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Efficacy and Antifungal Mechanism of Rosemary Essential Oil against *Colletotrichum gloeosporioides*

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Abstract: The antifungal activity and mechanism of rosemary essential oil against *Colletotrichum gloeosporioides*, the walnut anthracnose pathogen, were investigated using scanning electron microscopy (SEM), index determination and transcriptome technique. The results showed that rosemary essential oil could inhibit the growth of *C. gloeosporioides* with minimum inhibitory (MIC) and fungicidal (MFC) concentrations of 15.625 µL/mL and 31.25 µL/mL, respectively. Scanning electron microscopy revealed that the mycelium morphology became shriveled, twisted, and severely deformed after being treated with rosemary essential oil. The activity of chitinase, which decomposes fungal cell wall components in *C. gloeosporioides*, increased. The ergosterol content in the plasma membrane decreased, while the cell contents including nucleic acids, soluble protein and soluble reducing sugar were released resulting in the extracellular electrical conductivity being changed. For metabolic activity, the enzymes succinate dehydrogenase (SDH), malate dehydrogenase (MDH), ATPase and ATP decreased, whereas phosphofructokinase (PFK) increased. Transcriptome sequencing results showed that the antifungal mechanism of rosemary essential oil involves the destruction of the cell wall and membrane, inhibition of genetic material synthesis, and cell division and differentiation. The results are helpful to understand the efficacy and antifungal mechanism of rosemary essential oil against *C. gloeosporioides* and provide a theoretical basis for the development of rosemary essential oil as a biological control agent.

Keywords: *Colletotrichum gloeosporioides*; rosemary essential oil; antifungal mechanism; transcriptome

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1. Introduction

Walnuts are an excellent tree with ecological, economic and social benefits. While walnut anthracnose has become one of the key diseases that leads to a reduction in walnut yield and quality, anthracnose is one of the world's top ten plant fungal pathogens, with a wide range of hosts and highly destructive [1]. The main pathogen for walnut anthracnose is *Colletotrichum gloeosporioides*. Currently, many synthetic chemical fungicides, such as chlorothalonil and carbendazim, are used for walnut anthracnose control. The negative effects of the fungicides include the loss of efficiency in the control of phytopathogenic diseases, enhanced resistance of plant pathogens to active ingredients, environmental destruction, and severe harm to human health [2]. Alternatives to safe, effective and environmentally friendly fungicides have been explored, but there are currently no plant origin biochemicals available to control anthracnose. Plant essential oils have attracted attention due to their strong antifungal activity, low toxicity and environmental friendliness.

Essential oils are aromatic, oily liquids extracted from various plants [3]. They are complex mixtures of low-molecular weight compounds synthesized by plants [4] and mainly contain terpenes (monoterpenes and sesquiterpenes), alcohols, ketones, aldehydes and other volatile organic compounds that can be used as antibacterial agents, antiviral

drugs, and antioxidants [5,6]. In addition, essential oils are recognized by the Food and Drug Administration (FDA) as a "generally recognized safe" natural product and can be used in food preparations, drugs, and cosmetics [7].

Rosemary essential oil (REO) contains camphor, α-pinene, 1,8-cineole, Bicyclo (2.2.1) heptane-2-one, trimethyl, Eucalyptol, Camphene, b-Pinene, D-Limonene, a-Phellandrene, β-Caryophyllene, and other relative components [8–11]. Studies have shown that REO can inhibit the growth of *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* [12]. REO also has a strong inhibitory effect on some plant pathogens, such as *Sclerotinia sclerotiorum*, *Sclerotinia nivalis*, *Alternaria panax*, *Cylindrocarpon destructans* and *Fusarium oxysporum* [11,13]. REO has the potential to be used as a natural antimicrobial agent due to the presence of terpenes, such as pinene, camphene, limonene, and eucalyptol [11]. With the improvement of rosemary breeds, expansion of planting areas, optimization of the extraction process and multi-level processing and utilization, the cost of rosemary essential oil has rapidly decreased in recent years. Rosemary essential oil has shown great potential in the biocontrol of plant diseases due to its strong antifungal activity and extremely low cost.

The ability of *C. gloeosporioides* to recognize plant surface signals and overcome host defenses was enhanced because of the evolution and expansion of the gene families. During infection, the expression level of GH family genes in *C. gloeosporioides* was upregulated, which helped the adaptation of *C. gloeosporioides* to a variety of woody plants [14]. Understanding the adaptive evolution mechanism of *C. gloeosporioides* to hosts helped us explore the antifungal mechanism among different strains at the genetic level.

According to reports, the antifungal mechanism of essential oils involves components that could disrupt the microbial plasma membrane, which increases membrane fluidity and permeability, disrupts membrane proteins, inhibits respiration, changes the ion transport process of fungi, and induces the leakage of ions and other cell contents [15,16]. There are currently few studies on the antifungal activity and mechanism of REO. In this study, the effects of REO on the cell wall, plasma membrane and energy metabolism were evaluated in order to explore its inhibitory effect and antifungal mechanism on *C. Gloeosporioides*, providing a theoretical basis for the development of REO as a biological control agent.

2. Materials and Methods

Colletotrichum gloeosporioides strain P51 was isolated from the twigs of walnuts with anthracnose and identified by the Non-wood Forest Laboratory of Forestry College at Henan Agricultural University, which also provided the REO. Chitinase activity detection kit, succinate dehydrogenase activity detection kit, malate dehydrogenase activity detection kit, Na^+K^+-ATPase activity detection kit, ATP content detection kit and phosphofructokinase activity detection kit (Solarbio Science and Technology Co., Ltd., Beijing, China) were used to detect changes in physiological indicators.

Spore suspension (1×10^6 CFU/mL) of *C. gloeosporioides* was inoculated in PDB and cultured at 28 °C, 180 r/min for 48 h. The REO was then added to achieve a final concentration and continued oscillating for certain hours. An equal amount of sterile water was added to serve as the negative control.

2.1. Antifungal Activity of REO on C. gloeosporioides

The plate coating method was used to determine the inhibitory effect of REO on *C. gloeosporioides* [17]. REO was diluted with 0.5% Tween-80 to concentrations of 500 μL/mL, 400 μL/mL, 300 μL/mL, 200 μL/mL, 100 μL/mL, 10 μL/mL, 1 μL/mL, and 0.1 μL/mL. The different concentrations of REO (100 μL) were spread on a potato dextrose agar (PDA) medium. The cake of *C. gloeosporioides* (diameter 0.8 cm) was placed in the center of the medium and cultured in the dark at 28 °C for 7 days. In this study, 100 μL 0.5% Tween-80 and 100 μL sterile water were spread on PDA medium as negative controls. The inhibition rate (IR) of mycelium was calculated using Equation (1) [18]:

$$IR\ (\%) = (dc - dt)/(dc - 0.8) \times 100 \quad (1)$$

where dt is the average diameter (cm) of the REO-treated colony, and dc is the average diameter (cm) of the control colony.

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of REO against *C. gloeosporioides* were determined by microdilution method with some modifications [19]. REO was gradually diluted with potato dextrose broth (PDB) in a 96-well plate, and 100 µL of *C. gloeosporioides* suspension (1×10^6 CFU/mL) was added to each well and evenly mixed. PDB was used as a negative control and cultured in an incubator at 28 °C for 48 h. The mycelial growth and clarity of the bottom of the 96-well plate were observed. Compared to the negative control group, the minimum concentration of REO without any visible mycelial growth was MIC [20]. On day 7, the PDB in tubes were subcultured on PDA plates; the minimum concentration of REO without mycelial growth was MFC [21,22].

2.2. SEM Observation of Fungal Morphology

REO was added to cultured spore suspension to achieve a final concentration equal to MIC and continued oscillating culture for 24 h. The collected mycelium was then fixed with 2.5% glutaraldehyde solution and stored in a refrigerator at 4 °C overnight.

The mycelium was then rinsed with 0.1 mol/L PBS buffer solution three times for 15 min each time. Then, 30%, 40%, 50%, 60%, 70%, 80% and 90% ethanol were used to dehydrate the mycelium step by step for 15 min each time, and 100% ethanol was used to dehydrate the mycelium twice for 20 min each time [20]. Tert-butanol was used to rinse the mycelium three times for 15 min each time. Finally, the mycelium was dried with a critical point drier and observed under a scanning electron microscope (SEM).

2.3. Effects of REO on the Cell Wall

REO was added to cultured spore suspension to achieve a final concentration equal to MIC and continued oscillating culture. Mycelium was removed from each bottle at 0, 6, 12, 24, 36, 48 and 72 h after REO treatment. A sterile gauze was used to filter the mycelium, and then rinsed with sterile water. Chitinase activity was determined with a chitinase activity detection kit following the instructions [23].

2.4. Effects of REO on Plasma Membrane

2.4.1. Effects on Ergosterol Content

REO was added to cultured spore suspension to achieve a final concentration equal to 1/2MIC, MIC and 2MIC. Culture continued oscillating for 48 h at 28 °C and 180 r/min; the culture medium was removed by extraction and filtration and then washed thrice with 0.01 mol/L PBS buffer. The wet weight of the mycelium was weighed. Mycelium was then collected in a sterile centrifuge tube, and 3 mL of 25% potassium hydroxide alcohol solution was added. After vortexing for 2 min and placing in a water bath at 85 °C for 4 h, 2 mL deionized water and 5 mL *n*-heptane were added after cooling. Ergosterol was extracted from the solution, oscillated for 3 min, and stood at room temperature for 1 h to stratify the solution. The upper *n*-heptane solution was aspirated, and the absorbance was measured at 282 nm and 230 nm with a microplate reader. The *E* values (in percentages per cm) determined for crystalline ergosterol and 24(28) dehydroergosterol were 290 and 518, respectively. The ergosterol content (EC) was then calculated using Equation (2) [24]:

$$EC\ (\%) = (A282/290 - A230/518)/ww, \quad (2)$$

where ww is the average weight of wet mycelium after treatment by REO.

2.4.2. Effects on Extracellular Conductivity and Leakage of Nucleic Acids, Soluble Proteins and Soluble Reducing Sugar

The extracellular conductivity was measured according to Wang et al. [25] with some modifications. Mycelium (0.2 g) was taken from cultured spore suspension and resuspended in a conical flask containing 10 mL sterile water. REO was added to achieve a

final concentration equal to MIC, and sterile water was used as a negative control. The supernatant was cultured at 28 °C and 180 r/min at 0, 2, 4, 6 and 8 h, respectively, and the conductivity of the supernatant was determined by a conductivity meter.

The supernatant of *C. gloeosporioides* culture broth treated with REO of MIC was taken at 0, 6, 12, 24, 36, 48 and 72 h. The absorbance at 260 nm was measured by a microplate reader in order to characterize the leakage of nucleic acids [26]. The content of soluble protein leakage was determined by Coomassie brilliant blue G-250 staining, and the leakage of soluble reducing sugar in the fungal solution was detected using the colorimetric method of 3,5-dinitrosalicylic acid according to Liu et al. [27] with some modifications.

2.5. Effects of REO on Energy Metabolism of C. gloeosporioides

The mycelium of *C. gloeosporioides* treated with REO of MIC at 0, 6, 12, 24, 36, 48 and 72 h were taken, and the activities of succinate dehydrogenase (SDH), malate dehydrogenase (MDH), Na^+k^+-ATPase, ATP content and phosphofructokinase (PFK) were detected using their various detection kits according to Li et al. [28] and Wang et al. [29].

2.6. Transcriptome Sequencing

REO was added to cultured spore suspension to achieve a final concentration equal to MIC. Culture continued oscillating for 6 h and the mycelium was collected, frozen in liquid nitrogen immediately for 30 min, and stored in an ultra-low temperature refrigerator. The transcriptome sequencing was performed at Hangzhou Kaitai Biotech Co., Ltd. (Hangzhou, China) using the Illumina NovaSeq 6000 platform.

2.7. Data Analysis

All tests were conducted in triplicate. One-way analysis of variance (ANOVA) and Duncan's multiple range test in SPSS 25.0 software were used for variance analysis and significant difference comparison, with $p < 0.05$ as a significant difference. The Origin 2018 software was used for the charting.

3. Results

3.1. Antifungal Activity of REO against C. gloeosporioides

REO exhibited antifungal activity against *C. gloeosporioides*, as demonstrated by Table 1 and Figure 1 which represent the inhibition rate and antifungal activity of REO on the mycelium of *C. gloeosporioides*, respectively. With the increase in REO concentration, the inhibition of the mycelial diameter was more pronounced. When the REO concentration was 0.1 μL/mL, there was no significant difference in the mycelial diameter between the control groups, indicating that very low concentration did not have an inhibitory effect on *C. gloeosporioides*. The mycelium inhibition rate was 100% at an REO concentration of 300 μL/mL and above.

Table 1. Inhibition rate of REO on mycelium of *C. gloeosporioides*.

REO Concentration (μL/mL)	Mycelium Diameter (cm)	Inhibitory Rates (%)
0	8.25 ± 0.05 a	0.00 a
0.1	8.12 ± 0.13 a	1.79 a
1	7.82 ± 0.08 b	5.82 b
10	4.93 ± 0.08 c	44.52 c
100	4.10 ± 0.31 d	55.70 d
200	2.42 ± 0.18 e	78.30 e
300	0.80 f	100 f
400	0.80 f	100 f
500	0.80 f	100 f

Notes: Different letters in the same column indicate significant difference at $p < 0.05$.

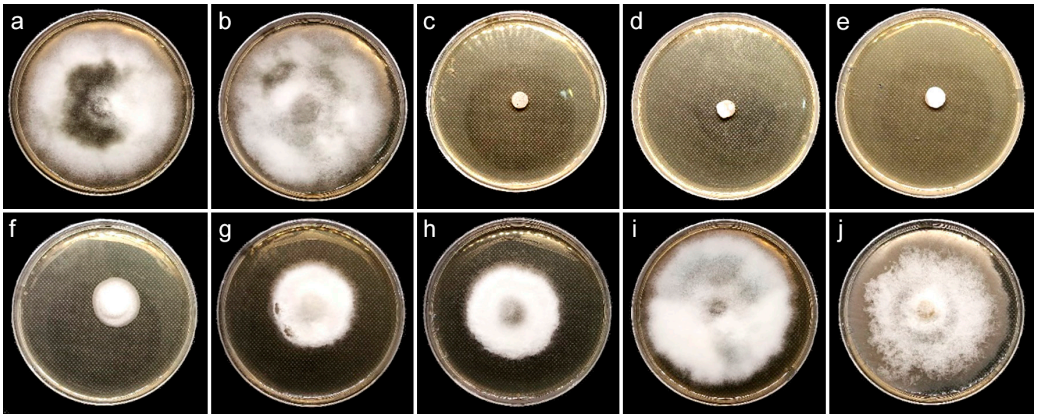

Figure 1. Antifungal activity of different concentrations of REO against *C. gloeosporioides*: (**a**) Tween-80; (**b**) sterile water; (**c**) 500 μL/mL; (**d**) 400 μL/mL; (**e**) 300 μL/mL; (**f**) 200 μL/mL; (**g**) 100 μL/mL; (**h**) 10 μL/mL; (**i**) 1 μL/mL; and (**j**) 0.1 μL/mL.

After being cultured in 96-well plates for 48 h, no mycelial growth and obvious spore precipitation were observed when the concentration of REO was 15.625 μL/mL and above. When cultured for 7 days, no mycelial growth was observed on PDA plates when the REO concentration was 31.25 μL/mL and above. Hence, the MIC and MFC were 15.625 μL/mL and 31.25 μL/mL, respectively.

3.2. Effects of REO on the Morphology of C. gloeosporioides

The surface of *C. gloeosporioides* mycelium is smooth and full under normal conditions (Figure 2a). After REO treatment, the morphology of *C. gloeosporioides* mycelium altered and became distorted, shriveled, and severely deformed (Figure 2b).

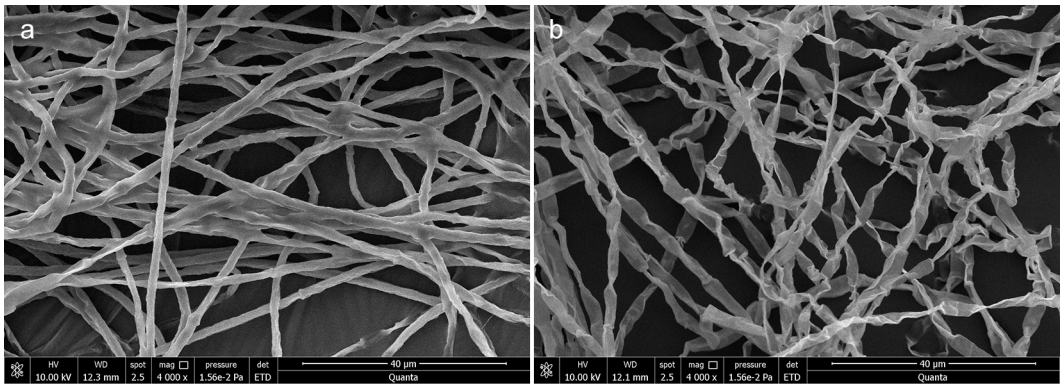

Figure 2. Morphology of *C. gloeosporioides* observed by SEM: (**a**) control group; (**b**) after REO treatment.

3.3. Effects of REO on Cell Wall of C. gloeosporioides

Chitinase can hydrolyze chitin, the main component of the fungal cell wall, and plays a significant role in the balance of carbon and nitrogen in ecosystems [30]. Chitinase is also involved in cell wall remodeling, hyphal development, branching and autolysis during fungal spore germination and contraction [31,32]. After REO treatment, the chitinase activity of *C. gloeosporioides* showed an overall increasing trend with time and increased rapidly within 12 h, after which the growth leveled off (Figure 3a). In addition, the chitinase activity was significantly higher in the treated group than in the control group. The results showed that REO significantly enhanced chitinase activity of *C. gloeosporioides* and may have affected other life activities of the fungi.

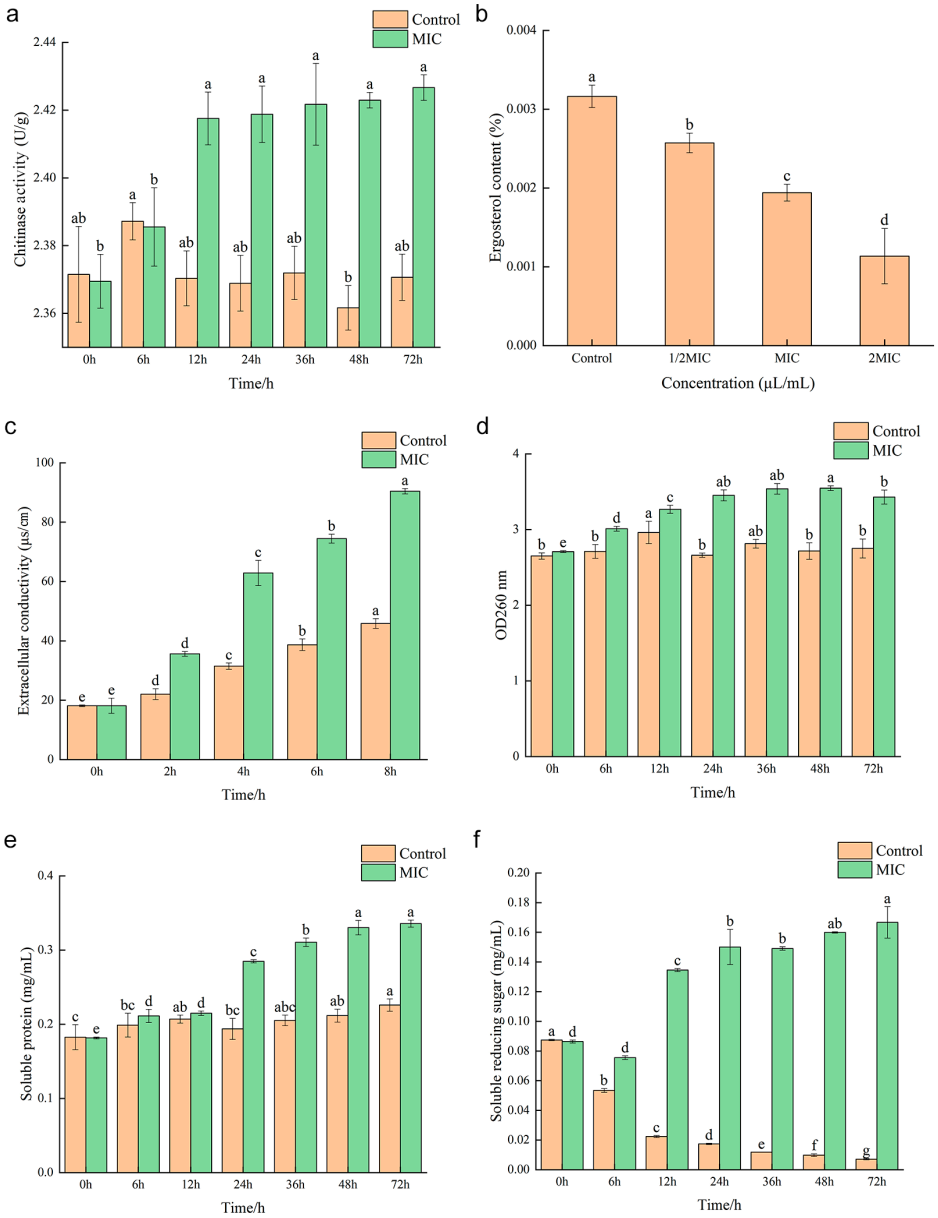

Figure 3. Effects of REO on cell wall and plasma membrane of *C. gloeosporioides*. (**a**) Chitinase activity; (**b**) ergosterol content; (**c**) extracellular conductivity; (**d**) nucleic acids leakage; (**e**) soluble protein leakage; and (**f**) soluble reducing sugar leakage. Different letters indicate significant difference at $p < 0.05$.

3.4. Effects of REO on Plasma Membrane of C. gloeosporioides

Ergosterol is the most abundant and most important sterol component in fungal membranes. It is involved in the regulation of membrane permeability and fluidity, regulation and distribution of integral membrane proteins, and control of the cell cycle [33]. Therefore, it is the key to the integrity of the plasma membrane structure. After REO treatment, the ergosterol content of *C. gloeosporioides* decreased, and the higher the concentration of REO, the lower the ergosterol content (Figure 3b). Compared to the control group, the content of ergosterol reduced by 18.75%, 40.63% and 65.63% after treatments with REO of 1/2MIC, MIC and 2MIC, respectively. The results suggested that REO was able to reduce the ergosterol content in the plasma membrane of *C. gloeosporioides* and may affect the integrity of plasma membrane.

The change in cellular conductivity is affected by the leakage of intracellular components and the outflow of electrolytes. Essential oils can change the homeostasis of

microorganisms by destroying microbial plasma membranes. Therefore, effects to material transport, energy production, and metabolic regulation can be observed after treatment with essential oils [34]. After the treatment of *C. gloeosporioides* with REO, the extracellular conductivity increased with prolonged treatment time, and was significantly higher in all the treated groups than in the control (Figure 3c). After treatment with REO for 8 h, the electrical conductivity was about two times higher than that of the control. It indicated that REO treatment increased the electrical conductivity of *C. gloeosporioides*, revealing that REO disrupted the plasma membranes leading to leakage of intracellular substances.

The amount of nucleic acids leakage which is characterized by the absorbance at 260 nm (OD260), along with soluble proteins leakage and soluble reducing sugar leakage, can reflect the permeability and integrity of the plasma membranes. As shown in Figure 3d, the nucleic acids leakage after the treatment of *C. gloeosporioides* with REO was higher than that of and the control and increased with prolonged treatment time. Likewise, after treatment with REO for 72 h, the soluble protein leakage of *C. gloeosporioides* increased consistently, whereas there was little change in the control (Figure 3e).

The soluble reducing sugar content showed a decreasing trend during the first 6 h of REO treatment; it then increased rapidly from 6 to 12 h, and then the increasing rate slowed down, while the soluble reducing sugar content was decreased consistently in the control (Figure 3f). The decrease in soluble reducing sugars in the control was due to absorption and utilization of *C. gloeosporioides*. In the first 6 h of REO treatment, a small amount of soluble reducing sugar was absorbed, but when the plasma membrane was disrupted, and the cell contents were leaked out, this lead to a consistent increase in soluble reducing sugar content. Moreover, REO treatment may have inhibited the absorption of soluble reducing sugars by *C. gloeosporioides*, also leading to the accumulation of soluble reducing sugars.

The leakage of nucleic acids, soluble proteins and soluble reducing sugar were all increased with REO treatment; it confirmed that the plasma membrane of *C. gloeosporioides* was disrupted after REO treatment.

3.5. Effects of REO on TCA Cycle and Energy Metabolism of C. gloeosporioides

Life relies not only on material metabolism but also on energy metabolism. The tricarboxylic acid (TCA) cycle is the pivot of material transformation and energy metabolism in the organism. Succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) are the key rate-limiting enzymes in the tricarboxylic acid cycle. Phosphofructokinase (PFK) can provide energy for organisms through glycolysis when the tricarboxylic acid cycle is affected. Adenosine triphosphate (ATP) is a high-energy phosphate compound that serves as the most direct source of energy for various physiological activities in cells. The normal metabolism of microorganisms could be affected by a rapid decline or loss of ATP content [35]. ATPase can hydrolyze ATP into ADP and phosphate ions, which provide energy for material transport, metabolism, and information transfer [35]. The activities of succinate dehydrogenase (SDH), malate dehydrogenase (MDH), Na+k+-ATPase, phosphofructokinase (PFK) and ATP content were detected to observe the effects of REO on the TCA cycle and energy metabolism in *C. gloeosporioides*.

As shown in Figure 4a–d, the activities of SDH, MDH, ATPase and PFK in the control group increased in the first 6 h, after which there was an overall trend of gradual decrease. The results showed that the TCA cycle and energy metabolism of *C. gloeosporioides* gradually enhanced during the first 6 h, after which the TCA cycle and energy metabolism were somewhat inhibited due to environmental constraints. After REO treatment, there was a decreasing trend in the activities of SDH, MDH, ATPase, while there was an increasing trend in the activity of PFK. It revealed that the TCA cycle and energy metabolism were inhibited by REO treatment, and PFK was increased to accelerate the glycolytic pathway. After REO treatment, ATP content increased for the first 6 h, and then decreased rapidly, whereas it showed a slight increase and then a slight decrease in the control group (Figure 4e). It suggested that the REO inhibited ATP synthesis in *C. gloeosporioides*.

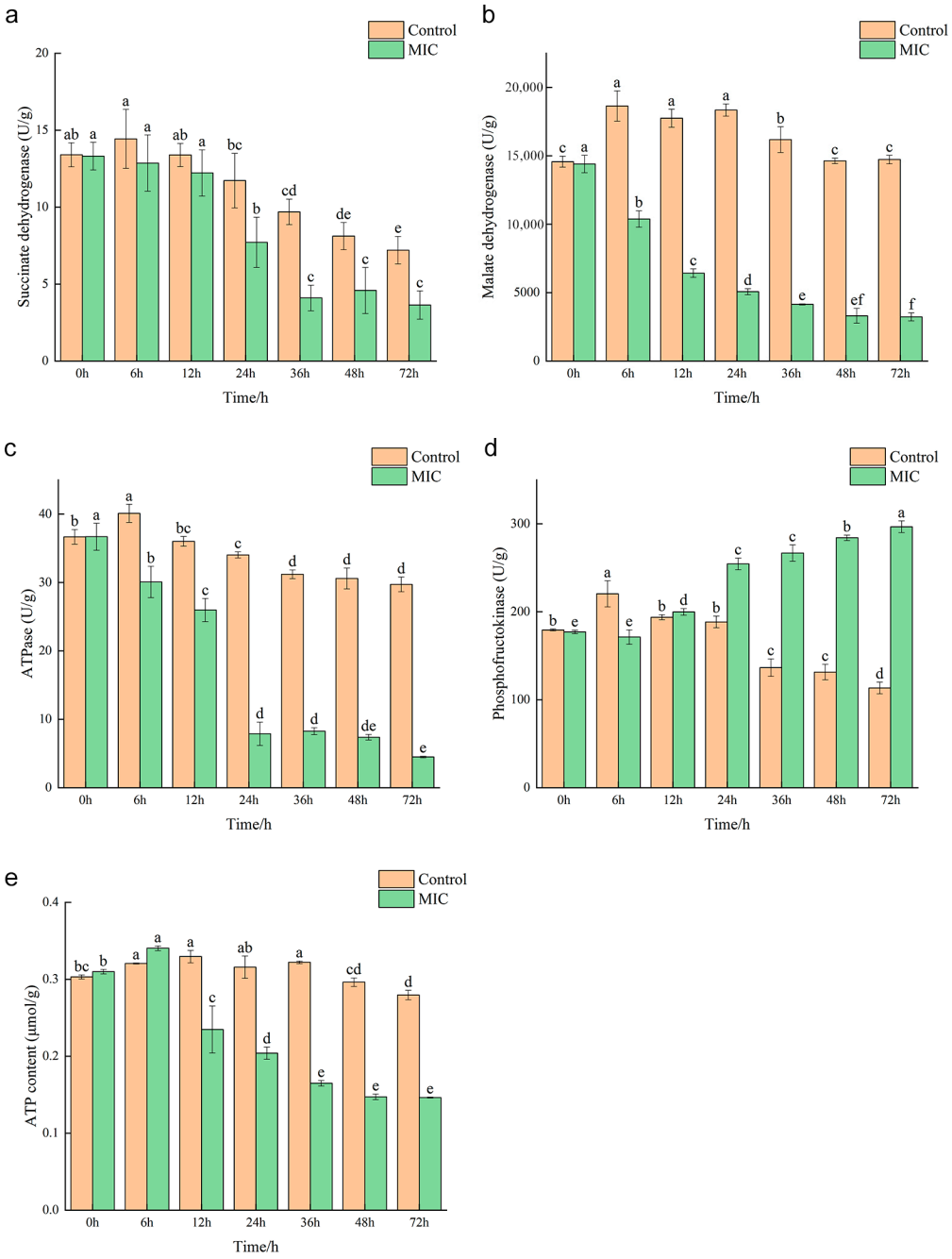

Figure 4. Effects of REO on activities of the enzymes in *C. gloeosporioides* after REO treatment. (**a**) SDH; (**b**) MDH; (**c**) ATPase; (**d**) PFK; and (**e**) ATP content. Different letters indicate significant difference at $p < 0.05$.

3.6. Molecular Mechanism of REO Inhibiting C. gloeosporioides

After filtering the original sequencing data, the processed data was compared with the reference genome (https://www.ncbi.nlm.nih.gov/assembly/GCA_011800055.1, accessed on 31 March 2020) to obtain the location information of the reads on the reference genome and the feature information of the sequencing samples. After the treatment of *C. gloeosporioides* with REO at MIC concentration, a total of 2956 differentially expressed genes were significant. As shown in Figure 5, there were 1845 genes that were significantly upregulated and 1111 genes that were significantly downregulated.

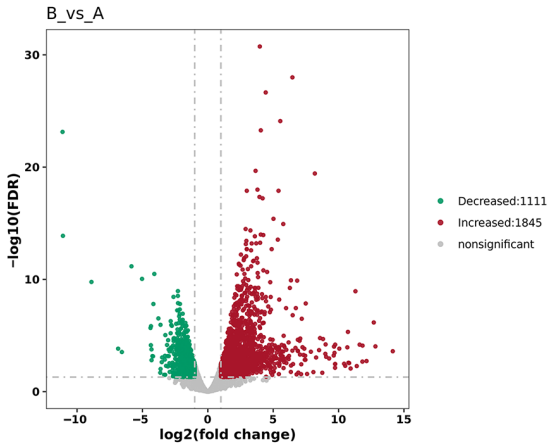

Figure 5. Volcano map of the differential gene expression. Each point in the figure represents a gene, and the abscissa represents the log2 (fold change) of the difference in gene expression in the two samples. The ordinate represents the negative logarithm of the FDR value of the gene expression change.

Using GO enrichment analysis, the functions of differentially expressed genes were classified according to biological process (BP), cellular component (CC) and molecular function (MF). After REO treatment (Figure 6), the differentially expressed genes mainly involved in the biological process include regulation of chromatin organization, response to nutrients, mitotic cell cycle phase transition, cell cycle phase transition, and other processes. In the cell components, the differentially expressed genes were mainly involved in the plasma membrane and cellular bud, etc. In terms of molecular function, the differentially expressed genes were involved mainly in the molecular function of regulators, enzyme regulatory activity, enzyme activator activity, and so on.

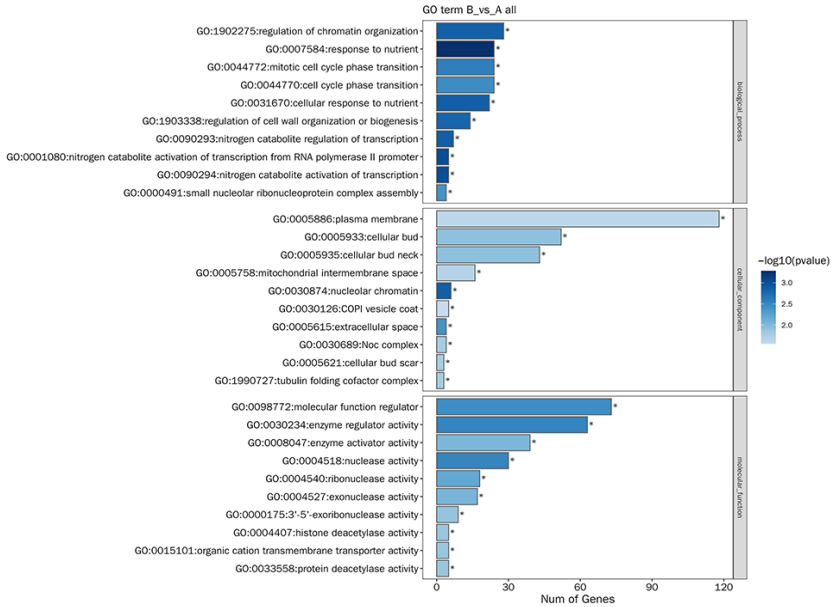

Figure 6. GO enrichment analysis. Ten GO functions with the most significant enrichment (p-value < 0.05) were selected from the three categories of BP, CC and MF, respectively. The ordinate is the GO category entry with significant differences, and the abscissa is the number of differentially expressed genes enriched in the GO term. The color represents the enrichment significance p-value, and the functions with $p < 0.05$ is marked with '*'.

The most significant biochemical metabolic and signal transduction pathways, in which differentially expressed genes were mainly involved, can be determined through

significant pathway enrichment analysis. The differentially expressed genes were mainly involved in ribosome biosynthesis, chaperones and folding catalysts, starch and sucrose metabolism, cell cycle-yeast, translation factors, and other pathways (Figure 7).

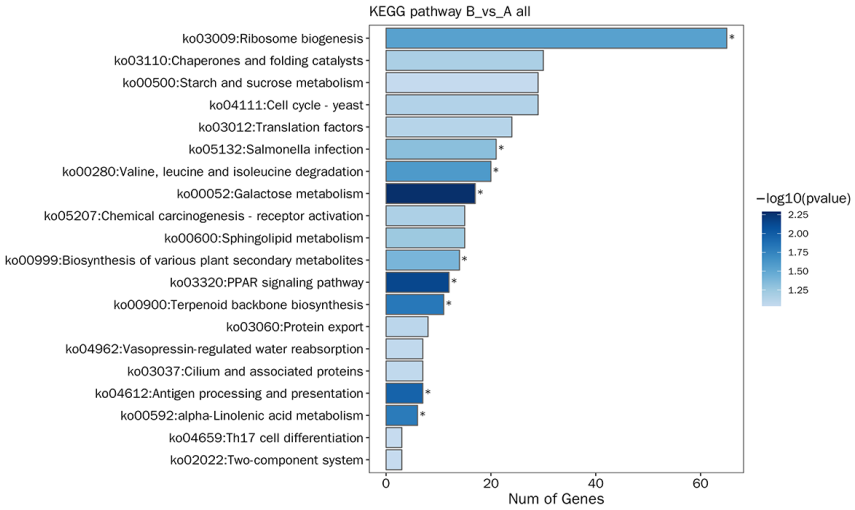

Figure 7. KEGG enrichment analysis. Twenty KEGG pathways with the most significant enrichment were selected. The ordinate is the KEGG pathway, and the abscissa is the number of differential genes enriched for the pathway. The color represents the enrichment significance *p*-value, and the pathway with $p < 0.05$ is marked with '*'.

After REO treatment, the expression of the cell wall integrity protein scw1 gene related to the cell wall was downregulated. The expression of ribosomal biosynthesis protein NOC1 gene, ribosomal RNA processing protein 12 gene and RNA 3'-terminal phosphocyclase-like protein gene related to ribosomal biosynthesis were also downregulated. The expression of chromosome structure maintenance protein 2 gene, G2/mitosis specific cyclin 4 gene and M phase induced phosphatase gene related to the cell cycle were downregulated. In addition, there was a downregulation of the expression of the developmental regulatory factor flbA gene. The above analysis showed that REO inhibited cell wall formation, genetic material synthesis, cell division and differentiation in *C. gloeosporioides*.

4. Discussion

In this study, REO could inhibit the growth of *C. gloeosporioides*. REO increased chitinase activity, which decomposes the fungal cell wall component chitin. Previous studies have shown that essential oils affect the synthesis of cell wall components and enzymes closely related to the cell wall [36,37]. The chitinase activity increased after lemongrass essential oil treatment of grape leaf blight and downy mildew [38]. *Litsea cubeba* essential oil (LCEO) destroyed the cell wall integrity of *Colletotrichum scovillei*, the main pathogen responsible for pepper anthracnose [39]. Treatment of apricot spoilage bacteria with 4% oregano essential oil nanocapsules significantly increased chitinase activity [40].

After treatment with REO, the content of plasma membrane component ergosterol decreased. Previous studies have revealed the inhibitory effect of essential oil on ergosterol production. For instance, the volatile oil of ginger leaves had an inhibitory effect on *Fusarium graminearum*, which inhibited ergosterol production at a concentration of 1000 μg/mL [41]. In another study, the treatment of *Penicillium digitatum* with citronella essential oil significantly decreased plasma membrane integrity and ergosterol content while accumulating lanosterol as a precursor of ergosterol biosynthesis. The addition of exogenous ergosterol reduced the inhibition rate of citronellal and restored ergosterol content and membrane structure to normal levels [33]. The reason could be that REO is lipophilic and can penetrate the plasma membrane and interact with enzymes and mem-

brane proteins responsible for ergosterol biosynthesis, leading to a decrease in ergosterol biosynthesis by disrupting the sterol biosynthesis pathway [42].

The content of extracellular conductivity and the leakage of nucleic acids, soluble protein and soluble reducing sugar increased with REO treatment. Similarly, treatment with zedoary oil caused serious damage to the plasma membrane of *Phytophthora capsici*, resulting in the leakage of cell contents and significantly improving the relative conductivity [25]. The treatment of *Aspergillus niger* with thyme essential oil will destroy its cell wall, cell membrane and organelle structure, thus causing leakage of intracellular substances and changes in intracellular and extracellular ion gradients, ultimately leading to the death of *Aspergillus niger* [43]. The essential oil components can be dissolved in the lipids of the plasma membrane, changing the proportion and structure of fatty acids in the plasma membrane [44], destroying the structure and integrity of the plasma membrane, impairing the function of the membrane barrier and the stability of the intracellular environment, increasing cell permeability and leaking cell contents (cytoplasm, soluble proteins, sugars and nucleic acids) into the medium, thereby increasing the conductivity of the medium [24]. For instance, the disruption of β-tubulin distribution leads to cell cycle arrest, resulting in damage to the mitotic spindle, eventually damaging the plasma membrane and causing leakage of cellular components [45]. Treatment with Martini essential oil destroyed 58 types of plasma membrane proteins in *Aspergillus flavus* and released 157 types of membrane proteins, severely damaging the integrity of the plasma membrane [46].

After treatment with REO, the activities of SDH, MDH, ATPase and ATP content decreased, which affected the TCA cycle and energy metabolism of *C. gloeosporioides*. Perilla essential oil inhibited the growth of *Aspergillus flavus* by blocking the antioxidant defense process and glycolysis pathway; thereby, an alternative energy production pathway was activated to compensate for unbalanced glycolysis [35]. Essential oils are capable of interfering with the absorption, transport and metabolism of microbial nutrients, resulting in the inhibition of microbial growth and reproduction. The energy-related pathways, including TCA, oxidative phosphorylation, gluconeogenesis/glycolysis, nitrogen metabolism and sulfur metabolism, were disrupted in the gray mold samples treated with (E) -2-hexenal fumigation [47]. Similarly, the main components of dill seed essential oil, carvone and limonene, exhibited antifungal activity on *Sclerotinia sclerotiorum*, and synergistically inhibited fungal growth, ergosterol synthesis, MDH and SDH [48]. According to Zhao et al., star anise essential oil can reduce the ATP content and cell viability of *Aspergillus niger* and inhibits its growth by blocking the synthesis pathway of NAD [49].

Transcriptome sequencing results showed that REO could inhibit cell wall formation, genetic material synthesis, cell division and differentiation in *C. gloeosporioides*. The antibacterial mechanism of turmeric natural essential oil against *Aspergillus flavus* is to destroy the membrane system of fungal cells and downregulate the relative expression of mycotoxin genes in the aflatoxin biosynthesis pathway [50]. The exogenous essential oil decanal could significantly inhibit the growth of *Penicillium expansum* in vitro, as well as the production of patulin and the expression of genes related to patulin biosynthesis. As a result, it can effectively control the penicilliosis of apple and pear fruits [51]. The essential oil of *Cedrus deodara* leaves, whose main components are camphor and anisol, downregulated the expression of *Candida albicans* biofilm biofilm-related genes (ECE1, ECE2, RBT1 and EED1), which reduced the formation of *Candida albicans* biofilms [52]. Previous studies showed that essential oils could interact with microbial DNA, causing damage to DNA and inhibiting microbial growth [53]. Techniques such as next-generation sequencing of genomes and transcriptomes can help to understand the molecular basis of the interaction between the pathogen and the plant. However, due to gene family expansion, there are differences among strains. Therefore, it is recommended to use the outcomes of these techniques on a case-by-case basis [54]. This allows the precise identification of factors that regulate gene function and expression and the integration of this information with other strategies to examine the exact effects of REO.

The formation of biofilms is not limited to the bacterial world but also includes fungal pathogens [55]. Fungal biofilms are heterogeneous, surface-associated colonies comprised of filamentous hyphae (chains of elongated cells), pseudohyphal cells, yeast-form cells, and various forms of extracellular matrix [56]. Biofilm formation is an important virulence factor for pathogenic fungi. Yeast and filamentous fungi can attach to both living and abiotic surfaces, developing highly organized communities that are resistant to antimicrobials and environmental conditions [57]. Zosteric acid is a secondary metabolite of seagrass, *Zostera marina*. Sublethal concentrations of zosteric acid can reduce the adhesion of fungi and play a key role in the thickness and morphology of fungal biofilms [55].

5. Conclusions

REO could inhibit the growth of *C. gloeosporioides* with MIC and MFC concentrations of 15.625 μL/mL and 31.25 μL/mL, respectively. After being treated with REO, the mycelium became shriveled, twisted, and severely deformed. REO could disrupt the plasma membrane and cell wall by increasing chitinase activity and decreasing ergosterol content, which then released the cell contents. REO inhibits the TCA cycle and energy metabolism of *C. gloeosporioides* by regulating the key enzymes activities related to the TCA cycle and energy metabolism. REO could inhibit the growth of *C. gloeosporioides* by regulating the expression of genes related to cell wall formation, genetic material synthesis, cell division and differentiation to inhibit. The results revealed the efficacy and antifungal mechanism of rosemary essential oil against *C. gloeosporioides* and provide a theoretical basis for rosemary essential oil as an effective fungicide and biocontrol agent.

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References

1. Dean, R.; Van Kan, J.A.; Pretorius, Z.A.; Hammond-Kosack, K.E.; Di Pietro, A.; Spanu, P.D.; Rudd, J.J.; Dickman, M.; Kahmann, R.; Ellis, J.; et al. The top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* **2012**, *13*, 414–430. [CrossRef]
2. Kumar, A.; Kudachikar, V.B. Antifungal properties of essential oils against anthracnose disease: A critical appraisal. *J. Plant Dis. Prot.* **2018**, *125*, 133–144. [CrossRef]
3. Cui, H.; Zhang, X.; Zhou, H.; Zhao, C.; Lin, L. Antimicrobial activity and mechanisms of Salvia sclarea essential oil. *Bot. Stud.* **2015**, *56*, 16. [CrossRef]
4. Silva, B.D.D.; Bernardes, P.C.; Pinheiro, P.F.; Fantuzzi, E.; Roberto, C.D. Chemical composition, extraction sources and action mechanisms of essential oils: Natural preservative and limitations of use in meat products. *Meat Sci.* **2021**, *176*, 108463. [CrossRef]
5. Chen, Y.; Liu, L.; Wang, H.; Ma, J.; Peng, W.; Li, X.; Lai, Y.; Zhang, B.; Zhang, D. Environmentally friendly plant essential oil: Liquid gold for human health. *Adv. Agron.* **2021**, *170*, 289–337.
6. Yadav, E.; Kumar, S.; Mahant, S.; Khatkar, S.; Rao, R. Tea tree oil: A promising essential oil. *J. Essent. Oil Res.* **2016**, *29*, 201–213. [CrossRef]
7. Dima, C.; Dima, S. Essential oils in foods: Extraction, stabilization, and toxicity. *Curr. Opin. Food Sci.* **2015**, *5*, 29–35. [CrossRef]
8. da Silva Bomfim, N.; Nakassugi, L.P.; Oliveira, J.F.P.; Kohiyama, C.Y.; Mossini, S.A.G.; Grespan, R.; Nerilo, S.B.; Mallmann, C.A.; Alves Abreu Filho, B.; Machinski, M., Jr. Antifungal activity and inhibition of fumonisin production by Rosmarinus officinalis L. essential oil in Fusarium verticillioides (Sacc.) Nirenberg. *Food Chem.* **2015**, *166*, 330–336. [CrossRef] [PubMed]
9. Becer, E.; Altundag, E.M.; Güran, M.; Vatansever, H.S.; Ustürk, S.; Hanoglu, D.Y.; Baser, K.H.C. Composition and antibacterial, anti-inflammatory, antioxidant, and anticancer activities of *Rosmarinus officinalis* L. essential oil. *S. Afr. J. Bot.* **2023**, *160*, 437–445. [CrossRef]
10. Mutlu-Ingok, A.; Devecioglu, D.; Dikmetas, D.N.; Karbancioglu-Guler, F.; Capanoglu, E. Antibacterial, antifungal, antimycotoxigenic, and antioxidant activities of essential oils: An updated review. *Molecules* **2020**, *25*, 4711. [CrossRef]

11. Hussein, K.A.; Lee, Y.D.; Joo, J.H. Effect of rosemary essential oil and *Trichoderma koningiopsis* T-403 VOCs on pathogenic fungi responsible for ginseng root rot disease. *J. Microbiol. Biotechnol.* **2020**, *30*, 1018–1026. [CrossRef]
12. Olivas-Méndez, P.; Chávez-Martínez, A.; Santellano-Estrada, E.; Asorey, L.G.; Sánchez-Vega, R.; Rentería-Monterrubio, A.L.; Chávez-Flores, D.; Tirado-Gallegos, J.M.; Méndez-Zamora, G. Antioxidant and antimicrobial activity of Rosemary (*Rosmarinus officinalis*) and Garlic (*Allium sativum*) Essential Oils and Chipotle Pepper Oleoresin (*Capsicum annum*) on beef hamburgers. *Foods* **2022**, *11*, 2018. [CrossRef]
13. Khanjani, R.; Dehghan, H.; Sarrafi, Y. Antifungal edible tomato coatings containing ajwain, neroli, and rosemary essential oils. *J. Food Meas. Charact.* **2021**, *15*, 5139–5148. [CrossRef]
14. Meng, F.L.; Tian, C.M. Gene Family Expansion during the Adaptation of *Colletotrichum gloeosporioides* to Woody Plants. *J. Fungi* **2023**, *9*, 1185. [CrossRef] [PubMed]
15. Zhang, J.H.; Sun, H.L.; Chen, S.Y.; Zeng, L.; Wang, T.T. Anti-fungal activity, mechanism studies on α-Phellandrene and Nonanal against *Penicillium cyclopium*. *Bot. Stud.* **2017**, *58*, 13. [CrossRef] [PubMed]
16. Bajalan, I.; Rouzbahani, R.; Pirbalouti, A.G.; Maggi, F. Antioxidant and antibacterial activities of the essential oils obtained from seven Iranian populations of Rosmarinus officinalis. *Ind. Crops Prod.* **2017**, *107*, 305–311. [CrossRef]
17. Cui, X.; Zhu, Q.J.; Hou, R.; Wan, J. Antibacterial activity and mechanism of eugenol, carvacrol and thymol against *Fusarium graminearum*. *Food Sci.* **2022**, *43*, 10–18.
18. Trabelsi, D.; Hamdane, A.M.; Said, M.B.; Abdrrabba, M. Chemical composition and antifungal activity of essential oils from flowers, leaves and peels of Tunisian *Citrus aurantium* against *Penicillium digitatum* and *Penicillium italicum*. *J. Essent. Oil Bear. Plants* **2016**, *19*, 1660–1674. [CrossRef]
19. Wu, T.; Cheng, D.; He, M.Y.; Pan, S.Y.; Yao, X.L.; Xu, X.Y. Antifungal action and inhibitory mechanism of polymethoxylated flavones from *Citrus reticulata* Blanco peel against *Aspergillus niger*. *Food Control* **2014**, *35*, 354–359. [CrossRef]
20. Hao, K.Y.; Xu, B.C.; Zhang, G.Y.; Lv, F.F.; Wang, Y.H.; Ma, M.X.; Si, H.B. Antibacterial activity and mechanism of *Litsea cubeba* L. essential oil against *Acinetobacter baumannii*. *Nat. Prod. Commun.* **2021**, *16*, 1934578X21999146. [CrossRef]
21. Irobi, O.N.; Daramola, S.O. Antifungal activities of crude extracts of *Mitracarpus villosus* (Rubiaceae). *J. Ethnopharmacol.* **1993**, *40*, 137–140. [CrossRef] [PubMed]
22. Rasooli, I.; Abyaneh, M.R. Inhibitory effects of Thyme oils on growth and aflatoxin production by *Aspergillus parasiticus*. *Food Control* **2004**, *15*, 479–483. [CrossRef]
23. Zhang, M.M.; Ke, Y.; Dong, T.J.; Wu, X.D.; Xiao, S.S.; Wang, X.D.; Ding, B.B. Inhibitory activity and mechanism of chitosan oligosaccharides on spoilage fungi. *J. Chin. Inst. Food Sci. Technol.* **2023**, *23*, 71–80.
24. He, J.L.; Wu, D.T.; Zhang, Q.; Chen, H.; Li, H.Y.; Han, Q.H.; Lai, X.Y.; Wang, H.; Wu, Y.X.; Yuan, J.; et al. Efficacy and mechanism of Cinnamon Essential Oil on inhibition of *Colletotrichum acutatum* isolated from 'Hongyang' kiwifruit. *Front. Microbiol.* **2018**, *9*, 1288. [CrossRef]
25. Wang, B.; Liu, F.; Li, Q.; Xu, S.; Zhao, X.Z.; Xue, P.L.; Feng, X. Antifungal activity of zedoary turmeric oil against *Phytophthora capsici* through damaging cell membrane. *Pestic. Biochem. Physiol.* **2019**, *159*, 59–67. [CrossRef]
26. Wang, W.; Chen, Y.F.; Wei, Z.F.; Jiang, J.J.; Peng, J.Q.; He, Q.T.; Xu, W.Y.; Liu, H.M. Microemulsion of cinnamon essential oil formulated with tea polyphenols, gallic acid, and tween 80: Antimicrobial properties, stability and mechanism of action. *Microorganisms* **2023**, *11*, 2. [CrossRef] [PubMed]
27. Liu, X.-Y.; Huo, Y.-Y.; Yang, J.; Li, T.-T.; Xu, F.-R.; Wan, H.-P.; Li, J.-N.; Wu, C.-H.; Zhang, Y.-H.; Dong, X. Integrated physiological, metabolomic, and proteome analysis of *Alpinia officinarum* Hance essential oil inhibits the growth of *Fusarium oxysporum* of *Panax notoginseng*. *Front. Microbiol.* **2022**, *13*, 1031474. [CrossRef] [PubMed]
28. Li, Q.; Zhao, Y.; Zhu, X.M.; Xie, Y.L. Antifungal effect of o-vanillin on mitochondria of *Aspergillus flavus*: Ultrastructure and TCA cycle are destroyed. *Int. J. Food Sci. Technol.* **2022**, *57*, 3142–3149. [CrossRef]
29. Wang, H.X.; Peng, Z.H.; Sun, H.M. Antifungal activities and mechanisms of trans-cinnamaldehyde and thymol against food-spoilage yeast *Zygosaccharomyces rouxii*. *J. Food Sci.* **2022**, *87*, 1197–1210. [CrossRef]
30. Gomaa, E.Z. Microbial chitinases: Properties, enhancement and potential applications. *Protoplasma* **2021**, *258*, 695–710. [CrossRef]
31. Thakur, D.; Bairwa, A.; Dipa, B.; Jhilta, P.; Chauhan, A. An overview of fungal chitinases and their potential applications. *Protoplasma* **2023**, *260*, 1031–1046. [CrossRef]
32. Kumar, M.; Chakdar, H.; Pandiyan, K.; Thapa, S.; Shaind, M.; Singh, A.; Srivastava, A.K.; Saxena, A.K. Bacterial chitinases: Genetics, engineering and applications. *World J. Microbiol. Biotechnol.* **2022**, *38*, 252. [CrossRef]
33. OuYang, Q.; Liu, Y.; Oketch, O.R.; Zhang, M.; Shao, X.; Tao, N. Citronellal exerts its antifungal activity by targeting ergosterol biosynthesis in penicillium digitatum. *J. Fungi* **2021**, *7*, 432. [CrossRef]
34. Bautista-Silva, J.P.; Seibert, J.B.; Amparo, T.R.; Rodrigues, I.V.; Teixeira, L.F.M.; Souza, G.H.B.; dos Santos, O.D.H. Melaleuca leucadendra essential oil promotes loss of cell membrane and wall integrity and inhibits bacterial growth: An in silico and in vitro approach. *Curr. Microbiol.* **2020**, *77*, 2181–2191. [CrossRef]
35. Hu, Z.Y.; Lu, C.; Zhang, Y.S.; Tong, W.J.; Du, L.H.; Liu, F. Proteomic analysis of *Aspergillus flavus* reveals the antifungal action of *Perilla frutescens* essential oil by interfering with energy metabolism and defense function. *LWT-Food Sci. Technol.* **2022**, *154*, 112660. [CrossRef]
36. Ju, J.; Xie, Y.F.; Yu, H.; Guo, Y.H.; Cheng, Y.L.; Qian, H.; Yao, W.R. Synergistic interactions of plant essential oils with antimicrobial agents: A new antimicrobial therapy. *Crit. Rev. Food Sci. Nutr.* **2022**, *62*, 1740–1751. [CrossRef]

37. Ju, J.; Xie, Y.F.; Guo, Y.H.; Cheng, Y.L.; Qian, H.; Yao, W.R. The inhibitory effect of plant essential oils on foodborne pathogenic bacteria in food. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 3281–3292. [CrossRef] [PubMed]
38. Maia, A.J.; Oliveira, J.S.B.; Schwan-Estrada, K.R.F.; Faria, C.M.R.; Batista, A.F.; Costa, W.F.; Batista, B.N. The control of isariopsis leaf spot and downy mildew in grapevine cv. Isabel with the essential oil of lemon grass and the activity of defensive enzymes in response to the essential oil. *Crop Prot.* **2014**, *63*, 57–67. [CrossRef]
39. Huang, J.L.; Liu, S.S.; Liu, R.K.; Yi, Y.J.; Li, C.Z.; Xiao, Z.H.; Tu, J.; Xiao, J.J. Mechanisms of Litsea cubeba essential oil in the control of *Colletotrichum scovillei* in pepper (*Capsicum annuum* L.): Cell membrane/wall perspective. *Physiol. Mol. Plant Pathol.* **2023**, *127*, 102103. [CrossRef]
40. Shi, Z.D.; Jiang, Y.P.; Sun, Y.J.; Min, D.D.; Li, F.J.; Li, X.A.; Zhang, X.H. Nanocapsules of oregano essential oil preparation and characterization and its fungistasis on apricot fruit during shelf life. *J. Food Process. Preserv.* **2021**, *45*, e15649. [CrossRef]
41. Ferreira, F.M.D.; Hirooka, E.Y.; Ferreira, F.D.; Silva, M.V.; Mossini, S.A.G.; Machinski, M. Effect of Zingiber officinale Roscoe essential oil in fungus control and deoxynivalenol production of *Fusarium graminearum* Schwabe in vitro. *Food Addit. Contam.* **2018**, *35*, 2168–2174. [CrossRef]
42. Brandao, R.M.; Ferreira, V.R.F.; Batista, L.R.; Alves, E.; Lira, N.D.; Bellete, B.S.; Scolforo, J.R.S.; Cardoso, M.D.G. Antifungal and antimycotoxigenic effect of the essential oil of Eremanthus erythropappus on three different *Aspergillus* species. *Flavour Fragr. J.* **2020**, *35*, 524–533. [CrossRef]
43. Rasooli, I.; Rezaei, M.B.; Allameh, A. Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from *Thymus eriocalyx* and *Thymus x-porlock*. *Food Control* **2006**, *17*, 359–364. [CrossRef]
44. Di Pasqua, R.; Hoskins, N.; Betts, G.; Mauriello, G. Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *J. Agric. Food Chem.* **2006**, *54*, 2745–2749. [CrossRef]
45. Shahina, Z.; El-Ganiny, A.M.; Minion, J.; Whiteway, M.; Sultana, T.; Dahms, T.E.S. *Cinnamomum zeylanicum* bark essential oil induces cell wall remodelling and spindle defects in *Candida albicans*. *Fungal Biol. Biotechnol.* **2018**, *5*, 3. [CrossRef]
46. Lin, L.; Chen, S.; Xia, W.; Li, T.; Dong, L.; Zhang, Y.; Zhang, Y.; Li, H.; Zhao, Y.; Fu, X. A new strategy: Inhibitory effect and mechanism of *Cymbopogon martinii* essential oil on Aspergillus flavus. *Food Biosci.* **2023**, *51*, 102315. [CrossRef]
47. Song, G.; Du, S.; Sun, H.; Liang, Q.; Wang, H.; Yan, M.; Zhang, J. Antifungal mechanism of (E)-2-hexenal against *Botrytis cinerea* growth revealed by transcriptome analysis. *Front. Microbiol.* **2022**, *13*, 951751. [CrossRef]
48. Ma, B.; Ban, X.; Huang, B.; He, J.; Tian, J.; Zeng, H.; Chen, Y.; Wang, Y. Interference and mechanism of dill seed essential oil and contribution of carvone and limonene in preventing sclerotinia rot of rapeseed. *PLoS ONE* **2015**, *10*, e0131733. [CrossRef]
49. Zhao, F.; Huang, J.; Qi, J.; Li, Q.; Wu, H.; Ju, J. Proteomic analysis of antifungal mechanism of star anise essential oil against *Aspergillus* niger and its application potential in prolonging bread shelf life. *LWT-Food Sci. Technol.* **2022**, *169*, 114023. [CrossRef]
50. Hu, Y.; Zhang, J.; Kong, W.; Zhao, G.; Yang, M. Mechanisms of antifungal and anti-aflatoxigenic properties of essential oil derived from turmeric (*Curcuma longa* L.) on *Aspergillus flavus*. *Food Chem.* **2017**, *220*, 1–8. [CrossRef]
51. Zhou, T.; Wang, X.H.; Ye, B.S.; Shi, L.; Bai, X.L.; Lai, T.F. Effects of essential oil decanal on growth and transcriptome of the postharvest fungal pathogen *Penicillium expansum*. *Postharvest Biol. Technol.* **2018**, *145*, 203–212. [CrossRef]
52. Manoharan, R.K.; Lee, J.H.; Lee, J. Antibiofilm and antihyphal activities of cedar leaf essential oil, camphor, and fenchone derivatives against *Candida albicans*. *Front. Microbiol.* **2017**, *8*, 1746. [CrossRef] [PubMed]
53. Chung, E.Y.; Byun, Y.H.; Shin, E.J.; Chung, H.S.; Lee, Y.H.; Shin, S. Antibacterial effects of vulgarone B from *Artemisia iwayomogi* alone and in combination with oxacillin. *Arch. Pharmacal Res.* **2009**, *32*, 1711–1719. [CrossRef] [PubMed]
54. Abd-Elgawad, M.M.M. Understanding Molecular Plant–Nematode Interactions to Develop Alternative Approaches for Nematode Control. *Plants* **2022**, *11*, 2141. [CrossRef] [PubMed]
55. Villa, F.; Cappitelli, F.; Cortesi, P.; Kunova, A. Fungal Biofilms: Targets for the development of novel strategies in plant disease management. *Front. Microbiol.* **2017**, *8*, 654. [CrossRef]
56. Lagree, K.; Desai, J.V.; Finkel, J.S.; Lanni, F. Microscopy of fungal biofilms. *Curr. Opin. Microbiol.* **2018**, *43*, 100–107. [CrossRef]
57. Costa-Orlandi, C.B.; Sardi, J.C.O.; Pitangui, N.S.; De Oliveira, H.C.; Scorzoni, L.; Galeane, M.C.; Medina-Alarcón, K.P.; Melo, W.C.M.A.; Marcelino, M.Y.; Braz, J.D.; et al. Fungal biofilms and polymicrobial diseases. *J. Fungi* **2017**, *3*, 22. [CrossRef]