J Agric and Vet Sci 9:19–27
8. Gotor-Vila A, Teixido N, Di Francesco A, Usall J, Ugolini L, Torres R, Mari M (2017) Antifungal effect of volatile organic compounds produced by *Bacillus amyloliquefaciens* CPA-8 against fruit pathogen decays of cherry. Food Microbio 64:219–225. doi: 10.1016/j.fm.2017.01.006
9. Gottel NR, Castro HF, Kerley M, Yang Z, Pelletier DA, Podar M, Karpinets T, Uberbacher E, Tuskan GA, Vilgalys R, Doktycz MJ, Schadt CW (2011) Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. Appl Environ Microb 77:5934–5944. doi: 10.1128/AEM.05255-11
10. Inderbitzin P, Subbarao KV (2014) *Verticillium* systematics and evolution: how confusion impedes *Verticillium* wilt management and how to resolve it. Phytopathology 104:564–574. doi: 10.1094/PHYTO-11-13-0315-IA
11. Kanchiswamy CN, Malnoy M, Maffei ME (2015) Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Front Plant Sci 6:151. doi: 10.3389/fpls.2015.00151
12. Luo X, Xie C, Dong J, Yang X, Sui A (2014) Interactions between *Verticillium dahliae* and its host: vegetative growth, pathogenicity, plant immunity. Appl Microbiol Biot 98:6921–6932. doi: 10.1007/s00253-014-5863-8
13. Minerdi D, Bossi S, Gullino ML, Garibaldi A (2009) Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. Environ Microbiol 11:844–854. doi: 10.1111/j.1462-2920.2008.01805.x
14. Mohamad OAA, Li L, Ma JB, Hatab S, Xu L, Guo JW, Rasulov BA, Liu YH, Hedlund BP, Li WJ (2018) Evaluation of the antimicrobial activity of endophytic bacterial populations from Chinese traditional medicinal plant licorice and characterization of the bioactive secondary metabolites produced by

*Bacillus atrophaeus* against *Verticillium dahliae*. Front Microbiol 9:924. doi: 10.3389/fmicb.2018.00924

15. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem 30:2785–2791. doi: 10.1002/jcc.21256
16. Mulero-Aparicio A, Cernava T, Turra D, Schaefer A, Di Pietro A, Lopez-Escudero FJ, Trapero A, Berg G (2019) The role of volatile organic compounds and rhizosphere competence in mode of action of the non-pathogenic *Fusarium oxysporum* FO12 toward *Verticillium* wilt. Front Microbiol 10:1808. doi: 10.3389/fmicb.2019.01808
17. Niu G, Chater KF, Tian Y, Zhang J, Tan H (2016) Specialized metabolites regulating antibiotic biosynthesis in *Streptomyces* spp. FEMS microbiol Rev 40:554–573. doi: 10.1093/femsre/fuw012
18. Niu QH, Huang X, Hui F, Huang S, Ke T, Zhang KQ, Zhang L (2012) Colonization of *Caenorhabditis elegans* by *Bacillus nematocida* B16, a bacterial opportunistic pathogen. J Mol Microb Biotech 22:258–267
19. Prieto P, Navarro-Raya C, Valverde-Corredor A, Amyotte SG, Dobinson KF, Mercado-Blanco J (2009) Colonization process of olive tissues by *Verticillium dahliae* and its in planta interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. Microb Biotechnol 2:499–511. doi: 10.1111/j.1751-7915.2009.00105.x
20. Wheatley RE (2002) The consequences of volatile organic compound mediated bacterial and fungal interactions. Ant Van Leeuwenhoek 81:357–364
21. Rybakova D, Rack-Wetzlinger U, Cernava T, Schaefer A, Schmuck M, Berg G (2017) Aerial warfare: a volatile dialogue between the plant pathogen *Verticillium longisporum* and its antagonist *Paenibacillus polymyxa*. Front Plant Sci 8:1294. doi: 10.3389/fpls.2017.01294
22. Schlaeppi K, Dombrowski N, Oter RG, van Ver Loren E, Schulze-Lefert P (2014) Quantitative divergence of the bacterial root microbiota in *Arabidopsis thaliana* relatives. P Natl Acad Sci USA 111:585–592. doi: 10.1073/pnas.1321597111
23. Solanki MK, Singh RK, Srivastava S, Kumar S, Kashyap PL, Srivastava AK (2015) Characterization of antagonistic-potential of two *Bacillus* strains and their biocontrol activity against *Rhizoctonia solani* in tomato. J Basic Microb 55:82–90. doi: 10.1002/jobm.201300528
24. Song R, Li J, Xie C, Jian W, Yang X (2020) An overview of the molecular genetics of plant resistance to the *Verticillium* wilt pathogen *Verticillium dahliae*. Int J Mol Sci 21. doi: 10.3390/ijms21031120
25. Sullivan RF, Holtman MA, Zylstra GJ, White JF, Kobayashi DY (2003) Taxonomic positioning of two biological control agents for plant diseases as *Lysobacter enzymogenes* based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. J Appl Microbiol 94: 1079–1086. doi: 10.1046/j.1365-2672.2003.01932.x
26. Toral L, Rodriguez M, Martinez-Checa F, Montano A, Cortes-Delgado A, Smolinska A, Llamas I, Sampedro I (2021) Identification of volatile organic compounds in extremophilic bacteria and their

effective use in biocontrol of postharvest fungal phytopathogens. Front Microbiol 12:773092. doi: 10.3389/fmicb.2021.773092

27. Torres MJ, Brandan CP, Petroselli G, Erra-Balsells R, Audisio MC (2016) Antagonistic effects of *Bacillus subtilis* subsp. *subtilis* and *B. amyloliquefaciens* against *Macrophomina phaseolina*: SEM study of fungal changes and UV-MALDI-TOF MS analysis of their bioactive compounds. Microbiol Res 182: 31–39. doi: 10.1016/j.micres.2015.09.005
28. Wan MGLG, Zhang JB, Jiang DH, Huang HC (2008) Effect of volatile substances of *Streptomyces* platensis F-1 on control of plant fungal diseases. Biol Control 46:552–559
29. Wei F, Fan R, Dong H, Shang W, Xu X, Zhu H, Yang J, Hu X (2015) Threshold microsclerotial inoculum for cotton verticillium wilt determined through wet-sieving and real-time quantitative PCR. Phytopathology 105:220–229. doi: 10.1094/PHYTO-05-14-0139-R
30. Wei F, Zhao L, Xu X, Feng H, Shi Y, Deakin G, Feng Z, Zhu H (2019) Cultivar-dependent variation of the cotton rhizosphere and endosphere microbiome under field conditions. Front Plant Sci 10:1659. doi: 10.3389/fpls.2019.01659
31. Wheeler DL, Dung JKS, Johnson DA (2019) From pathogen to endophyte: an endophytic population of *Verticillium dahliae* evolved from a sympatric pathogenic population. New Phytol 222:497–510. doi: 10.1111/nph.15567
32. Xie S, Vallet M, Sun C, Kunert M, David A, Zhang X, Chen B, Lu X, Boland W, Shao Y (2019) Biocontrol potential of a novel endophytic bacterium from mulberry (morus) tree. Front Bioeng Biotech 7:488. doi: 10.3389/fbioe.2019.00488
33. Yu J, Wu Y, He Z, Li M, Zhu K, Gao B (2018) Diversity and antifungal activity of endophytic fungi associated with *Camellia oleifera*. Mycobiology 46:85–91. doi: 10.1080/12298093.2018.1454008
34. Zhang J, Liu YX, Zhang N, Hu B, Jin T, Xu H, Qin Y, Yan P, Zhang X, Guo X, Hui J, Cao S, Wang X, Wang C, Wang H, Qu B, Fan G, Yuan L, Garrido-Oter R, Chu C, Bai Y (2019) NRT1.1B is associated with root microbiota composition and nitrogen use in field-grown rice. Nat Biotechnol 37:676–684. doi: 10.1038/s41587-019-0104-4
35. Zhang L, Tao Y, Zhao SY, Yin XY, Chen JM, Wang M, Cai YF, Niu QH (2020) A novel peroxiredoxin from the antagonistic endophytic bacterium *Enterobacter* sp. V1 contributes to cotton resistance against *Verticillium dahliae*. Plant Soil doi. https://doi.org/10.1007/s11104-020-04661-7
36. Zhao P, Li P, Wu S, Zhou M, Zhi R, Gao H (2019) Volatile organic compounds (VOCs) from *Bacillus subtilis* CF-3 reduce anthracnose and elicit active defense responses in harvested litchi fruits. AMB Express 9:119. doi: 10.1186/s13568-019-0841-2
37. Zhao P, Zhao YL, Jin Y, Zhang T, Guo HS (2014) Colonization process of Arabidopsis thaliana roots by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. Protein Cell 5:94–98. doi: 10.1007/s13238-013-0009-9

# Figures

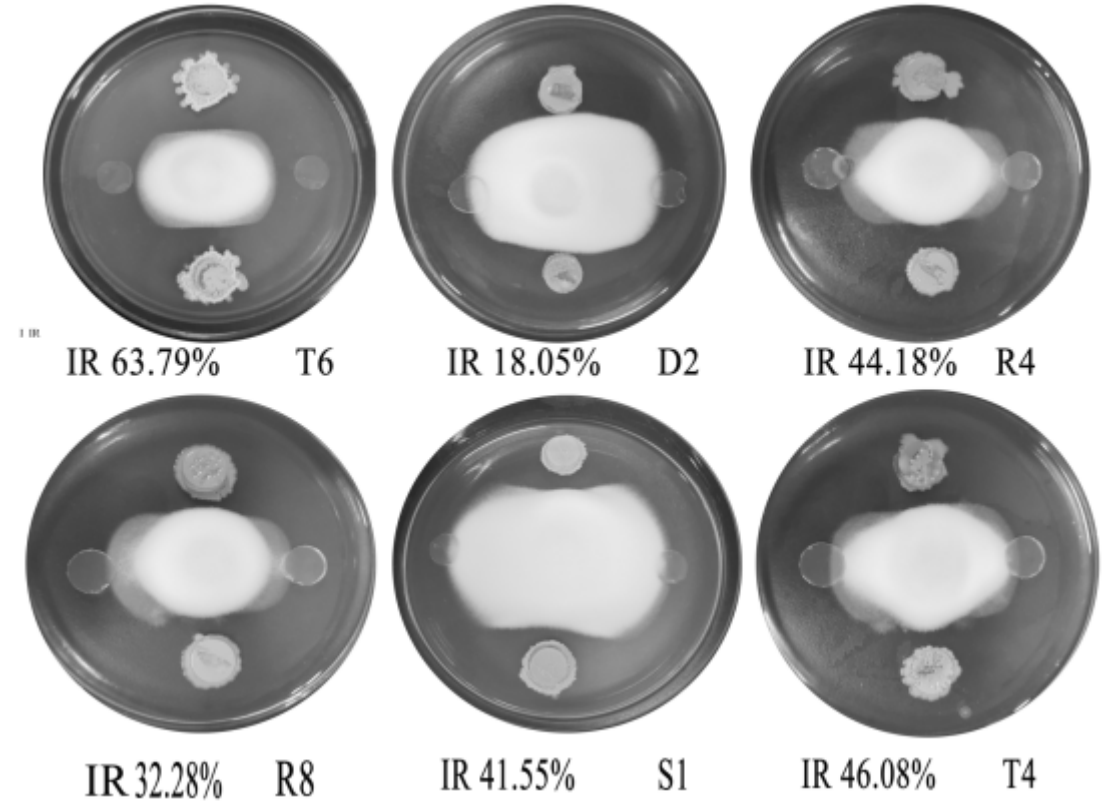



**Figure 1**

Antifungal activities of the six isolated strains with inhibition activities: The left and right sides of the plate were the culture medium control blocks, and the top and the bottom of the plate were the bacterial blocks, respectively.

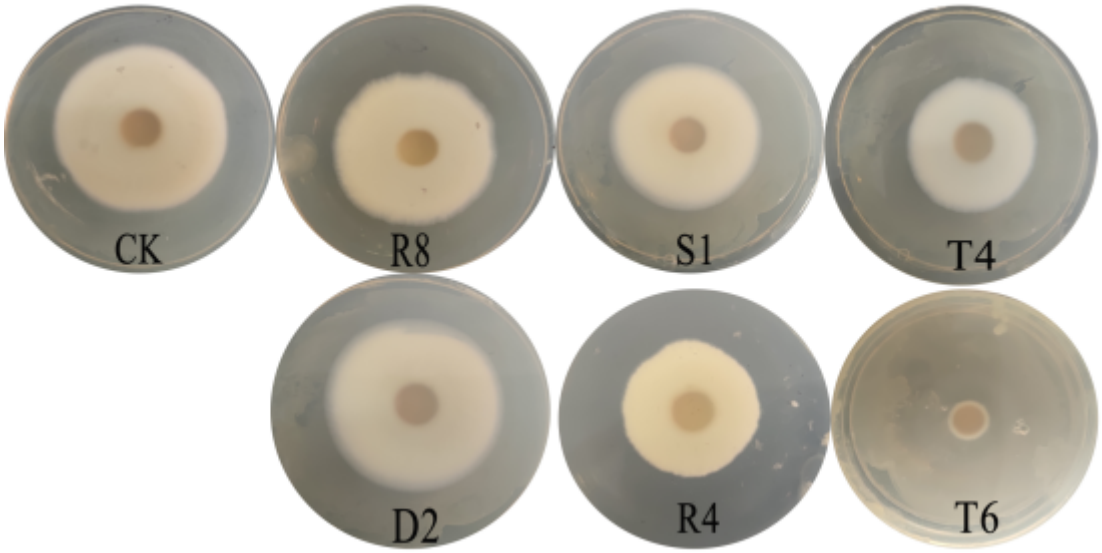



**Figure 2**

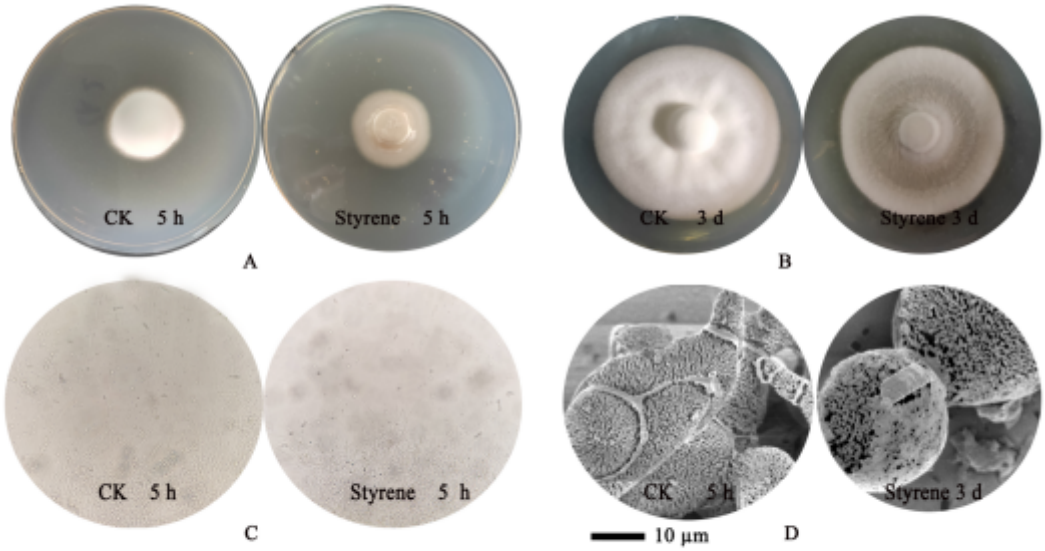
Pot experiments rescreening the strain T6 against *Verticillium* wilt.


## Figure 3

Anti-fungi capabilities of the tested bacteria against *V. dahliae* in soil.


## Figure 4

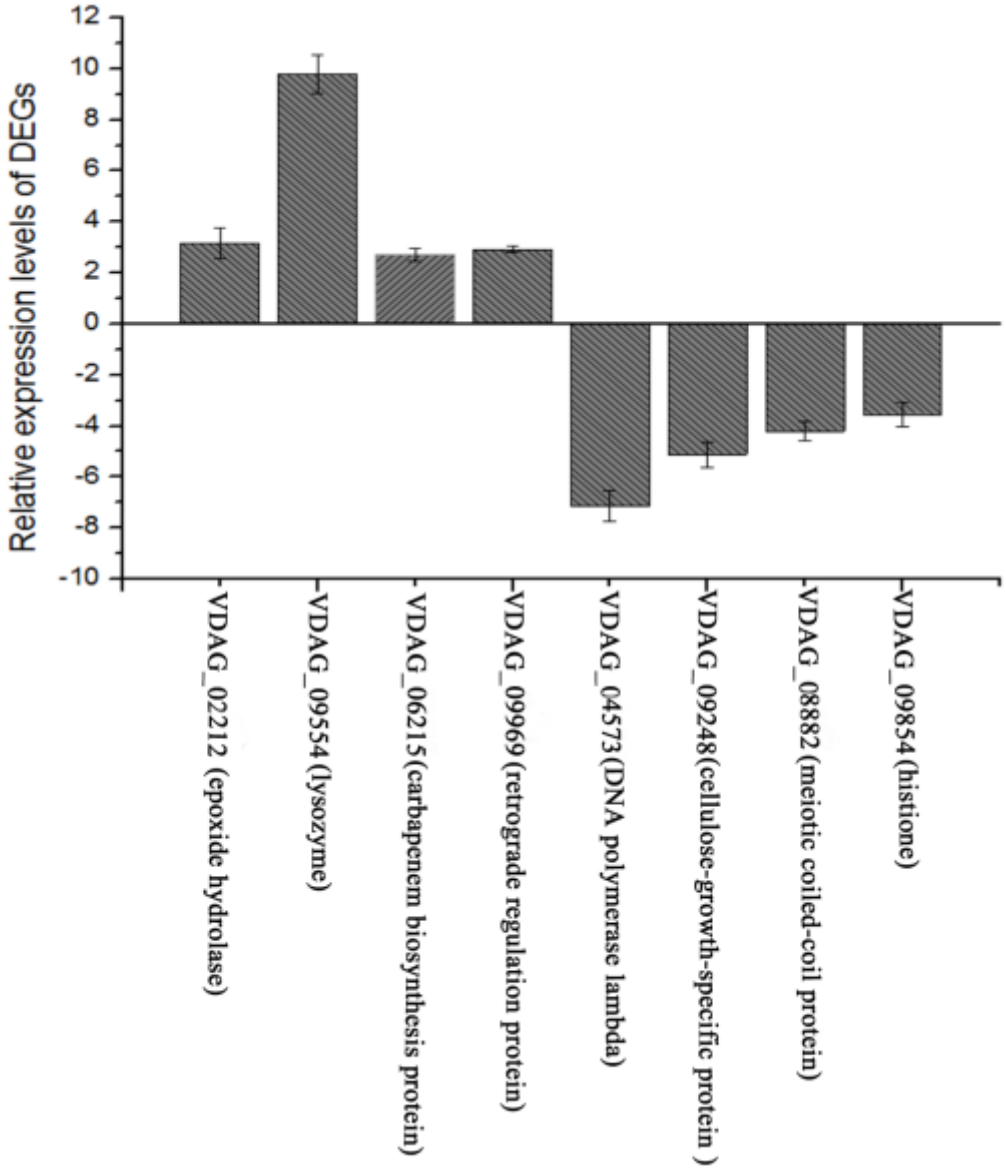
Anti-fungal activity results of volatile substances produced by the six strains tested by inverted plate experiments.




## Figure 5

Results of styrene inhibiting fungi *V. dahliae*. A. Antifungal activity test of styrene -treated for 5 h on plates; B. Antifungal activity test of styrene-treated for 3 d on plates; C. Microscopic observation of the

fungi after styrene -treated for 5 h; D. SEM observation of the fungi after styrene -treated for 3 d.




## Figure 6

Expression levels of genes related to growth by qRT-PCR. The x-axis indicates the gene names. The left y-axis indicated relative expression level of qRT-PCR. Error bars represent standard error of mean.