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Geraniol causes apoptosis in Colletotrichum gloeosporioides by inducing a burst of ROS

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Competing interests
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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Abbreviations
ROS, Reactive oxygen species; CAT, Catalase; POD, Peroxidase; SOD, Superoxide dismutase; GSH, Glutathione peroxidase; NIST, National Institute of Standards and Technology; PBS, Phosphate Buffered Saline; PDW, Platelet distribution width; DCHF-DA, 2,7-dichlorodihydrofluorescein diacetate; PCR, Polymerase Chain Reaction; DPI, Diphenyl iodonium chloride; NAC, N-acetylcysteine; TPB, Trypan blue; NADPH, Triphosphopyridine nucleotide; CEO, Clove essential oil; GC-MS, Gas Chromatography-Mass Spectrometry; MIC, Minimum inhibitory concentration; MLC, Minimum lethal concentration; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; PDB, Protein Data Bank; AEO, Authorized Economic Operator; DMSO, Dimethylsulfoxide.

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Abstract
Anthracnose is a fungal disease caused by *Colletotrichum gloeosporioides*, which is one of the main causes of mango rotting and deterioration, so it is important to control anthracnose. The calendula essential oil is a volatile constituent extracted from calendula officinalis, which has attracted attention from researchers worldwide because of its excellent antimicrobial and antioxidant activities. However, studies on the antifungal mechanism of calendula essential oil and its active constituents against *C. gloeosporioides* have not been reported. Therefore, this paper preliminarily evaluated the *anti-C. gloeosporioides* activity of calendula officinalis essential oil and its main active constituent geraniol. We found that geraniol induced a reactive oxygen species (ROS) burst by modulating the expression of the NADPH oxidase subunit *NoxR* gene. Moreover, the activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and glutathione peroxidase (GSH) increased with increasing geraniol concentration, which demonstrated that the pro-oxidant property of geraniol was one of the main reasons for its inhibition of *C. gloeosporioides* growth. Furthermore, we found that geraniol induced apoptosis in *C. gloeosporioides* in a dose-dependent manner. In conclusion, these results provide a new reference for future exploration of the antifungal mechanism of geraniol against *C. gloeosporioides*.

Keywords: Geraniol; Colletotrichum gloeosporioides; Mango; Reactive oxygen species

Introduction

Mango (*Mangifera indica L.*) is an important tropical subtropical fruit, and grapes, oranges, apples, and bananas are the world's top five fruits. Mango has a unique aroma and contains various nutrients such as vitamins, proteins, dietary fibers, and minerals. Additionally, it is effective in lowering cholesterol, preventing and treating diabetes, exhibiting anti-tumor properties, and preventing cardiovascular diseases. Therefore, it is favored by consumers in various countries [1, 2]. However, ripe mango's soft texture and rich nutritional value expose it to serious fungal disease threats during postharvest storage and transportation. Among them, anthracnose caused by *C. gloeosporioides* is the most serious, it can cause 30% to 50% of mango to rot during storage and transportation, accounting for more than 70% of the total fruit diseases. Mango anthracnose is not only spread by various means such as insects and rainwater, but also survives in unfavorable growing conditions through mycelium or spores. This has severely limited the development of the mango industry. Currently, the vast majority of producers tend to use chemical methods for post-harvest preservation of mangoes. However, the long-term use of chemical preservatives or chemical pesticides not only leads to the development of resistance to pathogenic bacteria but also pollutes the environment [3–6]. Therefore, the development of various natural, green, and environmentally friendly novel antimicrobial agents has become a hotspot and common goal in the field of food science research at home and abroad.

In recent years, plant essential oils, as a kind of natural extracts, have been extensively studied for their antimicrobial and antioxidant activities in the post-harvest preservation of fruits and vegetables and some promising results have been achieved [7]. For example, recently, a researcher developed a pickering emulsion preservative paper containing clove essential oil (CEO) and investigated its effect on the shelf-life of tomatoes. It was found that the incorporation of CEO in the cling paper significantly reduced the incidence of tomato rot within 12 days [8]. Similarly, Popescu et al. [9] developed a chitosan-based edible coating incorporating plant essential oils that significantly extended the postharvest shelf life of strawberry and apple slices. This coating positively influenced ascorbic acid levels, total phenolic content, and antioxidant activity in the fruits during refrigeration. Furthermore, the application of thyme essential oil on mangoes not only prolonged their storage period by 3 days but also maintained a relatively low decay rate [10]. The use of thyme essential oil coatings in mango preservation demonstrates considerable potential for enhancing fruit quality and preventing decay. The potential of essential oils in the postharvest preservation of fruits and vegetables has been confirmed by several studies.

Calendula essential oil, a volatile compound extracted from *Calendula officinalis*, has garnered significant attention from researchers worldwide due to its exceptional antimicrobial and antioxidant properties. According to the literature, calendula essential oil exhibits strong inhibitory effects against various common pathogenic fungi, including *Penicillium*, *Aspergillus niger*, and *Candida albicans* [11, 12]. Although studies have documented the antifungal effects of calendula essential oil against pathogenic fungi, to the best of our knowledge, the antifungal mechanisms of calendula essential oil and its active constituents against *C. gloeosporioides* remain unreported.

In this study, we first analyzed the active constituents of calendula essential oil and conducted a preliminary evaluation of its antifungal activity, specifically focusing on geraniol, its main active component, against *C. gloeosporioides*. The results indicated that geraniol exhibited stronger antifungal activity against *C. gloeosporioides* than calendula essential oil. Building on these findings, we further investigated the antifungal mechanisms of geraniol against *C. gloeosporioides*. This research aims to provide a theoretical foundation for understanding the antifungal mechanisms of calendula essential oil and its active constituents, as well as their potential applications in the food industry.

Materials and methods

Experimental materials and reagents

C. Gloeosporioides was obtained from the China Industrial Microbial Strain Preservation and Management Center. Calendula essential oil and geraniol, both with a purity greater than 98%, were purchased from Shanghai McLean Biochemical Co.

Compositional analysis of calendula essential oil

The method described by Gao et al. [13] utilized gas chromatography-mass spectrometry (GC-MS) to identify the active constituents in calendula essential oil. The GC conditions were as follows: the initial temperature was set at 40 ℃, which was then increased to 70 ℃ at a rate of 2.5 ℃/min and held for 2 minutes. Next, the temperature was raised to 150 ℃ at 5 ℃/min and maintained for an additional 2 minutes, followed by an increase to 280 ℃ at 10 ℃/min, where it was held for 5 minutes. The injection port temperature was maintained at 250 ℃, and high-purity helium was used as the carrier gas with a flow rate of 0.5 mL/min. The injection volume was 0.1 μL, with a splitting ratio of 200:1. For the mass spectrometry conditions, ionization was achieved via electron bombardment at an electron energy of 70 eV, with an ion source temperature of 230 ℃ and a quadrupole temperature of 150 ℃. The composition of the essential oil was analyzed by comparing it to the standard mass spectra provided by the NIST Mass Spectrometry Library.

Determination of inhibition zone diameter

The method described by Ju et al. [14] was adapted with appropriate modifications. For the solid phase inhibition test, calendula essential oil and geraniol were diluted to final concentrations of 200 μg/mL, 250 μg/mL and 300 μg/mL using acetone. A specific volume of each solution was then pipetted onto a sterile filter paper disc, which was placed in the center of a bacteria-containing agar plate in an aseptic environment. The plates were incubated at 28 °C for 4 days. Acetone served as the control. The diameter of the inhibition zone was measured using a vernier caliper with a crosshatch method. In the gas-phase inhibition test, the pretreatment method was identical to that of the solid-phase inhibition test, with the only difference being that the filter paper discs containing calendula essential oil and geraniol were affixed to the inner side of the plate cover. The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values of *C. gloeosporioides* were determined using a twofold dilution method.

Determination of cell viability of *C. gloeosporioides* by MTT

The method described by Gao et al. [15] was adapted with slight modifications. A 100 μL spore suspension at a concentration of 1 × 106 mL-1 was inoculated into PDB medium and incubated at 28 °C for 4 days. The MIC of geraniol against *C. gloeosporioides* was determined to be 0.09375 μg/mL based on the results of the preliminary test. The mycelium was then divided into fragments ranging from 100 to 300 μm and treated with 0.5 MIC, 1 MIC and 2 MIC of geraniol for 24 hours. The wet weight of the mycelium was recorded after washing it twice with PBS buffer. Subsequently, excess geraniol was removed using DMSO, and the mycelium was rinsed with PBS. The mycelium was then resuspended in MTT solution and incubated with shaking at 28 °C for 90 minutes. After incubation, the mycelial precipitate was collected by centrifugation, and 800 μL of DMSO was added to dissolve the residue. The absorbance was measured at 560 nm. The experiment was performed in triplicate, and the average value was calculated.

Determination of Taipan blue staining and ROS accumulation

The method described by Ju et al. [16] was slightly adapted. First, 2 mL of spore suspension was inoculated into 28 mL of PDW for expansion culture, which was maintained for 48 hours. Following this, geraniol was added, and the culture was continued for an additional 24 hours. After the culture period, the cell suspension was collected by centrifugation and diluted as needed. The cell suspension was then

mixed with 0.4% Taipan blue solution in a volume ratio of 9:1, stained for 3 minutes, and examined using an electron microscope.

Intracellular ROS levels were measured using 10 μM of 2,7-dichlorodihydrofluorescein diacetate (DCHF-DA). The excitation and emission wavelengths were set at 488 nm and 520 nm, respectively.

Determination of antioxidant enzyme activity and *NoxR* gene expression

The activities of CAT, POD, SOD, and GSH were measured at 240 nm, 470 nm, 420 nm and 412 nm, respectively, in accordance with the kit protocol. The method described by Ju et al. [17] was utilized for these determinations. A reverse transcription kit was employed to synthesize the first strand cDNA, which was subsequently analyzed using a real-time fluorescence quantitative PCR instrument.

Based on the primer design presented in Table S1, the PCR program was as follows: pre-denaturation at 90 ℃ for 30 seconds, denaturation at 90 ℃ for an additional 5 seconds, annealing and extension at 55 ℃ for 30 seconds, followed by a total of 30 cycles. Relative expression levels were obtained by the $2^{-\Delta\Delta Ct}$ method.

Determination of vigor recovery in *C. gloeosporioides*

Place 5 mm bacterial discs in a solid medium containing 0.1 mM diphenyl iodonium chloride (DPI) or 10 mM N-acetylcysteine (NAC). Cultures were incubated at 28 °C for 2 days and evaluated for growth. To detect mycelial apoptosis, mycelium was stained with Taipan blue as described in 2.5. The accumulation of ROS in DPI-affected B. glabrata was then determined. 0.1 mM DPI or 10 mM NAC and different working concentrations of geraniol were added to the cultures, respectively, and incubation was continued for 24 h. Mycelium was collected for DCFH-DA staining.

Statistical analyses

All the experimental data were performed in triplicate, and then the average was calculated. Data were analyzed with Origin Lab 9.0 s. Means were compared by Duncan's new multiple-range test. Statistically significant differences were set at $P < 0.05$.

Results and discussion

Compositional analysis of calendula essential oil

A total of 20 major compounds were identified in the calendula essential oil by GC-MS, among which the top five major chemical constituents were geraniol (69.15%), 1,2-cyclohexanedicarboxylic acid (6.87%), camphor (4.28%), eucalyptol (4.21%), and 2,4,6-tribromophenylethyl decanoate (3.77%) (Table S2). The highest content of geraniol was found in calendula essential oil, which is consistent with the findings of Ložienė et al. which is consistent with the findings of Ložienė et al. [18]. Given that the geraniol content in calendula essential oil exceeded 50%, we evaluated the inhibition zone diameter of both calendula essential oil and geraniol against *C. gloeosporioides* under gas phase and solid phase conditions in subsequent experiments.

Inhibition of *C. gloeosporioides* by calendula essential oil and geraniol

As illustrated in Figure 1, the inhibitory effect of calendula essential oil on *C. gloeosporioides* was not pronounced in the gas-phase inhibition test, and no significant inhibition zone was observed. In contrast, the inhibition zone diameter for *C. gloeosporioides* increased continuously with increasing concentrations of geraniol. These results indicate that geraniol was more effective than calendula essential oil in inhibiting *C. gloeosporioides* in the gas-phase inhibition assay. In the solid-phase bacteriostatic test, both calendula essential oil and geraniol demonstrated significant inhibitory effects against *C. gloeosporioides*. As the inhibition concentration increased, both agents exhibited more pronounced inhibition zones. Notably, similar to the findings in the gas-phase inhibition test, the inhibition zone diameter in the geraniol treatment group was significantly larger than that in the calendula essential oil treatment group at the same concentration. This shows that geraniol has a better inhibitory effect than calendula essential oil. This is similar to the results of previous studies, for example, the inhibitory effect of geraniol present in oregano essential oil and thyme essential oil on *E. coli* was superior to that of essential oil mixtures under the same concentration conditions [19]. Based on this, we delved into the mechanism of action of carvacrol against *C. gloeosporioides* in the following experiments.

Effect of geraniol on the cell viability of *C. gloeosporioides*

Previous research indicated that anise essential oil reduced the cellular activity of *A. niger* as measured by MTT analysis [20]. Similarly, the effect of geraniol on the cellular activity of *C. gloeosporioides* was assessed using the same method. As illustrated in Figure 2, MTT absorbance decreased following treatment with various concentrations of geraniol, indicating that geraniol exposure reduced the viability of *A. niger*. Notably, at a concentration of 2 MIC, the absorbance of the sample decreased by 67.7% compared to the control. This shows that geraniol decreased the viability of *C. gloeosporioides* in a dose-dependent manner. This is similar to previous findings regarding Sharma et al. [21]. They found that geraniol also showed a dose-dependent reduction in Candida albicans cell viability.

Mechanism of action of geraniol on *C. gloeosporioides*

Effect of geraniol on apoptosis in *C. gloeosporioides*

The structural integrity of the cell membrane allows living cells to repel TPB, preventing its entry. In contrast, inactive cells or those with compromised membranes exhibit increased permeability, resulting in blue staining by TPB. As illustrated in Figure 3, the color intensity of the mycelium deepened with increasing concentrations of geraniol, indicating a corresponding rise in the degree of mycelial apoptosis. This effect was particularly pronounced at a geraniol concentration of 2 MIC. These findings suggest that geraniol induces apoptosis in *C. gloeosporioides* in a dose-dependent manner. The results align with the findings discussed in section 3.3 regarding cell viability. However, while geraniol can induce apoptosis in *C. gloeosporioides*, the precise mode of action remains unclear.

Effect of geraniol on the accumulation of ROS

High accumulation of ROS can lead to oxidative stress and ultimately induce apoptosis. DCFH-DA staining serves as an indicator of ROS accumulation in cells, with stronger green fluorescence correlating to higher levels of ROS production. As illustrated in Figure 4A, the green fluorescence within the mycelium of *C. gloeosporioides* increased with escalating concentrations of geraniol. This effect was particularly pronounced at a geraniol concentration of 2 MIC, indicating that geraniol induces ROS accumulation in *C. gloeosporioides* cells in a dose-dependent manner. A previous study reported that geraniol induced a dose-dependent increase in ROS in *Biomphalaria glabrata* [22]. This finding is further corroborated by the fluorescence spectra presented in Figure 4B. Additionally, previous research has demonstrated that citral can induce apoptosis in *A. niger* by triggering a burst of ROS [16]. Similarly, curcumin has been shown to induce apoptosis in *Botrytis cinerea* through a ROS burst [23].

Effect of geraniol on antioxidant enzyme activity

Given that geraniol stimulated the production of ROS in *C. gloeosporioides*, we further investigated the activities of antioxidant enzymes in the mycelium. As illustrated in Figure 5, the activities of CAT, SOD, GSH and POD significantly increased with rising concentrations of geraniol in a dose-dependent manner. Specifically, at a concentration of 2 MIC of geraniol, the activities of CAT, SOD, GSH, and POD were enhanced by 1143.2%, 864.9%, 675.1%, and 87.2%, respectively, compared to the blank control group. Previous studies have shown that these antioxidant enzymes are induced by H_2O_2 and reflect its accumulation [24]. Our current findings regarding geraniol support these earlier reports; however, the precise mechanism underlying geraniol-induced ROS generation remains to be fully elucidated.

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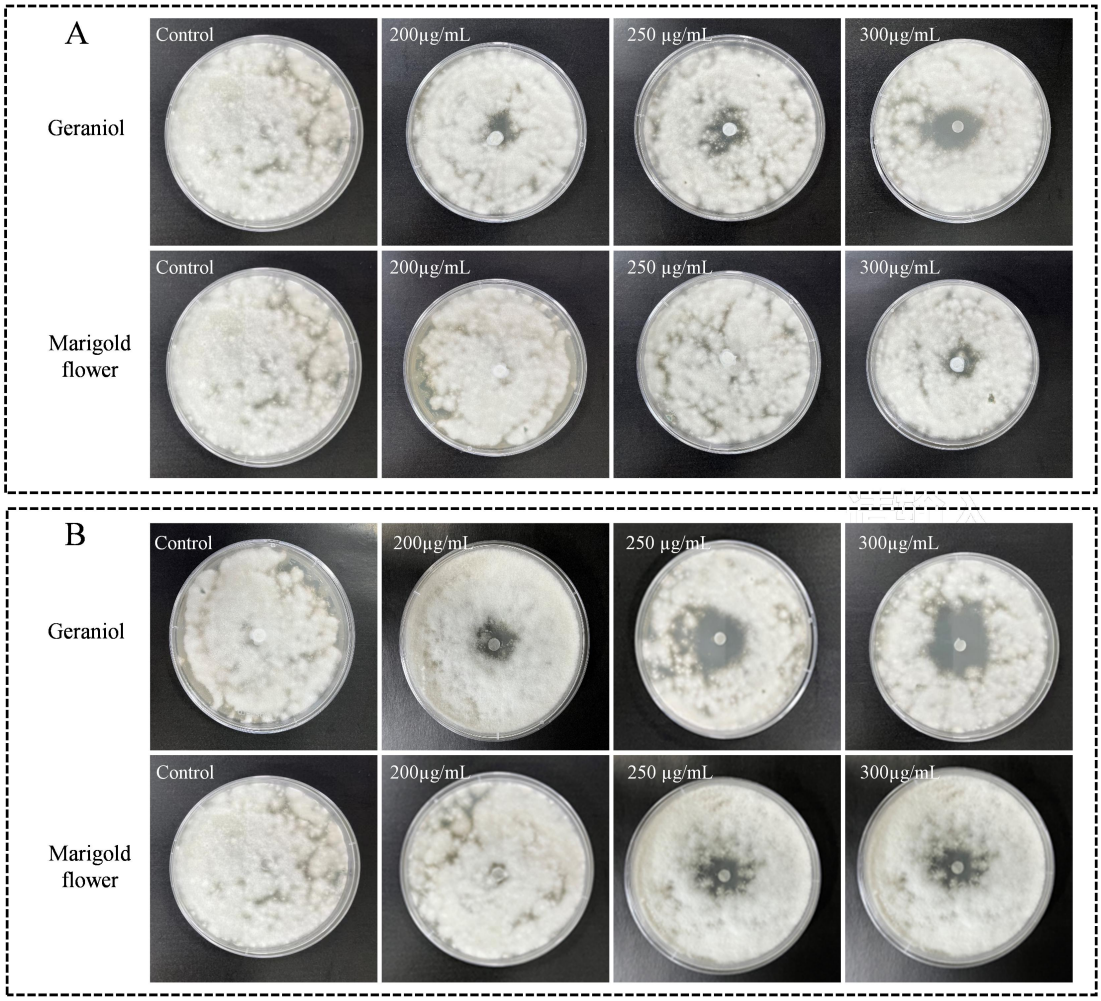

Figure 1. A: Changes in the inhibition zone diameter under the gas phase bacteriostatic experiment. B: Changes in the inhibition zone diameter under solid-phase antibacterial experimental conditions.

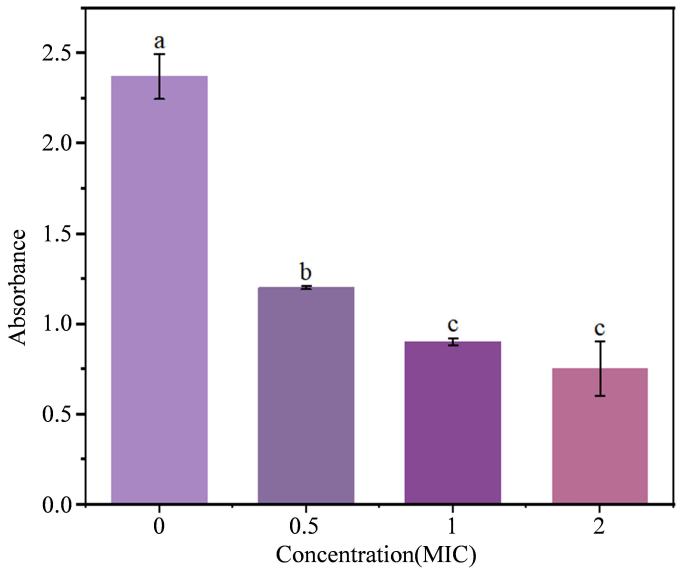

Figure 2. MTT-formamide production in *C. gloeosporioides* cells treated with geraniol for 24 h.

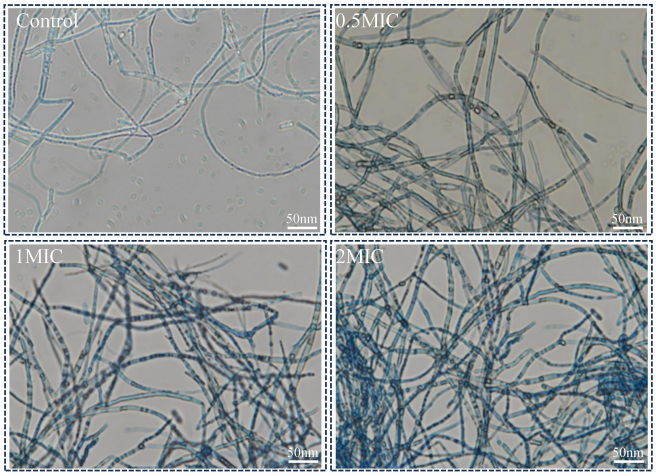

Figure 3. Effect of geraniol on apoptosis of *C. gloeosporioides*.

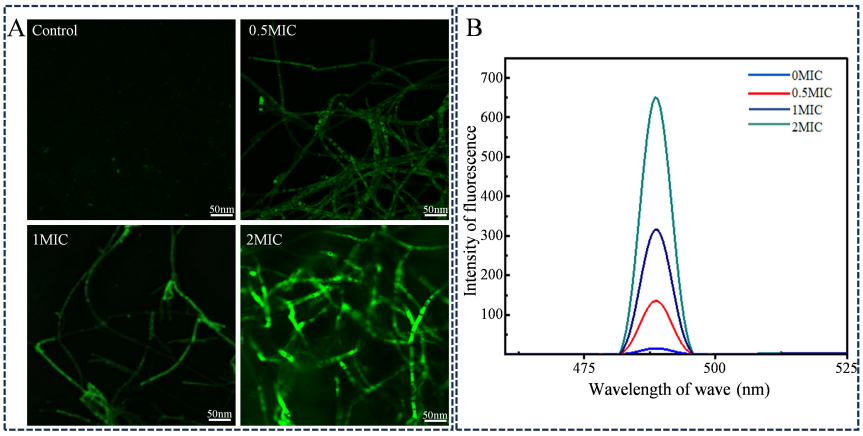

Figure 4. Effect of geraniol on the accumulation of ROS. A, Effect of geraniol on the accumulation of ROS in *C. gloeosporioides*. B, Fluorescence spectrum analysis.

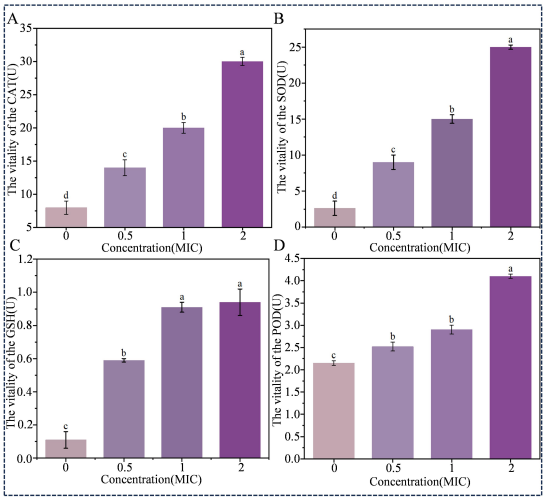

Figure 5. Effect of geraniol on antioxidant enzyme activity. A, B, C and D are changes in CAT, SOD, GSH, and POD activities, respectively.

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Effects of DPI and NAC on the Recovery of cell viability in geraniol-treated *C. gloeosporioides*

This study investigated the effects of ROS scavenger (NAC) and NADPH oxidase inhibitor (DPI) on geraniol-treated mycelium. By measuring the diameter of mycelial growth on the medium, we found that the medium supplemented with NAC partially restored the inhibitory effect of geraniol on *C. gloeosporioides* compared to the control (Figure 6A, B). This finding was further confirmed by Taipan blue staining experiments, This was also confirmed in Taipan blue staining experiments. The number of blue mycelium was significantly reduced in the samples with NAC added compared to the control, indicating that NAC restored the inhibitory effect of geraniol on *C. gloeosporioides* and prevented the inhibition of *C. gloeosporioides* by geraniol (Figure 6C). This reveals that the pro-oxidant property of geraniol is the main reason for its inhibition of *C. gloeosporioides* growth. In contrast, the number of blue mycelia in the DPI-treated samples did not decrease compared to the control, indicating that DPI did not enhance the inhibitory effect of geraniol on *C. gloeosporioides*.

Therefore, we further investigated the effect of DPI on geraniol-induced ROS accumulation in *C. gloeosporioides*. ROS are mainly produced within the mitochondrial respiratory chain of the cell, whereas NADPH oxidase is produced at the cell's plasma membrane. The fluorescence staining results in Figure 6D showed a tendency to decrease the staining intensity of mycelia with the addition of both DPI and NAC. Also, geraniol induced the expression of the NADPH oxidase subunit *NoxR* (Figure 6E). This suggests that geraniol-induced ROS accumulation in *C. gloeosporioides* is mediated by NADPH oxidase. This is consistent with previous findings on *C. gloeosporioides*. Hua et al. [23] found that curcumin significantly induced ROS production and triggered apoptosis in *Staphylococcus griseus*. It was confirmed by further studies that curcumin-induced ROS burst in *S. griseus* was mainly associated with the upregulation of the *NoxR* gene.

Conclusion

In the present study, the primary constituents of calendula essential oil were analyzed using GC-MS, revealing that geraniol comprised the highest content at 69.15% among the main components. The inhibitory mechanism of geraniol on *C. gloeosporioides* was further investigated. The results demonstrated that geraniol induced a ROS burst by regulating the expression of the NADPH oxidase subunit *NoxR* gene. The activities of CAT, POD, SOD and GSH increased with rising concentrations of geraniol, indicating that the pro-oxidant property of geraniol is a significant factor in its inhibition of *C. gloeosporioides* growth. Furthermore, geraniol induced apoptosis in *C. gloeosporioides* in a dose-dependent manner. In conclusion, the findings of this study provide new insights into the antifungal mechanism of geraniol against *C. gloeosporioides*.

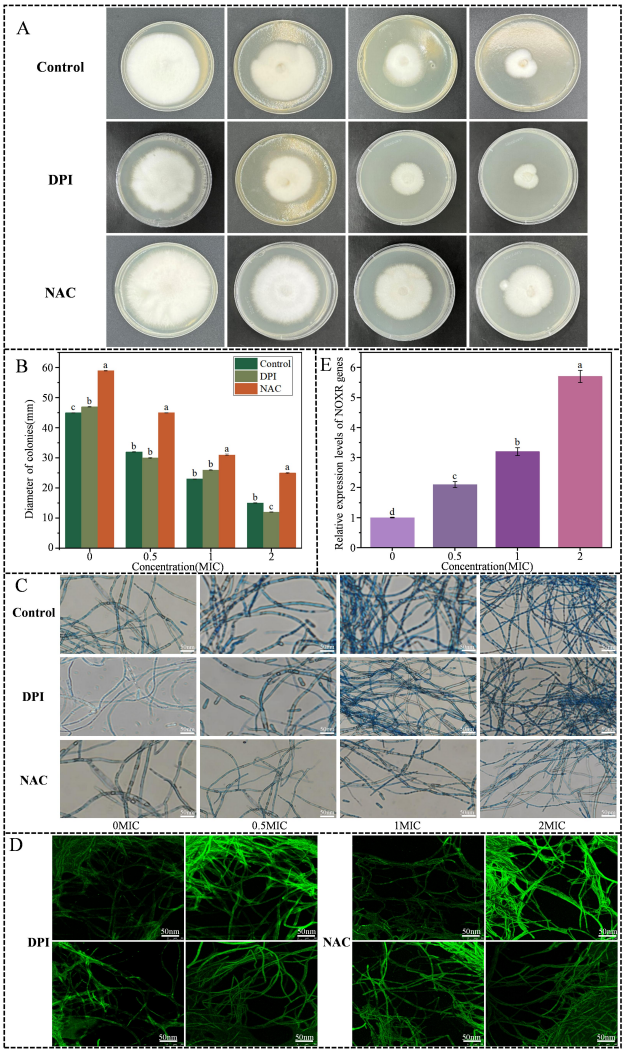

Figure 6. A: Visualization of the change in mycelial diameter of geraniol-treated *C. gloeosporioides* corrected by DPI and NAC. B: Statistical plots of changes in the mycelial diameter of geraniol-treated *C. gloeosporioides* amended by DPI and DPI. C: Effect of DPI and NAC on the mycelium of *C. gloeosporioides* treated with Geraniol. D: Accumulation of ROS in geraniol-treated *C. gloeosporioides* filaments by DPI and NAC. E. Effect of geraniol on *C. gloeosporioides NoxR* gene expression.

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