

## The effect of terpinen-4-ol in controlling blueberry fruit rot causing by *Alternaria tenuissima*

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### ABSTRACT

Terpinen-4-ol (T4O) is the main ingredient of tea tree essential oil with a light aroma. This study examined its inhibition on *Alternaria tenuissima*, and controlling effects on blueberry *Alternaria* fruit rot (AFR). The results demonstrated that 0.2  $\mu\text{L mL}^{-1}$  T4O suppressed colony growth and increased relative conductivity due to nucleic acids and soluble proteins leakage and 0.4  $\mu\text{L mL}^{-1}$  T4O suppressed spore germination of *A. tenuissima*. Furthermore, fumigation with 0.4  $\mu\text{L mL}^{-1}$  T4O lowered the disease index in blueberries by 33.5 %, enhanced the accumulation of secondary metabolites and stimulated chitinase, and  $\beta$ -1, 3-glucanase enzyme activities and gene expression. Hydrogen peroxide was surged during early storage, inducing the production of reactive oxygen species. The T4O-treated group responded this issue earlier, and exhibited higher antioxidant enzyme activities and gene expressions than the control group. So that enhanced the ability of blueberries to scavenge reactive oxygen species. Such protective changes safeguarded blueberries against oxidative stress to combat AFR.

### 1. Introduction

Blueberry, possessing both sour and sweet tastes, is also rich in anthocyanins, vitamins, and other bioactive substances (Bilbao-Sainz et al., 2024; Bei et al., 2024). However, its delicate skin makes it easily susceptible to fungal infections, diminishing its commercial value (Wang et al., 2023; Wu et al., 2025). Currently, the main fungi causing post-harvest rot in blueberries are *Botrytis cinerea*, *Alternaria* spp., and *Penicillium* spp. Among these pathogens, *Alternaria* spp. played a dominant role in northern China (Sehim et al., 2023). It not only had wide environmental adaptability but also produced toxins (Ali et al., 2023; He et al., 2024). Therefore, exploring effective measures to control AFR is crucial to prolong the storage life of blueberries.

The use of chemical fungicides to control AFR not only poses health risks but also promotes fungal resistance (Wang et al., 2023). Recently, biological control has become the preferred approach to mitigate AFR (Liu et al., 2021). At present, natural compounds or additives that are safe, environmentally friendly, and effective are being explored to combat AFR. Various natural extracts, such as burdock fructooligosaccharide, tannic acid, L-arginine, and L-lysine, have been used for

postharvest preservation purposes (Wang et al., 2023; Liu et al., 2024). Additionally, Liu et al. (2024) found that thyme essential oil inhibited *Staphylococcus aureus* and *Escherichia coli* while maintaining cherry tomato firmness and total soluble solids (TSS) (Liu et al., 2024). Menthol has been suggested to inhibit *Colletotrichum* spp. *in vitro* and enhance strawberry resistance (Cai et al., 2023). Liu et al. (2024) effectively suppressed the growth of *Penicillium* spp. and prolonged the shelf life of sugar oranges through the application of cubeba oil nanoemulsion. Although the preservative effect was achieved, the strong odor of many plants essential oils limited their further application. Through our preliminary screening, tea tree essential oil (TTO) was selected with not only effective on *A. tenuissima*, but also little unpleasant odor.

Terpinen-4-ol (T4O), the main constituent of tea tree essential oil (TTO), is a cyclic monoterpenoid compound with a light aroma (Liu et al., 2022). TTO has been scientifically proven to exhibit antifungal activity against different pathogens such as *E. coli*, *Botrytis cinerea*, *Pseudomonas aeruginosa* as well as *Bacillus subtilis* (Li et al., 2025). Furthermore, it has been observed that TTO and its sustained-release preparations can effectively enhance the quality of postharvest fruits and vegetables like pineapple, strawberry, tomato, banana, and red bell

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**Table 1**

Primers required for real-time fluorescence quantitative PCR.

Gene	Forward	Reverse
VcGADPH	5'-ATGACTTTGGTGAGGCTGAG-3'	5'-GGAGGAAGGAGGGAGGA-3'
VcSOD	5'-ATGCCAGAGGCTGAAGGTG-3'	5'-GGAGGAGGAGGGAGGAAG-3'
VcPOD24	5'-ATGAGCTAGGCGTGAAGGAG-3'	5'-GAAAGGAAGGAAGGAAGGAG-3'
VcAPX	5'-ATGGTCGGTTGGTGTG-3'	5'-GACTTGTGCCAGCGTCTG-3'
VcGLU	5'-ATGAGGCGGTGCTCCCTG-3'	5'-GACTGACGCCAACGCGCT-3'
VcCHI	5'-ATCCGCTATCGCTTACA-3'	5'-CACTCCAATGCACCGTTA-3'

pepper, thereby exhibiting excellent preservation potential. However, due to variations in extraction processes and differences in the origin materials, the types and concentrations of key components in TTO may vary, leading to uncertainty in its antifungal and freshness-preserving efficacy (Cai et al., 2023). Given this research background, we explored the potential use of T4O for managing postharvest blueberry AFR.

## 2. Materials and methods

### 2.1. Blueberry, essential oils, and target fungus

'Northland' blueberry fruits from Shenyang Agricultural University, which were free from diseases and pests and had uniform size, color, and maturity, were selected for tests. T4O (99 %) was obtained from Yuanye Biotechnology Co., LTD. The target fungus *A. tenuissima* (B20190712E1), previously isolated from decaying blueberries, was identified through morphological characteristics and molecular phylogenetic trees and cultured on Potato Dextrose Agar medium (PDA) at 28 °C for subsequent experiments.

### 2.2. In vitro experiment

#### 2.2.1. Suppressing effect of T4O on colony growth of *A. tenuissima*

Under aseptic conditions, a target fungus disc was placed on PDA, and put a sterile filter paper (6 mm) on the center of the petri dish lid. Various quantities of T4O (5, 10, 15, 20, and 25 µL) were added to the filter paper, achieving the concentrations as 0.05, 0.1, 0.15, 0.2, and 0.25 µL mL⁻¹ in a 100 mL petri dish space. Sterile water served as the control and incubated the dishes at 28 °C.

$$\text{The inhibition rate} (\%) = (D_1 - D_2) / D_1 \times 100$$

D1: the diameter of control group, D2: the diameter of treatment group.

#### 2.2.2. Inhibition effect of T4O on spore germination of *A. tenuissima*

Under aseptic conditions, spores from *A. tenuissima* colonies, grown at 28 °C for 7 d, were suspended in potato glucose water medium (PDB) to make a  $1 \times 10^5$  CFU mL⁻¹ suspension. The spore suspension and varying amounts of T4O were placed on hollow slides to reach the final concentrations as 0.05, 0.1, 0.2, 0.4, and 0.8 µL mL⁻¹; sterile water was the control. Incubation continued at 28 °C for 8 h before examining spore germination rates.

$$\text{Spore germination rate} (\%) = (\text{the number of germinated spores} / \text{the number of total spores}) \times 100$$

#### 2.2.3. Scanning electron microscopy (SEM)

The mycelia samples were preserved overnight at 4 °C in a 3 % (v/v) glutaraldehyde solution prepared with 0.06 mol L⁻¹ phosphate buffer, then washed for 20 min. Placed the samples on an ultra-low-temperature

freezer for 2 h with freeze-drying for 8 h. Sputter-coated the samples with a 20–30 nm layer of gold-palladium (45 s, 1.6 mA, 3 kV) to serve as a conductive medium (Polaron, Watford, UK). The morphology of mycelia was examined using a JSM-6610LV SEM (JEOL, Japan) with 2000 × magnification.

#### 2.2.4. Extracellular nucleic acid content of *A. tenuissima*

Various amounts of T4O (2.5, 5, 10, and 20 µL) were added to 50 mL PDB medium, creating concentrations of 0.05, 0.1, 0.2, and 0.4 µL mL⁻¹. Sterile water was served as the control. Then, *A. tenuissima* spore suspension (0.15 mL of  $1 \times 10^5$  CFU mL⁻¹) was introduced to each concentration treatment and incubated at 28 °C for 8 h and centrifuged 10 min at 10,000 × g, reported the absorbance at 260 nm.

#### 2.2.5. The intracellular soluble protein content of *A. tenuissima*

The control and treated groups were as in Section 2.2.4. Mycelia were harvested after centrifugation (10 min at 10,000 × g). Approximately 0.5 g of mycelia was pulverized with 4.5 mL normal saline using liquid nitrogen, and then centrifuged 10,000 × g at 4 °C. Added Coomassie Brilliant Blue solution (4.5 mL) to the supernatant, reacted for 10 min, then recorded the absorbance at 595 nm.

#### 2.2.6. Effect of T4O on malondialdehyde (MDA) content of *A. tenuissima*

The mycelia was prepared as the 2.2.4 section. Added different T4O concentrations (0.1, 0.2, and 0.4 µL mL⁻¹) to the mycelia. After incubation for 8 h, the samples were mixed with 3 mL of 0.8 % thiobarbituric acid (V/V). The mixture was subsequently heated in a boiling water bath for 30 min and centrifuged at 4 °C, 12,000 × g for 20 min. Recorded the absorbance at 450, 532, and 600 nm.

$$\text{MDA content (mmol kg}^{-1}\text{)} = [6.45(A_{532} - A_{600}) - 0.564A_{450}] \times 3 \times 0.5$$

#### 2.2.7. Effect of T4O on conductivity in *A. tenuissima*

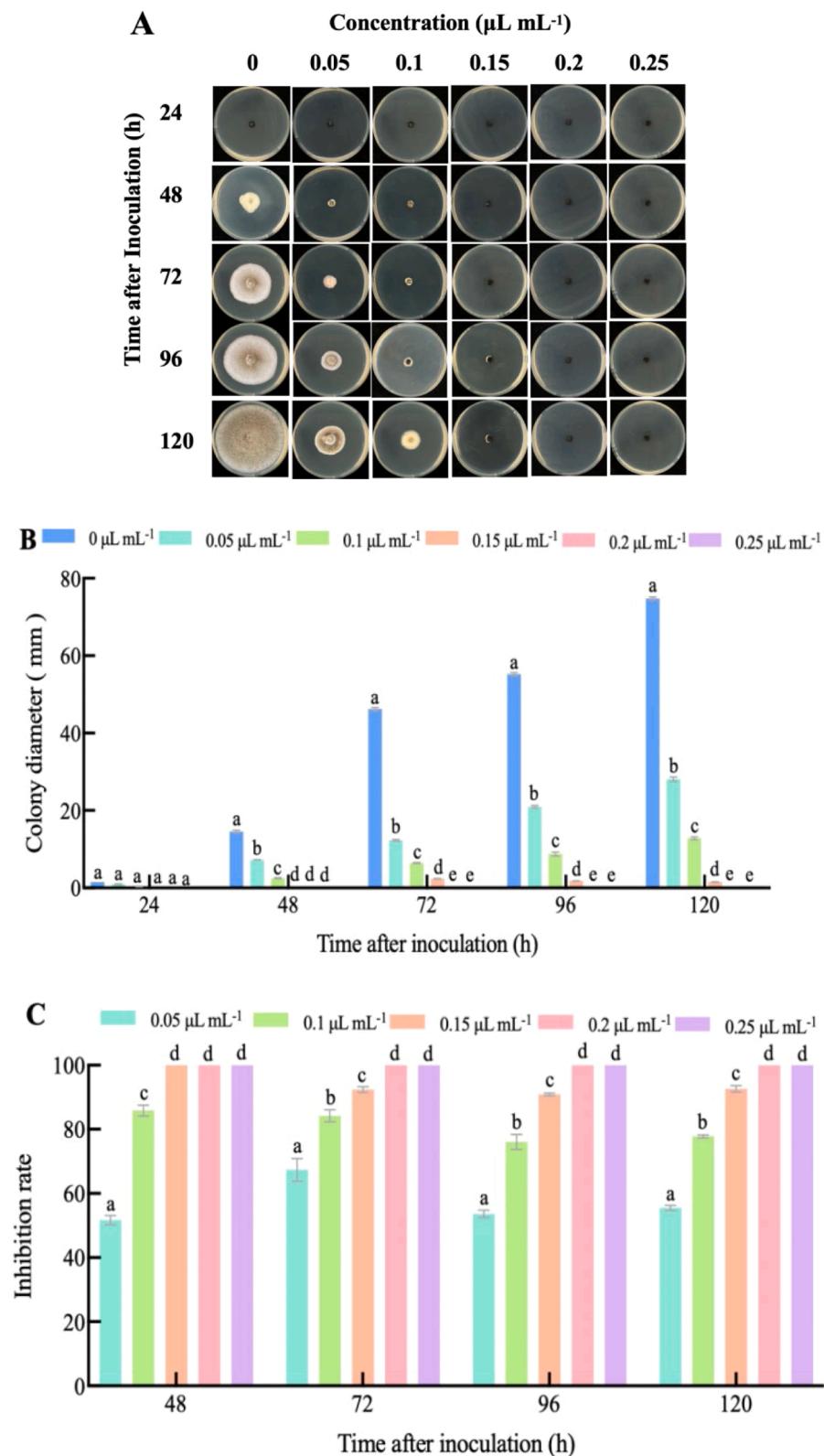
The control and treated groups were as in Section 2.2.4. Their conductivities were detected immediately after T4O was added (named L<sub>1</sub>). The samples were centrifuged at 10,000 × g for 20 min. The conductivities of these supernatants (named L<sub>2</sub>) were measured. After boiling all the supernatants for 30 min, their conductivities (L<sub>0</sub>) were assessed. The formula for relative electric conductivity was as follows:

$$\text{Relative electric conductivity (mS cm}^{-1}\text{)} = (L_2 - L_1) / L_0 \times 100$$

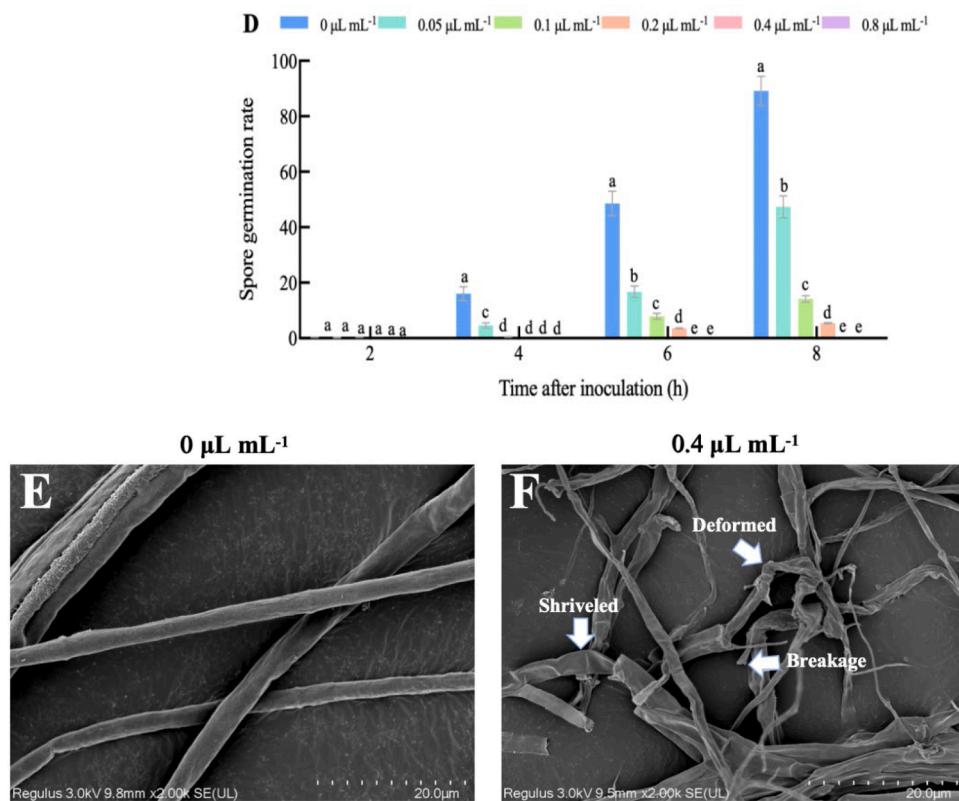
## 2.3. In vivo experiment

### 2.3.1. Biofumigation and disease assessment

Disinfected blueberry fruits were washed and placed in a sterile crisper (8 L). A sterile filter paper, soaked with 3.2 mL T4O, was positioned at the center of the lid, achieving a space concentration of 0.4 µL mL⁻¹. Distilled water served as the control. After 3 h of fumigation, the blueberries were moved to 96-well ice cells, inoculated with a 10 µL *A. tenuissima* spore suspension ( $1 \times 10^5$  CFU mL⁻¹) at the pedicle, and cultured on 85–90 % RH at 25 °C. Subsequently, the fruits were quick-



**Fig. 1.** Colony growth and spore germination of *Alternaria tenuissima* with 4-terpinol treatment. Colony of *A. tenuissima* on PDA with different concentrations of 4-terpinol (A). Statistical analysis of colony diameter (B). Statistical analysis of inhibition rate (C). Statistical analysis of spore germination (D). SEM images of the morphology of *A. tenuissima* in the control group (E). SEM images of the morphology of *A. tenuissima* in the treatment of  $0.4 \mu\text{L mL}^{-1}$  4-terpinol group (F). Magnifications,  $\times 2000$ . Bars=20  $\mu\text{m}$ . Approximately 200 spores were observed in 5 fields of each treatment. All target fungi were treated by 5 concentration of 4-ol by repeated 3 replicates. Vertical bars represented standard error of the mean. Columns marked with different letters indicated significant differences (\* $P < 0.05$ ).



**Fig. 1. (continued).**

frozen and stored at  $-80^{\circ}\text{C}$  for further tests. The disease index was calculated as follows:

#### 2.3.4. Measurement of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content in blueberry fruits

For  $\text{H}_2\text{O}_2$  measurements, the blueberry samples (3 g) were prepared

$$\text{Disease index} = [(\text{Level1 numbers of rot fruits} \times 1 + \text{Level2 numbers of rot fruits} \times 2 + \text{Level3 numbers of rot fruits} \times 3 + \text{Level4 numbers of rot fruits} \times 4) / \text{total fruits number}] \times 100$$

Disease severity levels (1–4 level): colony diameter  $\leq 1$  mm as Level 1; 1 mm  $<$  colony diameter  $\leq 5$  mm as Level 2; 5 mm  $<$  colony diameter  $\leq 10$  mm as Level 3; colony diameter  $> 10$  mm as Level 4. A higher level means more disease severity.

#### 2.3.2. Measurement of fruit firmness and TSS

Fruit firmness was assessed with TA.XTC-18 Texture Analyzer (Jiangsu, China) and expressed as Newtons (N).

The total soluble solids (TSS) content was tested by the Brix refractometer (DLX-SDJ1527, China).

#### 2.3.3. Measurement for the secondary metabolites

The procedure was performed following the method of Wang et al. (2024), with minor modifications. The blueberry samples (3 g) were mixed with 4 mL HCl-methanol with ice bath, and centrifuged at 12,000  $\times g$  for 10 min. The absorbance values at 280 nm and 325 nm were utilized to assess the overall content of phenols and flavonoids, respectively, while the difference in absorbance between 530 nm and 600 nm was used to quantify the anthocyanin content.

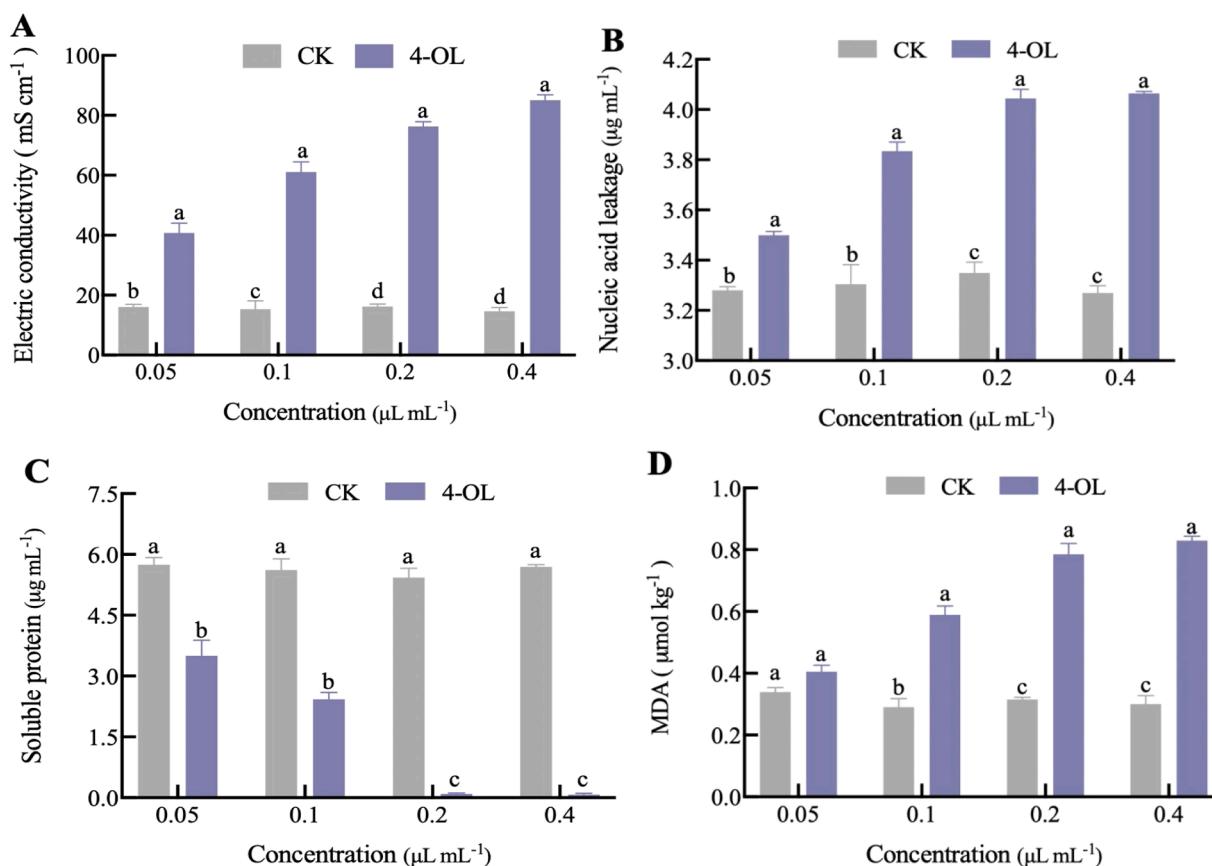
as mentioned in Section 2.3.3, following the method of Li et al. (2024).

#### 2.3.5. Measures of disease resistance enzyme activities

The activities of  $\beta$ -1,3-glucanase (GLU) and chitinase (CHI) were measured with the specific ELISA kits. The absorbances were recorded at 540 and 630 nm, respectively. One unit (U) of enzyme activity was defined as the change in absorbance per minute, with the results expressed as U kg $^{-1}$ .

#### 2.3.6. Analysis of antioxidant enzyme activities

Following the methods of Xu et al. (2024) for catalase (CAT) and superoxide dismutase (SOD), and Han et al. (2023) for ascorbate peroxidase (APX) and peroxidase (POD), the enzyme activities were measured with an automated microplate reader. Absorbances for the CAT, POD, APX, and SOD were read at 240, 470, 290, and 560 nm, respectively. A change of absorbance per minute indicated an enzyme activity unit (U), and the results were presented for U kg $^{-1}$ .



**Fig. 2.** Relative conductivity, nucleic acid material leakage, soluble protein, MDA content of *Alternaria tenuissima* mycelium. (A) Relative conductivity. (B) Nucleic acid material leakage. (C) Soluble protein. (D) MDA content. Vertical bars represented standard error of the mean. Columns marked with different letters indicated significant differences (\* $P < 0.05$ ).

#### 2.4. Quantitative real-time PCR of key enzyme genes

Blueberry RNA was isolated using the Kangwei Omini Plant RNA Kit, the cDNA was synthesized with the HiFi Script cDNA Synthesis Kit, and qPCR was measured with the Kangwei Ultra SYBR Mixture (Low Rox) Kit. The primers were designed using Primer Premier 5.0 software (Table 1) and manufactured by Sangon Bioengineering Co., Ltd.

#### 2.5. Data analysis

Each group was set with three parallels and the experiment was repeated three times. GraphPad Prism 9 software was employed for data plotting. Data analysis was performed using SPSS Statistics 25, and the Analysis of Variance (ANOVA) was used to determine the significant differences followed by Duncan's test or t-test. The data were presented as mean  $\pm$  standard deviation, with statistical significance considered at  $P < 0.05$ .

### 3. Results

#### 3.1. The inhibition effect of T4O on *A. tenuissima*

As the concentration of T4O increased, it significantly inhibited the colony growth (Fig. 1A-C). After 120 h, the colony growth of *A. tenuissima* was reduced by 50 % at  $0.05 \mu\text{L mL}^{-1}$  T4O, and  $0.2 \mu\text{L mL}^{-1}$  T4O completely suppressed the growth. Furthermore, T4O also suppressed spore germination (Fig. 1D). Within 8 h, treatments with  $0.05$ ,  $0.1$ , and  $0.2 \mu\text{L mL}^{-1}$  T4O reduced the germination rate from 95.8 % to 41.3 %, 18.6 %, and 5.7 %, respectively. The concentrations of  $0.4$  and  $0.8 \mu\text{L mL}^{-1}$  T4O fully inhibited spore germination. SEM

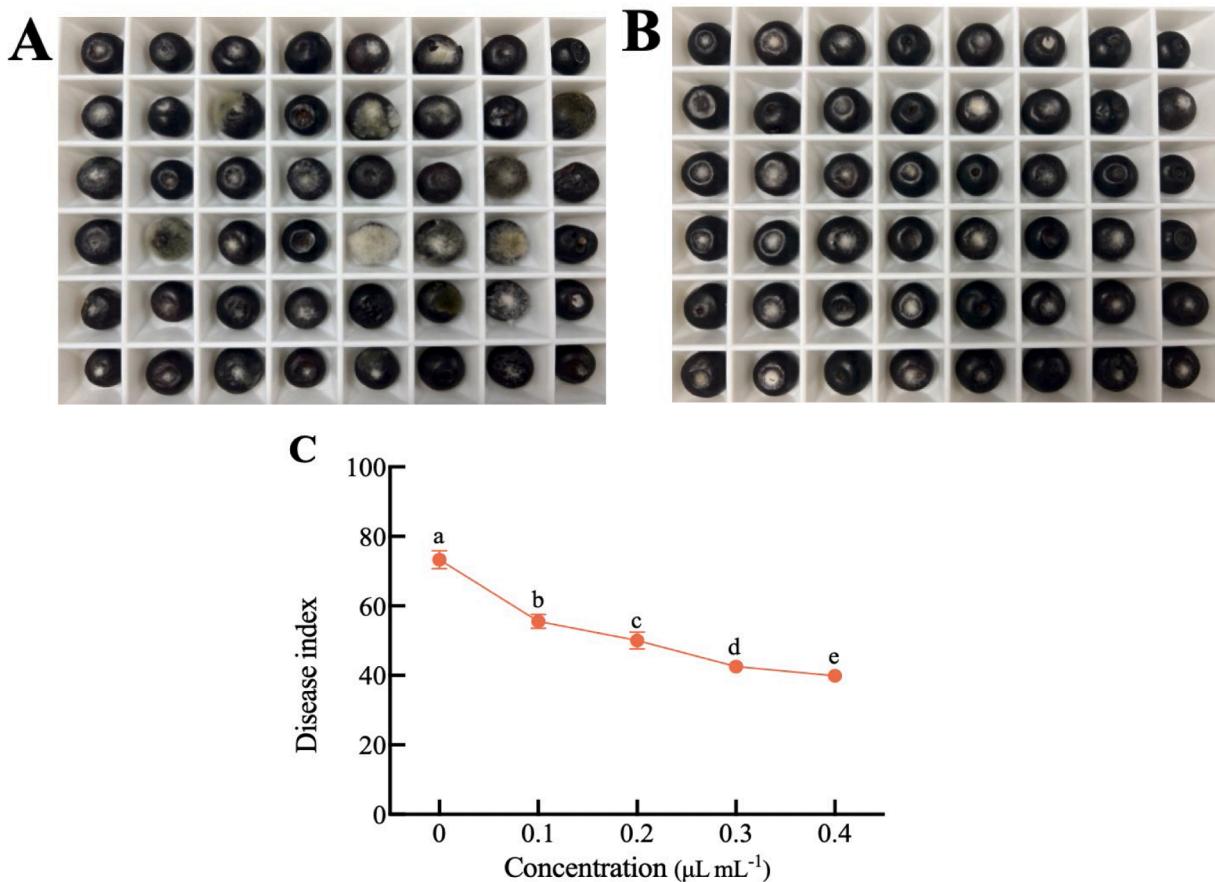
observations implied that the mycelia without T4O treatment were smooth and intact, free of visible damage (Fig. 1E), while the mycelia exposed to  $0.4 \mu\text{L mL}^{-1}$  T4O exhibited shriveled, deformed, and broken (Fig. 1F).

#### 3.2. Effect of T4O on structure and membrane permeability of *A. tenuissima*

The relative electrical conductivity in the control group remained stable at approximately 18 %, while it increased in the T4O-treated groups ( $0.05$ ,  $0.1$ ,  $0.2$ , and  $0.4 \mu\text{L mL}^{-1}$ ) by 51.2 %, 67.4 %, 88.3 %, and 90.6 %, respectively (Fig. 2A). After treating with  $0.2 \mu\text{L mL}^{-1}$  T4O, a significant release of  $4.08 \mu\text{L mL}^{-1}$  nucleic acids was observed with minimal soluble proteins detected in *A. tenuissima* (Fig. 2B-C). MDA levels were assessed to evaluate membrane lipid peroxidation in *A. tenuissima*. Compared to the control, MDA levels in the  $0.1$ ,  $0.2$ , and  $0.4 \mu\text{L mL}^{-1}$  T4O treatment groups increased by 0.47, 1.38, and 1.41 times, respectively (Fig. 2D), indicating that T4O disrupts cell membrane permeability and induced lipid peroxidation, leading to electrolyte leakage in a dose-dependent manner.

#### 3.3. T4O reduced blueberry disease index

After 5 d, the disease index in the control group was 73.4 %, while it in the treatment group was only 39.9 %. This indicated that T4O treatment decreased both the incidence rate and severity of disease in blueberries (Fig. 3A-C).



**Fig. 3.** Symptoms of blueberry in control (A) and 4-terpinol treatment groups (B) at 5 d Disease index at 5 d (C). Vertical bars represented standard error of the mean. Columns marked with different letters indicated significant differences (\* $P < 0.05$ ).

#### 3.4. T4O treatment increased GLU and CHI activities of blueberries

GLU and CHI are pathogenesis-related proteins (PRs) that participate in plant resistance against pathogens (Han et al., 2024). The GLU activity in blueberries initially increased and then declined in both groups. Overall, GLU activity was higher in the T4O-treated group. At 2 d, GLU activity in the T4O-treated group peaked at 2.2 times higher than in the control (Fig. 4A). Similarly, CHI activity was greater in the T4O group at 1 and 4 d (Fig. 4B).

*VcGLU* expression in blueberry fruits followed a similar initial increase and subsequent decrease in both groups. However, the peak expression in the T4O group at 0.5 d reached the 1-d peak of the control group, which was 47.6 % higher on day 2 (Fig. 4C). *VcCHI* expression remained relatively stable in the control group, but in the T4O group, it enhanced by 24.6 % at 1 d (Fig. 4D). This highlighted the potential of T4O in enhancing blueberry resistance and emphasized the significance of T4O in plant defense mechanisms studies.

#### 3.5. T4O treatment maintained the quality of inoculated blueberry

The T4O treatment group exhibited a delay in both hardness and water loss of blueberry fruits, without negatively affecting the TSS of the fruits (Fig. 5A-C). In addition, T4O treatment also enhanced the accumulation of the secondary metabolite in inoculated blueberries. Generally, the content of total phenols, flavonoids, and anthocyanins in blueberry fruits increased during the storage (Fig. 5D-F). Specifically, the total phenol content of the treatment blueberry fruits exhibited higher content in 2–5 d. Additionally, at 1 d, the flavonoid content showed a significant increase of 22.37 %.

The anthocyanin content in both groups achieved its peak at 2 d,

then a sharp decline of 37.2 % occurred in the control group at 3 d, while a slight decrease of 7.4 % was observed in the T4O treatment group. This indicated that T4O treatment promoted the accumulation of secondary metabolites in blueberries, enhancing blueberry resistance to *A. tenuissima* and maintaining quality.

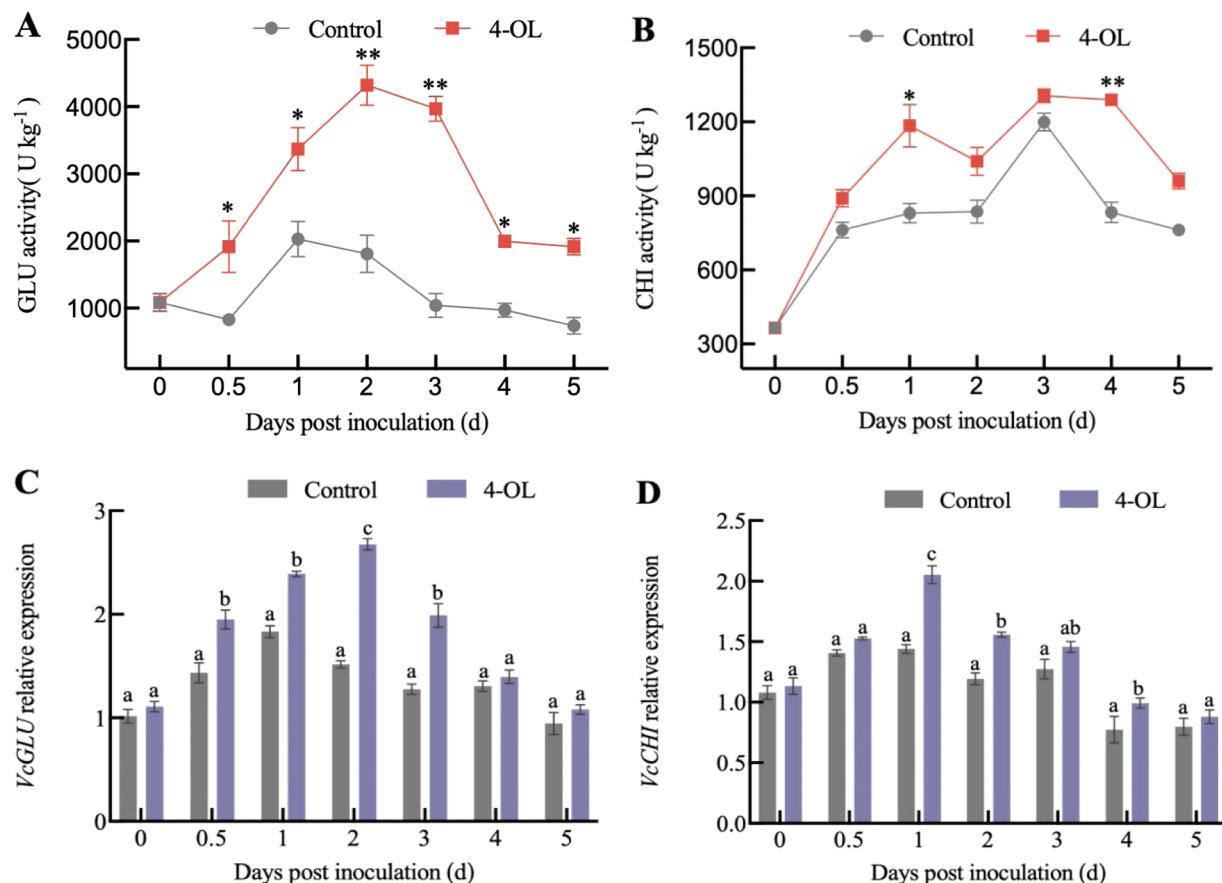
#### 3.6. T4O treatment induced early oxidation outbreak in inoculated blueberries

As a byproduct of plant aerobic metabolism,  $\text{H}_2\text{O}_2$  functions as a signal in managing oxidative stress in plants (Lin et al., 2023). Here, the  $\text{H}_2\text{O}_2$  content exhibited a similar trend in both groups (Fig. 6A). However, at 1 d, it was elevated in the T4O treatment group by 19.3 % compared to day 0 and subsequently declined rapidly, suggesting that T4O treatment facilitated an early onset of oxidation in blueberry. The hydrogen peroxide levels in blueberries from 2–5 d in the T4O group were reduced by up to 21.1 % compared to the control group.

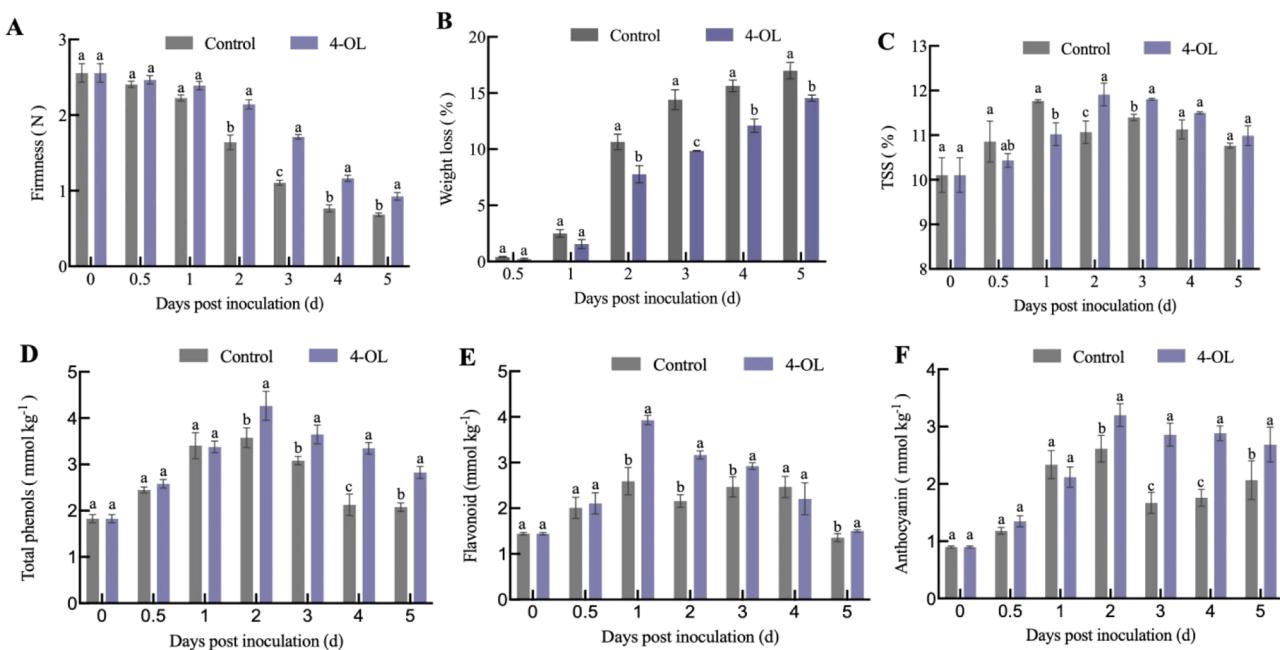
In addition, the MDA levels in treatment blueberries remained consistently lower compared to the control (Fig. 6B), it was 44.62 %, 41.71 %, and 27.26 % lower at 2, 3, and 5 d, respectively, suggesting that T4O treatment effectively mitigated membrane lipid peroxidation in blueberries.

#### 3.7. T4O activated the inoculated blueberry antioxidant defense system

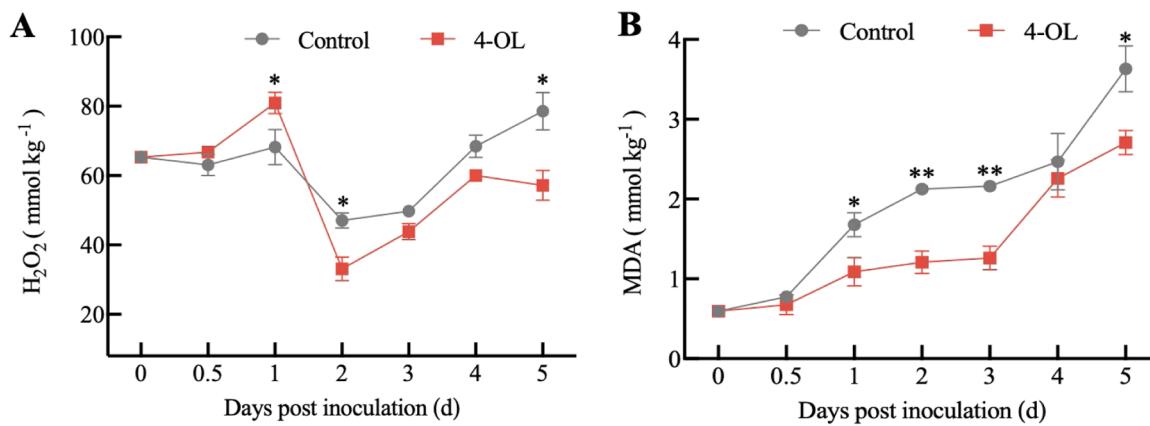
The SOD activity in the control group exhibited a 58 % decrease compared to 0 d and then reached the lowest value, about 31 % of the 0 d level, on 1 d. The T4O group maintained the 0 d level during 0.5 d to 1 d, which was approximately 72 % higher than the control (Fig. 7A). The POD activity of blueberries in the control group exhibited a 35.4 %



**Fig. 4.** Enzyme activities and gene expressions of  $\beta$ -1,3-glucanase (GLU) and chitinase (CHI) of blueberry in control and 4-terpinol groups. The activities of GLU and CHI were determined on a fresh weight basis. Asterisks denote significant differences between the two groups at each sampling time, which was determined by an independent sample *t*-test (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ).



**Fig. 5.** The firmness (A), TSS (B), weight loss (C), total phenols (D), flavonoid (E) and anthocyanin contents (F) of blueberry in control and 4-terpinol group. Vertical bars represented standard error of the mean. Columns marked with different letters indicated significant differences (\* $P < 0.05$ ).



**Fig. 6.**  $\text{H}_2\text{O}_2$  (A) and MDA (B) contents of blueberry in control and 4-terpinol group. Asterisks denote significant differences between the two groups at each sampling time, which was determined by an independent sample t-test (\*  $P < 0.05$ ).

decline from 0 d to 2 d. In contrast, T4O upregulated the POD enzyme activity up to 50.25 % during the same period. At 2 d, it in the T4O group reached its peak value, which was 2.4 times higher than the group without T4O (Fig. 7B). Furthermore, APX enzyme activity generally decreased in both groups (Fig. 7C). However, the decline in the T4O group was much slower during 1 d to 3 d compared to the other group. Additionally, CAT enzyme activity on days 2, 4, and 5 exhibited an increase of 26.5 %, 27.1 %, and 28.4 %, respectively (Fig. 7D).

The gene expression level of antioxidant enzymes was assessed using qPCR. In the control group, there was minimal fluctuation observed in the VcSOD gene expression from 0.5 d to 3 d (Fig. 7E). However, in the T4O treatment group, a slight upregulation was observed at 0.5 d, followed by a peak at 1 d, occurring 0.5 days earlier than the control blueberries. The expression of the VcPOD24 gene in the control group showed a down-regulation at 1 d, gradually recovering to the 0 d level by 2 d. In contrast, the T4O group sustained a higher level from 0.5 d to 2 d, not downregulating until 3 d, and was 35.7 % greater than the control group at 2 d (Fig. 7F). The expression of the VcAPX gene showed the same trend in both groups (Fig. 7G). Among them, the T4O treatment group showed a higher and earlier response compared to the control group from 2 d to 4 d. In the control group, upregulation of the VcCAT2 gene was observed on 1 d, followed by peak expression level and subsequent decrease (Fig. 7H). Conversely, in the treatment group, upregulation of the VcCAT2 gene occurred earlier, at 0.5 d than the control group. It reached the peak level on 2 d and started down-regulating on 3 d, which was 1 day later than in the control group. Notably, even at 4 d, it remained improved by 44.6 % compared to the control group.

#### 4. Discussion

*A. tenuissima* has been identified as the main pathogen causing AFR during storage time (Mao et al., 2023). T4O has a light aroma and was reported for mite removal and treating skin inflammation in humans (Li et al., 2025). However, its inhibitory effect on *A. tenuissima*, as well as its ability in controlling blueberry AFR has not been reported. In this study, the *in vitro* inhibitory activity of T4O against *A. tenuissima* was examined by spore germination, colony growth, mycelium morphology, and mycelium conductivity, nucleic acid and protein leakage. Further, the blueberries were fumigated with 0.4  $\mu\text{L mL}^{-1}$  T4O, the disease index, GLU and CHI enzyme activities as well as gene expressions of blueberries were measured to assess blueberry disease resistance. The firmness, TSS, and secondary metabolite content were measured to evaluate blueberry quality. Meanwhile, SOD, POD, CAT, and APX enzyme activities and gene expressions of blueberries were determined to assess the antioxidant ability of blueberries.

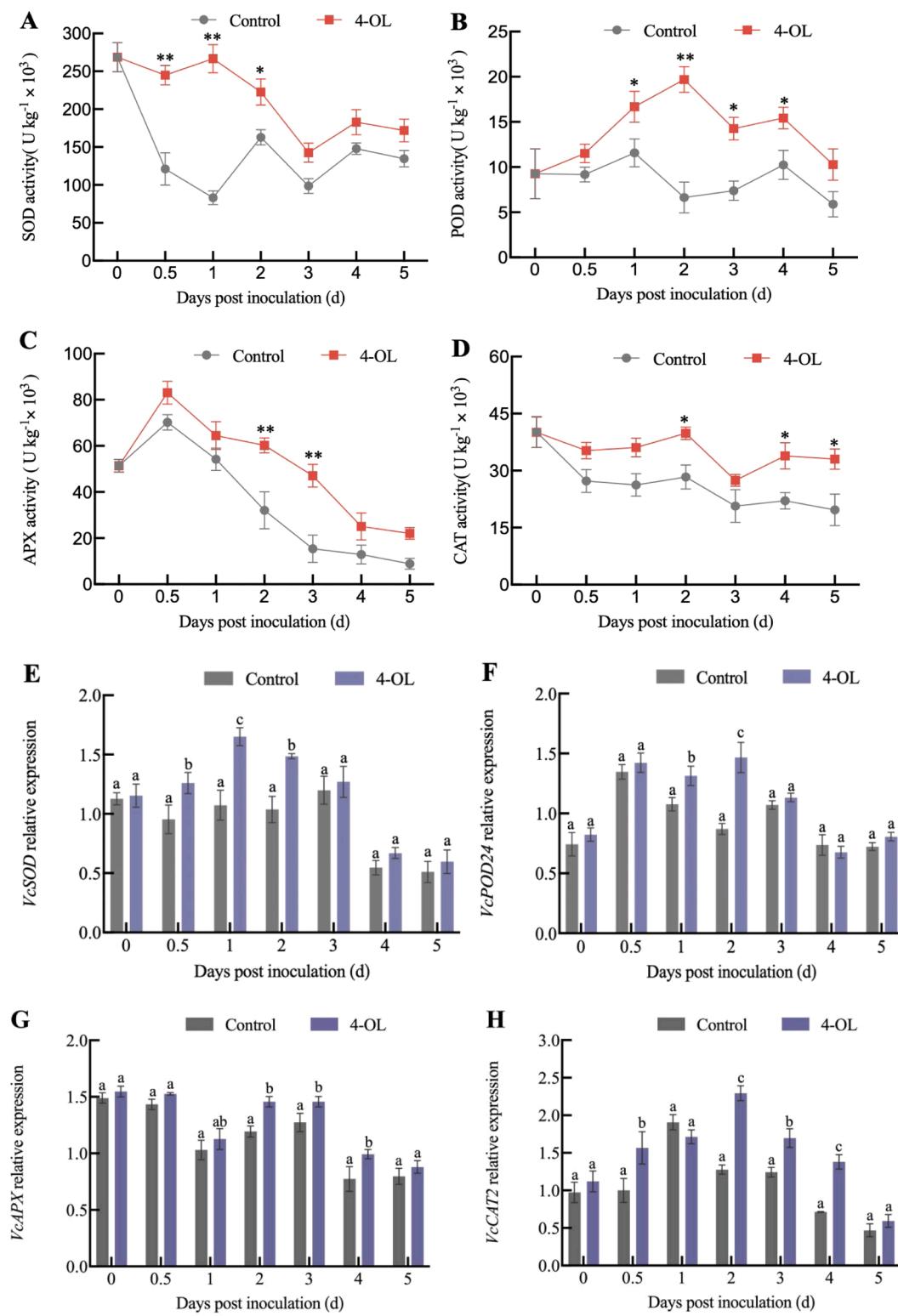
The inhibition effect of T4O was evident on the colony growth and spore germination of *A. tenuissima*. Colony growth was completely

ceased at 0.2  $\mu\text{L mL}^{-1}$  of T4O. At 0.4  $\mu\text{L mL}^{-1}$ , no spore germinated, indicating a higher sensitivity of colony growth to T4O than spore germination. The cell membrane acts as a critical barrier, controlling the entry and exit of substances to maintain intracellular balance and support biochemical processes (Xue et al., 2023). Treatment with 0.2  $\mu\text{L mL}^{-1}$  T4O increased relative conductivity, causing significant leakage of nucleic acids and proteins, suggesting that T4O compromised the cell membrane permeability in *A. tenuissima*. Cordeiro et al. (2020) reached a similar conclusion with 0.5  $\mu\text{L mL}^{-1}$  T4O treatment on *Staphylococcus aureus*.

As mentioned, T4O effectively inhibited *A. tenuissima* *in vitro*. However, its ability to control postharvest AFR in blueberries was uncertain due to the complex surface microenvironment of the fruit, which was conducive to pathogenic fungi. Cai et al. (2023) reported that 6.4  $\text{mmol L}^{-1}$  menthol completely inhibited *Geotrichum citri-aurantii* growth, while at least ten-times concentration was needed to defense citrus diseases caused by *G. citri-aurantii*. Our findings showed that 0.4  $\mu\text{L mL}^{-1}$  T4O effectively controlled blueberry AFR, which was only twice than the concentration of colony growth inhibition. GLU and CHI, two proteins related to disease resistance, are known to enhance plant defense from the fungi (Liu et al., 2022). Wang et al. (2023) discovered that L-arginine improved the resistance in blueberry by increasing GLU and CHI activities. In our study, T4O treatment enhanced the activities of GLU and CHI and promoted the earlier expression of *VcGLU* and *VcCHI*. This was consistent with previous findings on blueberry and grape treatments (Wang et al., 2023; Claudiane da Veiga et al., 2024). Therefore, we concluded that T4O enhanced blueberry resistance against *A. tenuissima* by boosting GLU and CHI activities and gene expression.

Previous studies have shown that a limited quantity of ROS generated in the early stages acts as signaling molecules (Qi et al., 2024). They trigger defense responses and stimulate the synthesis of disease-resistant compounds in fruits, thereby improving the resistance (Ali et al., 2023). However, excessive ROS may accelerate lipid peroxidation and fruit senescence (Jhanji et al., 2024). In this study,  $\text{H}_2\text{O}_2$  content in the T4O group increased on 1 d, causing an early oxygen burst in blueberry fruits. Then, a decrease of blueberry  $\text{H}_2\text{O}_2$  and MDA levels from 2 d to 5 d indicated that T4O treatment activated the oxidative defense system of the fruit, reducing damage from pathogens. Similar phenomena were observed with treatments using potassium cinnamate and burdock fructooligosaccharides on blueberries (Li et al., 2024; Wang et al., 2023).

Antioxidant enzymes such as POD, SOD, APX, and CAT were crucial for disease resistance in plants (Zhao et al., 2024). The addition of T4O activated the activity of these enzymes and increased the expression of *VcSOD*, *VcCAT2*, *VcPOD24*, and *VcAPX*. In the control group, the SOD activity of blueberries decreased 47.1 % at 1 d due to *A. tenuissima* stress. In contrast, the T4O group maintained initial SOD levels, with



**Fig. 7.** Enzyme activities and gene expressions of blueberry in control and 4-terpinol groups. The activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activities were determined on a fresh weight basis. Asterisks denote significant differences between the two groups at each sampling time, which was determined by an independent sample *t*-test (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ).

undisturbed gene expression. Li et al. (2025) found that the SOD activity of blueberry without *A. tenuissima* inoculation was declined only 16.8% at 1 d. This indicated that *A. tenuissima* might inhibit the synthesis of antioxidant enzymes in blueberries, led to the reduction of antioxidant activity after inoculation. POD activity in the control group fluctuated slightly, while that in the T4O group exhibited 43% increase on 1 d. In

T4O treatment group, *VcCAT2* was enhanced at 0.5 d, which was made earlier response to the oxidative stress damage.

Overall, T4O seemed vital for maintaining homeostasis by scavenging excessive ROS and preventing oxidative stress damage in blueberry fruits, suggesting that fumigation with 0.4  $\mu\text{L mL}^{-1}$  T4O could be used as an effective treatment to controlling blueberry AFR.

## 5. Conclusion

The treatment of  $0.2 \mu\text{L mL}^{-1}$  T4O effectively suppressed the colony growth of *A. tenuissima* and induced plasma membrane damage, leading to the release of nucleic acids and soluble proteins from the membrane and an increase in relative conductivity. On the other hand, fumigation treatment with  $0.4 \mu\text{L mL}^{-1}$  T4O mitigated the disease index of inoculated blueberries, and thus preserved their quality and stimulated the accumulation of secondary metabolites. Treatment with T4O enhanced both the activity and expression of PRs in inoculated blueberries, thereby increasing fruit resistance. Furthermore, T4O induced an initial outbreak of ROS, which triggered an early defense response to the disease. T4O treatment also activated the antioxidant defense system in blueberries, increased the enzyme activity and gene expression of SOD, POD, CAT, and APX, and protected blueberries from oxidative stress. Therefore, we suggested that T4O could serve as a natural antifungal agent for enhancing disease resistance in blueberries through the activation of PRs and ROS pathways.

## CRediT authorship contribution statement

**Yuxuan Li:** Methodology, Writing – review & editing, Writing – original draft, Software, Formal analysis, Data curation. **Jiaqi Wang:** Software, Methodology, Formal analysis, Data curation. **Sixu Lin:** Software, Methodology, Formal analysis. **Ling Yang:** Software, Methodology. **Bingxin Sun:** Software, Resources. **Yunhe Zhang:** Software, Resources. **Yufeng Xu:** Resources, Software. **Xuerui Yan:** Writing – review & editing, Resources, Investigation, Funding acquisition, Formal analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data availability

Data will be made available on request.

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