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Efficacy and Potential Mechanism of Essential Oils of Three Labiatae Plants against the Pathogenic Fungi of Root Rot Disease in *Atractylodes chinensis*

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Abstract: *Atractylodes chinensis* has a high medicinal value and is widely cultivated. However, root rot disease seriously affects the yield and quality of *A. chinensis*. To develop green and safe pesticides, the inhibitory effect of essential oils (EOs) of three Labiatae plants on the pathogenic fungi that causes root rot disease in *Atractylodes chinensis* was investigated. The results showed that the *Origanum vulgare* EO and *Thymus mongolicus* EO exhibited strong inhibitory effects on *Fusarium oxysporum*, *Fusarium solani*, and *Fusarium redolens*, with 100% inhibition rate. The low MIC values of EOs and their main components against the three pathogenic fungi indicated that all of them showed strong fungicidal effects. The MIC values of *O. vulgare* EO against *F. oxysporum*, *F. solani*, and *F. redolens* were 2.60 mg/mL, 3.13 mg/mL, and 1.56 mg/mL, respectively. Analyses using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) showed that the *O. vulgare* EO severely damaged the cell wall and cell membrane of mycelial cells. The *O. vulgare* EO increased cell permeability, leading to a large leakage of cell contents (DNA and proteins). In addition, *O. vulgare* EO inhibited *F. oxysporum* by inducing ROS production and reducing the amount of intracellular GSH, leading to a large accumulation of ROS. This study showed that plant EOs have excellent fungicidal activity and can be used as novel natural and environmentally friendly pesticides for the control of root rot in *A. chinensis*.

Keywords: *Atractylodes chinensis*; root rot; essential oils; *Origanum vulgare*; antifungal activity; mechanisms of action

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1. Introduction

Atractylodes chinensis is a perennial herbal plant of the Asteraceae family, mainly distributed in East Asian countries [1]. *A. chinensis* is one of the commonly used bulk herbs, and its dried rhizomes are used as a medicine, and *A. chinensis* has a high medicinal value [2,3]. *A. chinensis* has been traditionally used to treat digestive disorders, rheumatic diseases, and visual disorders [4]. The main active constituents of *A. chinensis* are atractylodin, atractylon, and β-eudesmol [5]. Modern pharmacological studies have shown that these substances have excellent pharmacological properties, such as anti-inflammatory, anticancer, anti-tumor, hypoglycemic, and neuroprotective [2,6–8]. In addition to its medicinal value, *A. chinensis* has been widely used in food, feed, and cosmetic industries [3].

Root rot is a highly prevalent disease in agricultural production and is known as "plant cancer" [9,10]. It is highly infectious, lethal, and difficult to control [11,12]. Root rot

is the most threatening disease observed in *A. chinensis* and can occur at all stages of its growth. In recent years, the market demand for *A. chinensis* has been expanding, and wild *A. chinensis* resources have not been able to meet people's needs [13]. However, with the increasing planting area and cultivation density, the root rot disease of *A. chinensis* has been becoming more and more serious in each cultivation area. Previous studies have shown that root rot of *A. chinensis* is mainly caused by *Fusarium* spp. [14]. The pathogenic fungus first invades the roots and keeps spreading to the above-ground parts, and at a later stage, the plant wilts completely [15]. Root rot causes 20% economic losses to the *A. chinensis* industry each year, with yield reductions of up to 50–80% in areas with severe disease [16].

Currently, the main method of controlling root rot of *A. chinensis* is chemical control, which mainly relies on pesticides, such as carbendazim and hymexazol [17]. Although there are obvious benefits of their use in the short term, the long-term use of chemicals will make the pathogens resistant and reduce the effectiveness of control [18–20]. Meanwhile, synthetic pesticides cause large amounts of residues and environmental pollution, which result in serious harm to the ecology, non-target organisms, and even human beings [21,22]. There is an urgent need to find safe and harmless methods of plant disease control.

Plant-derived pesticides have received extensive attention from scholars due to their easy degradation, low toxicity, and non-induced resistance [23,24]. Plant essential oils are a group of secondary metabolites of plants, which are fat-soluble natural mixtures with a volatile, strong aroma [25]. Plants naturally use these ingredients to fight off various pests and pathogens [26]. Plant EOs are fully degradable, leaving no residue and are highly selective and have no toxic effects on non-target organisms [27]. Therefore, plant EOs can be used as natural plant protectors as an alternative to synthetic pesticides [28].

Labiatae are annual or perennial herbs with a wide global distribution [29]. There are more than 7000 species in 236 genera of the family Labiatae that are rich in EOs, such as *Origanum vulgare*, *Thymus mongolicus*, *Mentha canadensis*, and *Lavandula angustifolia* [29]. Previous studies have shown that EOs of Labiatae have excellent inhibitory activities against bacteria and fungi [30–32]. Xiao et al. found that *O. vulgare* and *T. mongolicus* significantly inhibited *Staphylococcus aureus* [33]. A previous study showed that the EO of *M. piperita* and its major volatile components inhibited the growth of *Fusarium sambucinum*, effectively controling soft rot of *Capsicum pubescens* [34]. Nafis et al. found that lavender essential oil could inhibit a wide range of foodborne bacteria [35]. Currently, EOs of Labiatae have been widely used in food, feed, and pharmaceuticals and for crop protection [36]. However, there are no previous reports on controlling the root rot of *A. chinensis* by using Labiatae EOs.

In the study, the inhibitory activities of EOs from three Labiatae plants against root rot pathogens of *A. chinensis* were evaluated. The *O. vulgare* EO was selected to further investigate the mechanism of action against *F. oxysporum*. This study provides a theoretical basis for the development of green and environmentally friendly pesticides for the control of root rot disease of *A. chinensis*.

2. Materials and Methods

2.1. Essential Oils and Chemicals

O. vulgare EO, *T. mongolicus* EO, and *P. cablin* EO were purchased from Jiangxi Jianmin Natural Spice Co., Ltd. (Ji'an, China). Carbendazim (≥97%), carvacrol (≥98%), thymol (≥98%), and NAC (≥99%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). O-Cymene (≥98%) and γ-terpinene (≥97%) were purchased from Sigma Aldrich Co., Ltd. (St. Louis, MO, USA). Hymexazol (≥98%) was purchased from Shanghai Acmec Biochemical Co., Ltd. (Shanghai, China). MAN (≥98%) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). GSH (≥98%) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Reactive Oxygen Species (ROS) Fluorometric Assay Kit was purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Total Glutathione Assay Kit was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China).

2.2. Pathogenic Fungi

The pathogenic fungi were isolated from rotten roots of *A. chinensis* and identified as *F. oxysporum*, *F. solani*, and *F. redolens* by Tsingke Biotechnology Co., Ltd. (Beijing, China).

2.3. Measurement of In Vitro Antifungal Activity of Three EOs

The in vitro antifungal activity of three EOs was determined according to the Oxford cup method [37]. A mixture of 1% DMSO and 0.1% Tween-80 (1-DMSO-T) was used to dissolve three EOs and two chemical pesticides. The final concentrations of EOs and chemical pesticides were 50 mg/mL and 5 mg/mL, respectively. Under sterile conditions, all solutions were filtered through a 0.22 μm microporous filter membrane. After the activation of the pathogenic fungi, they were incubated at 28 °C for 7 days. Mycelial plugs were taken along with a 5 mm sterile punch and placed in the center of a new PDA plate. Four sterilized Oxford cups were placed symmetrically at 2 cm from the mycelial plug, and 200 μL of EO dilution was added to each Oxford cup. 1-DMSO-T was used as a negative control, and two chemical pesticides (carbendazim and hymexazol) were used as positive controls. Each group was repeated five times. The PDA plates were incubated in a constant temperature incubator at 28 °C for 7 days. The diameter of the colony was determined based on the average of two vertical diameters. The inhibition rate was calculated as follows:

The inhibition rate = (the diameter of negative control − the diameter of treatment group)/the diameter of negative control × 100%

2.4. Measurement of In Vitro Antifungal Activity of Three EOs

The fungal inhibitory activity of EO volatiles was determined with minor modifications according to the methods previously reported in the literature [26]. A 5 mm fungal mycelial plug was placed in the center of the PDA plate. EOs (20 μL) were added to the Petri dish lids, and the PDA plates with mycelial plugs were placed upside down on the lids. The control plates were not amended with EO. The control group was not treated with EO. Each group was repeated five times. The PDA plates were incubated in a constant temperature incubator at 28 °C for 7 days. The diameter of the colony was determined based on the average of two vertical diameters. The inhibition rate was calculated as described in Section 2.3.

2.5. GC-MS Determination of O. vulgare EO and T. mongolicus EO

The chemical compositions of the O. vulgare EO and the T. mongolicus EO were analyzed using GC-MS. The GC apparatus was a Thermo TRACE 1300 with a nonpolar Agilent DB-5 capillary column (30 m × 0.25 mm × 0.25 μm). The ionization potential was 70 ev. The inlet temperature, gasification temperature, and ion source temperature were all set to 280 °C. Samples were injected manually. The carrier gas was helium at a flow rate of 1.5 mL/min. The split ratio was 10:1. The scanning range was 30–550 AMU. The retention indices (RI) were determined according to previous literature methods [38]. Based on the RI, the chemical composition was determined by comparing with n-alkanes. The chemical composition of the essential oils was determined by comparing GC-MS mass spectra with previous data from the literature and computerized databases (Wiley/NIST) [39]. The relative content of each component was then calculated based on the relative peak area ratio [40].

2.6. Measurement of Fungal Inhibitory Activities of Main Components

The inhibitory activities of carvacrol, thymol, γ-terpinene, and o-cymene against the three pathogenic fungi were determined. The four main components were dissolved using 1-DMSO-T to a final concentration of 5 mg/mL. Under sterile conditions, all the solutions were passed through a 0.22 μm microporous filter membrane to obtain a sterile filtrate. A Ø5 mm mycelial plug was placed in the center of the PDA plate, and four sterilized Oxford cups were placed symmetrically at 2 cm from the plug, and 200 μL of sterile filtrate

was added to each Oxford cup. The PDA plates were incubated in a constant temperature incubator at 28 °C for 7 d. The colony diameters and inhibition rates were calculated as described in Section 2.3.

2.7. Determination of MIC

The pathogen was incubated at 28 °C for 7 days, and the spores were rinsed with 15 mL of 1/4 Potato Dextrose Broth (PDB). Spore suspensions were counted with a hemocytometer plate and diluted to a concentration of 1×10^5 spores/mL with 1/4 PDB. The *O. vulgare* EO and the *T. mongolicus* EO were dissolved using a mixture of 2% DMSO and 0.1% Tween-80 (2-DMSO-T). The initial concentration of the EOs were 50 mg/mL, and their solution was diluted 9 times with 2-DMSO-T using double dilution to obtain a concentration range of 50–0.098 mg/mL. Carbendazim and the main components (carvacrol and thymol) of the *O. vulgare* EO were also dissolved and diluted using the same method to obtain 10 concentrations ranging from 5 to 0.0098 mg/mL. All solutions were filtered using a 0.22 μm microporous membrane to obtain sterile filtrates. The 150 μL spore suspension and 50 μL sterile filtrate were added to the 96-well plate. A mixture of 150 μL spore suspension and 50 μL 2-DMSO-T was used as a negative control. The 96-well plates were incubated in a constant temperature incubator at 28 °C for 36 h. The absorbance at 595 nm of each well was detected using a multimode microplate reader (Tecan, Spark 30086376, Männedorf, Switzerland). Each group was repeated eight times, and the experiment was performed three times.

2.8. Effect of O. vulgare EO on the Mycelial Morphology of F. oxysporum

2.8.1. Optical Microscope Observation

The *F. oxysporum* colonies were rinsed with sterile water, and the spore concentration was adjusted to a concentration of 1×10^6 spores/mL. PDB (24 mL) and the spore suspension (1 mL) were added to a 50 mL centrifuge tube and incubated at 28 °C at 180 rpm for 3 d. The EO of *O. vulgare* was diluted with 2-DMSO-T and added to centrifuge tubes to produce a final concentration of 1/4 MIC and MIC, and incubated at 28 °C at 180 rpm for 24 h, with 2-DMSO-T as a negative control. The mycelial suspension was centrifuged at 10,000× *g* for 10 min and then rinsed three times with PBS. The morphology of the mycelia was observed using an optical microscope (Leica, DM6000B, Wetzlar, Germany).

2.8.2. Scanning Electron Microscopy (SEM)

The *F. oxysporum* colonies were treated with 1/4 MIC and MIC of *O. vulgare* EO for 6 h. Mycelium was fixed using 3% glutaraldehyde, post-fixed using 1% osmium tetroxide, and then dehydrated using gradient ethanol. The samples were glued to the sample holders with conductive adhesive, and the holders were placed into the ion sputterer for the spray treatment. Samples were observed using a scanning electron microscope (JEOL, JSM-IT700HR, Akishima, Japan).

2.8.3. Transmission Electron Microscopy (TEM)

The mycelial preparation method for TEM was the same as described in Section 2.8.1. The mycelium was pretreated with 3% glutaraldehyde, post-treated with 1% osmium tetroxide, dehydrated in a series of acetone treatments, infiltrated for a longer period in Epox 812, and then embedded. Ultrathin sections were sliced with a diamond knife and stained with uranyl acetate and lead citrate. Sections were observed using a transmission electron microscope (JEOL, JEM-1400-FLASH, Akishima, Japan).

2.9. Effect on Leakage of Cell Contents of F. oxysporum

A 20 mL volume of 1/4 MIC and MIC of the *O. vulgare* EO was added to 50 mL centrifuge tubes, and 0.5× *g* of mycelia was added to the tubes, respectively. Centrifuge tubes were incubated at 28 °C at 180 rpm for 12 h. 2-DMSO-T was used as a negative control. The mycelial suspensions were centrifuged at 10,000× *g* for 5 min. The DNA

concentration and soluble protein concentration in the supernatant were measured using a spectrophotometer (Thermo scientific, ND-2000C, Waltham, MA, USA). Each treatment was replicated five times.

2.10. Measurement of Intracellular Reactive Oxygen Species (ROS) Production

A 24 mL volume of PDB with 1 mL of *F. oxysporum* spore suspension were added to a 50 mL centrifuge tube and incubated at 28 °C at 180 rpm for 3 days. The *O. vulgare* EO diluted with 2-DMSO-T was added to the centrifuge tube to adjust the concentration of the solution to 1/2 MIC and incubated for 3 h. Mycelia were collected and washed three times using pre-cooled PBS. The mycelia were incubated with 10 μM DCFH-DA at 37 °C for 30 min in the dark. Mycelia were washed three times with PBS and observed under a high-resolution laser confocal microscope (Leica, TCSSP8, Wetzlar, Germany).

2.11. Effects of Exogenous ROS Scavengers on the Activity of O. vulgare EO

ROS scavengers (GSH, NAC, and MAN) were used to further analyze the relationship between the inhibition mechanism of the *O. vulgare* EO and ROS accumulation. A 5 mm fungal mycelial plug was placed on the PDA plate supplemented with 2 mM ROS scavengers. The *O. vulgare* EO (2 μL) was added to the Petri dish lids, and the PDA plates with mycelial plugs were placed upside down on the lids. Each group was repeated five times. The PDA plates were incubated in a constant temperature incubator at 28 °C for 7 days. The diameter of the colony was determined based on the average of two vertical diameters. The inhibition rate was calculated according to a previous method reported in the literature [41].

2.12. Intracellular GSH Detection

F. oxysporum mycelia were collected and treated with 1/2 MIC *O. vulgare* EO for 0.5 h, 1 h, and 2 h. 2-DMSO-T was used as negative control. A total of 1 g of mycelia was obtained, and the intracellular glutathione content was measured using a Total Glutathione Assay Kit [41]. Each group was repeated five times.

3. Results

3.1. Inhibitory Effect of EOs on Pathogenic Fungi

The inhibitory effects of the three EOs on the growth of root rot pathogens are shown in Figure 1. Both the *O. vulgare* EO and the *T. mongolicus* EO showed 100% inhibition of the three fungi, indicating that both EOs exhibited strong antifungal activities. The fungal inhibitory effect of the *P. cablin* EO was weak, with 35.47%, 11.48%, and 12.90% inhibition rates against *F. oxysporum*, *F. solani*, and *F. redolens*, respectively. Both chemical pesticides showed a certain inhibitory activity against the three pathogenic fungi. The inhibition rates of carbendazim against *F. oxysporum*, *F. solani*, and *F. redolens* were 65.59%, 6.81%, and 40.32%, respectively. Hymexazol exhibited the strongest inhibitory effect on *F. oxysporum* (76.52%), followed by *F. redolens* (50.21%) and *F. solani* (30.15%). The inhibitory effect of chemical pesticides on *F. solani* was weak.

3.2. Inhibitory Effects of EO Volatiles

The fungal inhibitory effects of the volatiles of the *O. vulgare* EO and the *T. mongolicus* EO were further measured to evaluate the indirect activity of the EOs (Figure 2). Volatiles from the *O. vulgare* EO inhibited 100% of the mycelial growth in the three pathogenic fungi. The volatiles of *T. mongolicus* EO inhibited the mycelial growth of *F. oxysporum*, *F. solani*, and *F. redolens* by 53.79%, 27.25%, and 30.86%, respectively.

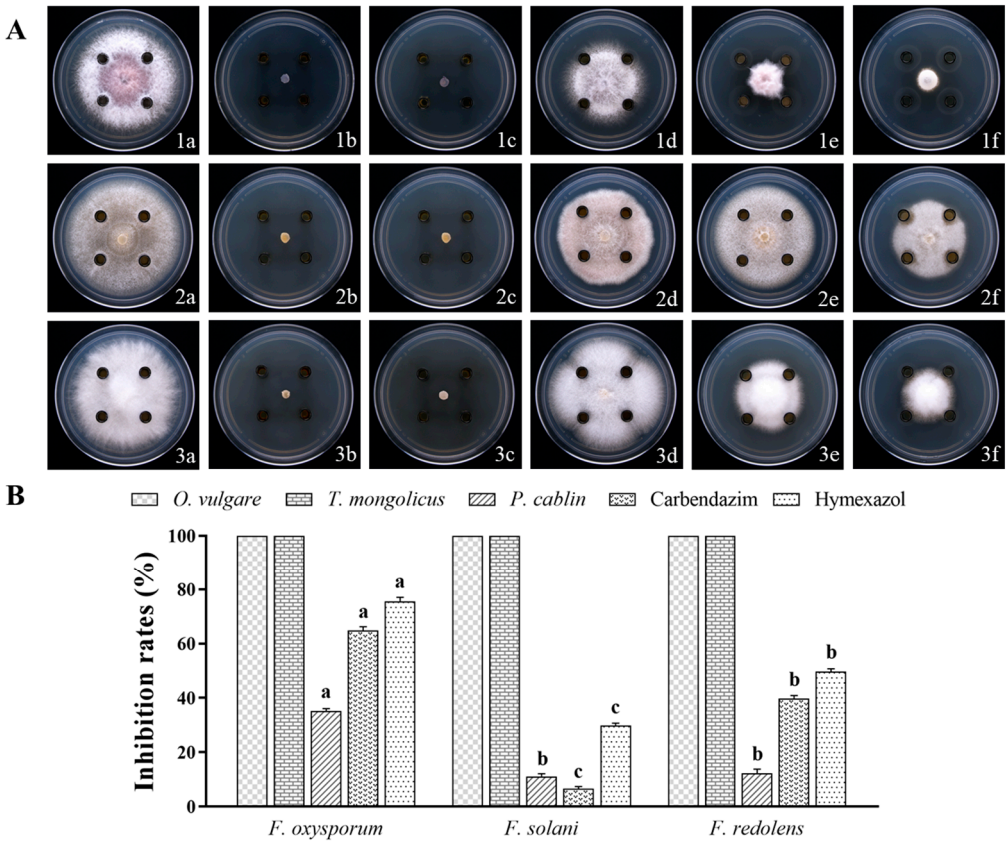

Figure 1. (**A**) The inhibitory effect of *O. vulgare*, *T. mongolicus*, and *P. cablin* essential oils (EOs) on three pathogenic fungi. The three pathogenic fungi were (1) *F. oxysporum*, (2) *F. solani*, and (3) *F. redolens*. The different treatments were (a) 1-DMSO-T, negative control, (b) *O. vulgare*, (c) *T. mongolicus*, (d) *P. cablin*, (e) carbendazim, positive control, and (f) hymexazol, positive control. (**B**) The inhibitory rates of *O. vulgare*, *T. mongolicus*, and *P. cablin* EOs in three pathogenic fungi. Different lowercase letters for the same treatment indicate significant differences ($p < 0.05$) among different fungi.

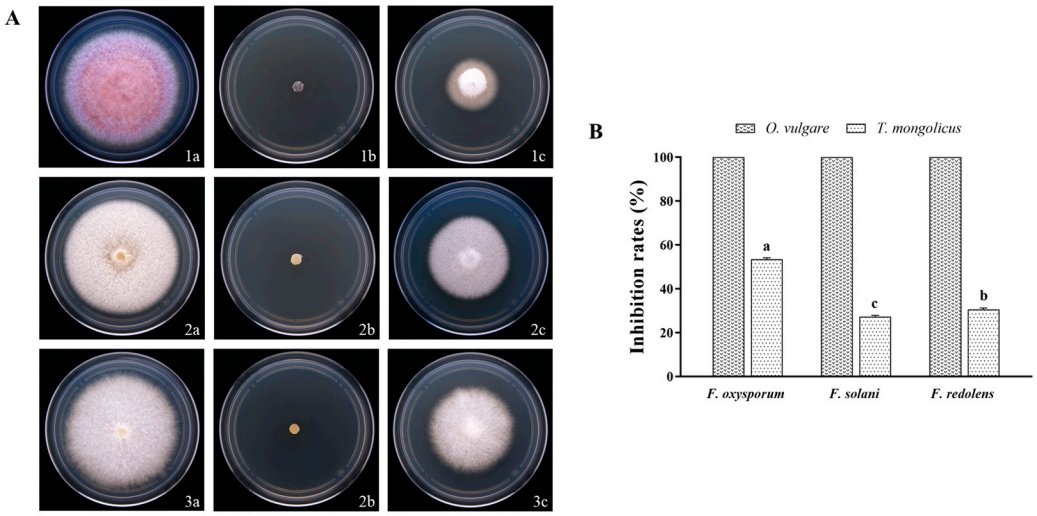

Figure 2. (**A**) Antifungal activities of EO volatiles in three pathogenic fungi. The three pathogenic fungi were (1) *F. oxysporum*, (2) *F. solani*, and (3) *F. redolens*. The different treatments were (a) control, (b) *O. vulgare*, and (c) *T. mongolicus*. (**B**) The inhibitory rates of EO volatiles in the three pathogenic fungi. Different lowercase letters for the same treatment indicate significant differences ($p < 0.05$) among different fungi.

3.3. Chemical Compositions

The chemical compositions of the *O. vulgare* EO and the *T. mongolicus* EO were analyzed with GC-MS, and the results are shown in Tables S1 and S2. The GC-MS chromatograms of the *O. vulgare* EO and the *T. mongolicus* EO are shown in Figures S1 and S2. The main components of the *O. vulgare* EO were carvacrol (86.23%) and thymol (12%). The most abundant component of the *T. mongolicus* EO was o-cymene (56.63%), followed by γ-terpinene (21.96%) and thymol (12.05%). The main component common to both EOs was thymol.

3.4. Antifungal Activities of Predominant Constituents

The results revealed that carvacrol and thymol had excellent inhibitory effects against the three pathogenic fungi (Figure 3). The inhibition rates of carvacrol against *F. oxysporum*, *F. solani*, and *F. redolens* were 67.52%, 54.46%, and 75.29%, respectively. Thymol showed the strongest inhibitory effect on *F. redolens* (73.99%), followed by *F. oxysporum* (63.59%), and *F. solani* (50.36%). The inhibition rates of γ-pinene against *F. oxysporum*, *F. solani*, and *F. redolens* were 9.84%, 5.55%, and 3.81%, which were significantly lower than those of the two phenols. The inhibitory effects of o-cymene on *F. oxysporum*, *F. solani*, and *F. redolens* were very weak, with inhibition rates of 4.11%, 3.75%, and 8.09%, respectively.

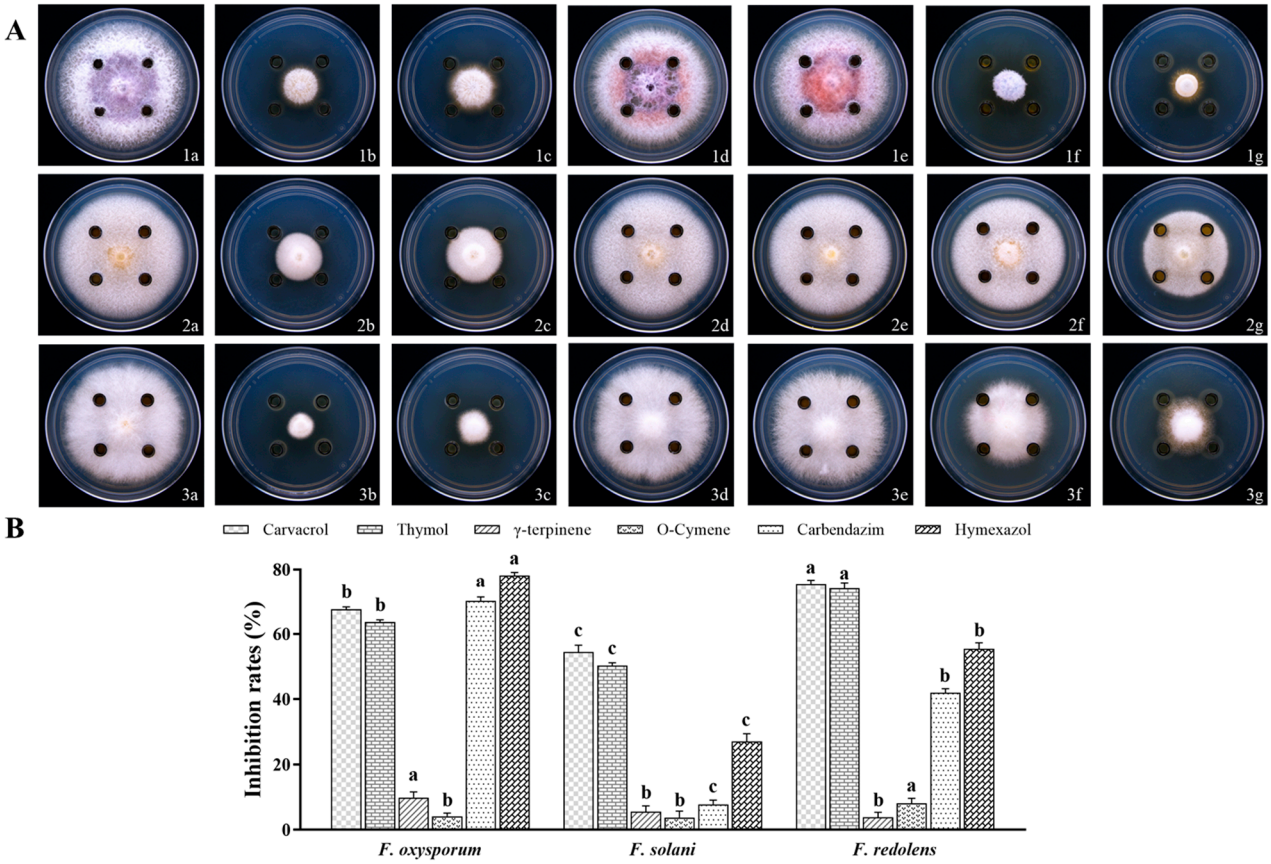

Figure 3. (**A**) Inhibitory effects of the main components on three pathogenic fungi. The three pathogenic fungi were (1) *F. oxysporum*, (2) *F. solani*, and (3) *F. redolens*. The different treatments were (a) 1-DMSO-T, negative control, (b) carvacrol, (c) thymol, (d) γ-terpinene, (e) o-cymene, (f) carbendazim, positive control, and (g) hymexazol, positive control. (**B**) The inhibitory rates of the main components on the three pathogenic fungi. Different lowercase letters for the same treatment indicate significant differences ($p < 0.05$) among different fungi.

3.5. Determination of MIC

The MIC values of the EOs and the main compounds against the fungi are shown in Table 1. The MIC values of the *O. vulgare* EO against *F. oxysporum*, *F. solani*, and *F. redolens* were 2.60 mg/mL, 3.13 mg/mL, and 1.56 mg/mL, respectively. The MIC values of the *T. mongolicus* EO against the three pathogenic fungi were 3.13 mg/mL, 2.60 mg/mL, and 2.60 mg/mL, respectively. The main components of the *O. vulgare* EO exhibited a higher antifungal activity than the *O. vulgare* EO and had a lower MIC value. The MIC values of carvacrol and thymol against the three pathogenic fungi were 0.83–1.04 mg/mL and 0.83–1.25 mg/mL, respectively. Carbendazim had a strong antifungal effect, with low MIC values of 0.05–0.07 mg/mL.

Table 1. MIC [1] values of the EOs [2] and compounds against fungi (mg/mL).

	F. oxysporum	***F. solani***	***F. redolens***
O. vulgare	2.60 ± 0.90^{a}	3.13 ± 0.00^{a}	1.56 ± 0.00^{b}
T. mongolicus	3.13 ± 0.00^{a}	2.60 ± 0.90^{a}	2.60 ± 0.90^{a}
Carvacrol	0.83 ± 0.36^{bc}	1.04 ± 0.36^{b}	0.63 ± 0.00^{cd}
Thymol	1.04 ± 0.36^{b}	2.60 ± 0.90^{a}	1.25 ± 0.00^{bc}
Carbendazim	0.07 ± 0.02^{c}	0.05 ± 0.02^{c}	0.07 ± 0.02^{d}

[1] MIC: minimal inhibitory concentration; [2] EOs: essential oil. Different letters on the same column represent significant differences ($p < 0.05$).

3.6. Effects of O. vulgare EO on Morphology and Ultrastructure of F. oxysporum

3.6.1. Optical Microscopy Observations

The optical microscopy showed that that the surface of mycelium in the control group was smooth, and the structure of mycelium was intact (Figure 4A). However, after treatment with 1/4 MIC *O. vulgare* EO, cavities appeared on the surface of the mycelium, and mycelia were locally deformed (Figure 4B). The mycelium treated with MIC *O. vulgare* EO degraded, and a large number of cavities appeared on the surface of the mycelium (Figure 4C). These results indicated that the *O. vulgare* EO might cause damage to the cell membrane of *F. oxysporum*.

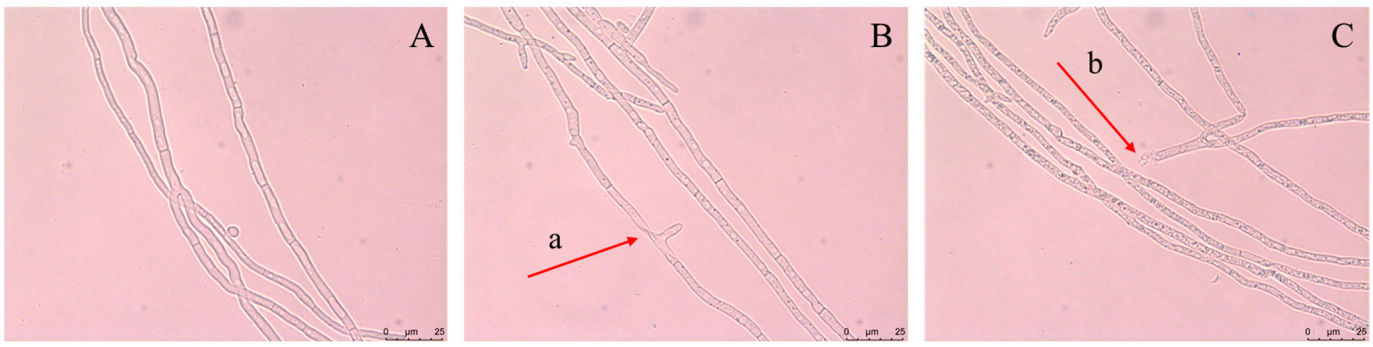

Figure 4. Optical microscopy observation of mycelial morphology of *F. oxysporum*. (**A**) Control, (**B**) 1/4 MIC *O. vulgare* EO treatment, and (**C**) MIC *O. vulgare* EO treatment. The red arrows indicate the point of abnormality in the hyphae: (a) hyphae folding and (b) hyphae fracture.

3.6.2. Scanning Electron Microscopy (SEM)

Scanning electron microscopy showed that the mycelium of the control group had a smooth surface and a complete structure (Figure 5(1A,2A)). However, after treatment with 1/4 MIC concentration of *O. vulgare* EO, the mycelium was deformed and appeared folded and wrinkled (Figure 5(1B,2B)); after treatment with MIC concentration of *O. vulgare* EO, the mycelium deformation was more severe, and some mycelia were ruptured (Figure 5(1C,2C)).

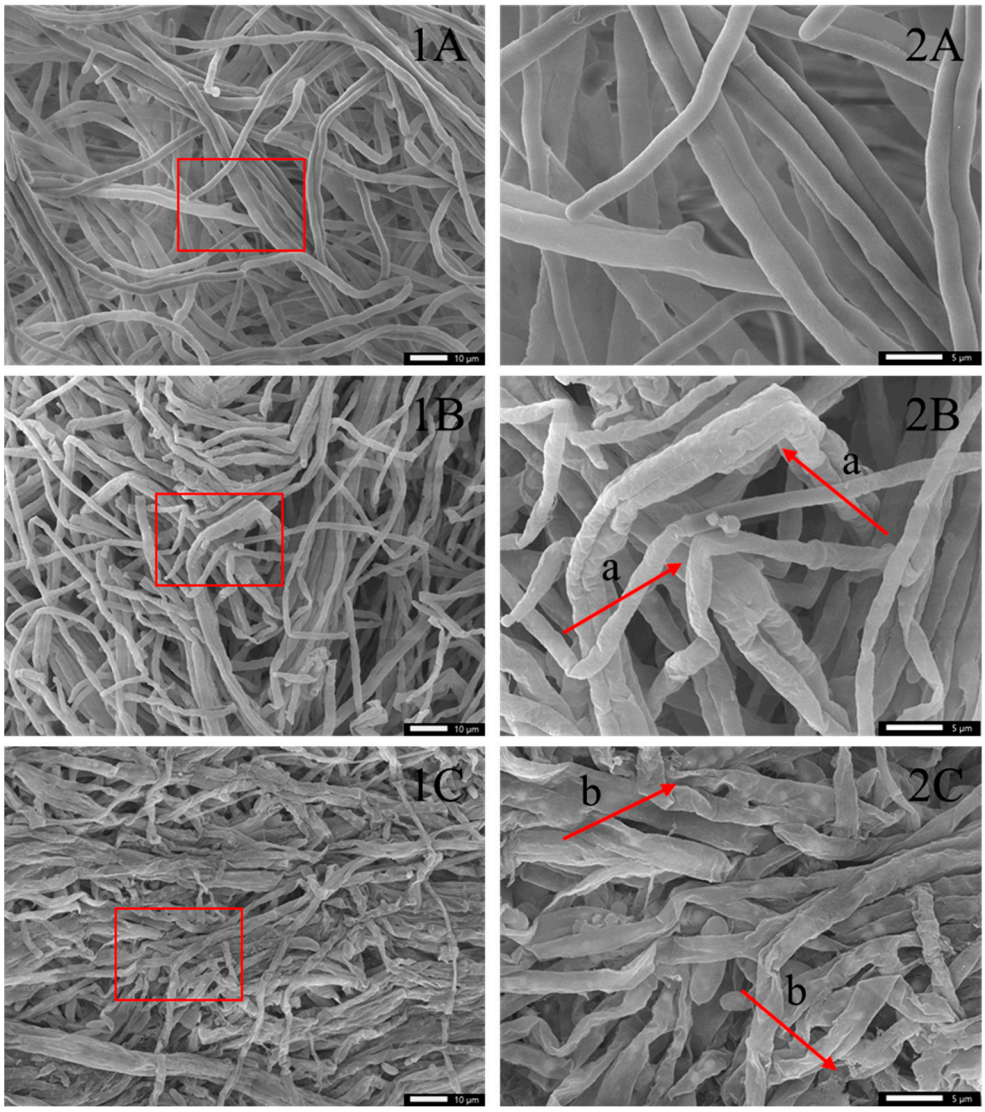

Figure 5. Scanning electron microscopy of mycelial morphology of *F. oxysporum*. (**A**) Control, (**B**) 1/4 MIC *O. vulgare* EO treatment, and (**C**) MIC *O. vulgare* EO treatment. (**1**) Magnification of 1000× and (**2**) magnification of 3000×. The red arrows indicate the point of abnormality in the hyphae: (a) hyphae deformation and (b) hyphae rupture.

3.6.3. Transmission Electron Microscopy (TEM)

The ultrastructure of mycelium was observed with transmission electron microscopy (TEM) (Figure 6). In the control group, the mycelial cell wall and plasma membrane were intact. Mycelial cell walls were thick and uniform, regular in shape, round or oval. The organelles were structurally intact and evenly distributed in the cytoplasm. After treatment with 1/4 MIC *O. vulgare* EO, the mycelial cell morphology was found to be highly irregular, and the cell wall deformation appeared as protrusions or depressions. Mycelial cells appeared to be adherent, and the cytoplasm was unevenly distributed. Moreover, the hyphae treated with MIC *O. vulgare* EO exhibited significant structural changes. Mycelial cell walls were thinner, and even their lysis occurred. The mycelial cells exhibited obvious plasmolysis, and the internal organelles were blurred and unrecognizable.

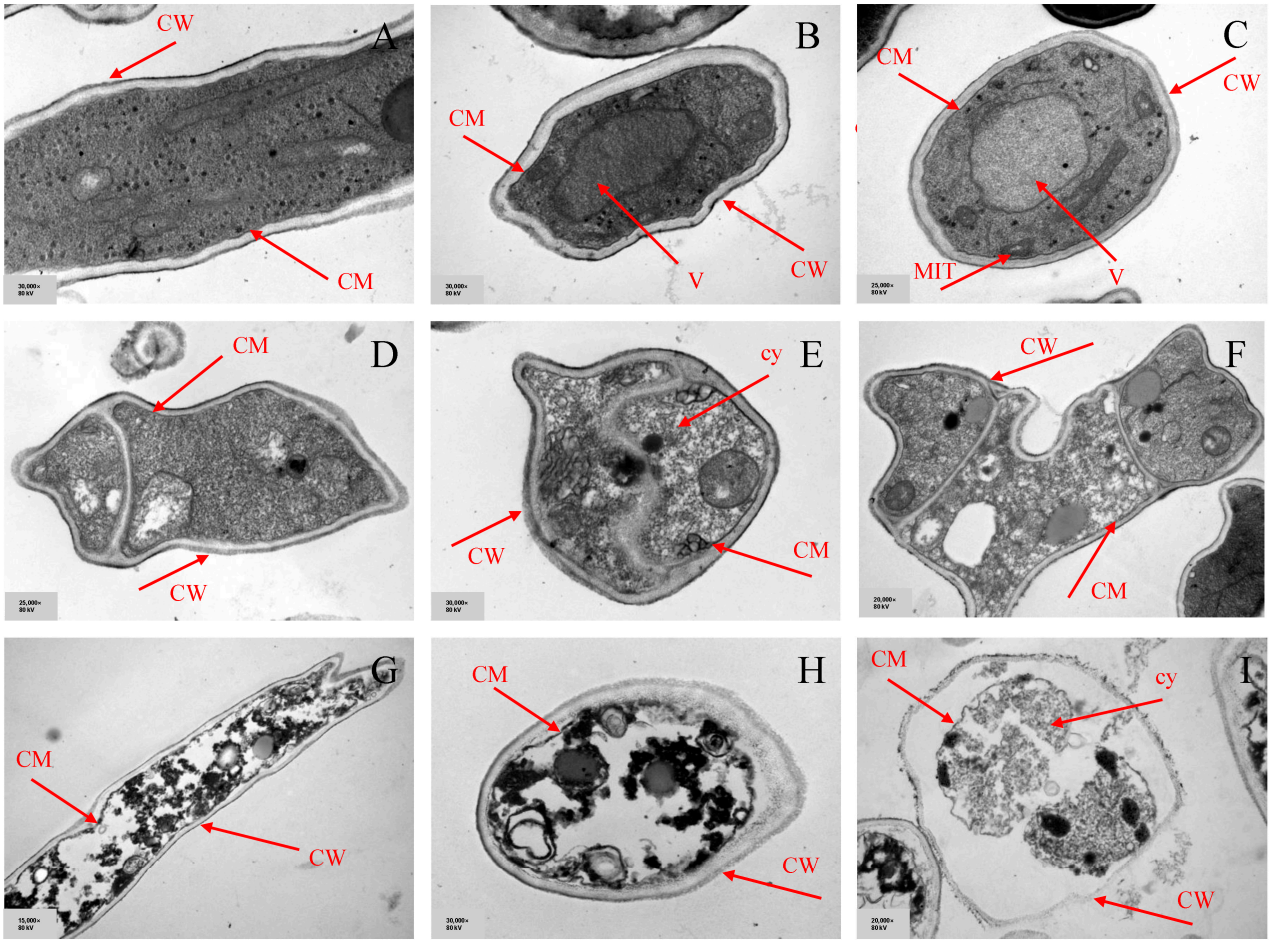

Figure 6. Ultrastructure of *F. oxysporum* observed with transmission electron microscope. (**A**–**C**) represent control group; (**D**–**F**) represent 1/4 MIC *O. vulgare* EO treatment group; (**G**–**I**) represent MIC *O. vulgare* EO treatment group. CW: cell wall; CM: cell membrane; MIT: mitochondria; V: vacuoles; cy: cytoplasm.

3.7. Effect on Leakage of Cell Contents of F. oxysporum

As shown in Figure 7, the *O. vulgare* EO caused a significant leakage of intracellular DNA and soluble proteins, and this effect exhibited concentration dependence. The DNA contents of 1/4 MIC and MIC were 179.48 μg/mL and 737.46 μg/mL, respectively, while that of the control group was only 8.1 μg/mL (Figure 7A). When treated with 1/4 MIC and MIC *O. vulgare* EO, the soluble protein contents were 51.07 and 162.22 times higher than the control group, respectively (Figure 7B).

3.8. The Effect of O. vulgare EO on Endogenous ROS in F. oxysporum

The DCFH-DA fluorescent probe was used to determine the intracellular ROS content of mycelium. As shown in Figure 8, the mycelium in the control group did not fluoresce. When treated with 1/2 MIC *O. vulgare EO*, clear fluorescence appeared within the mycelium. The results showed that the *O. vulgare* EO caused the production of ROS in the mycelial cells of *F. oxysporum*.

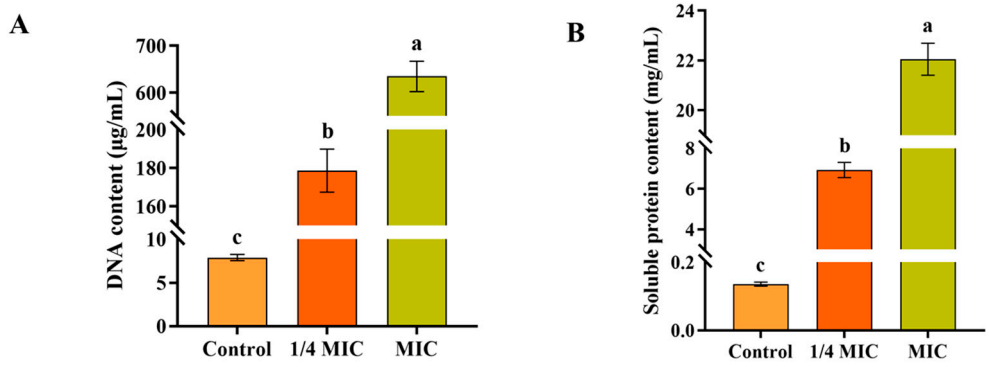

Figure 7. Effects of different concentrations of the *O. vulgare* EO on the DNA content (**A**) and the soluble protein content (**B**) of *F. oxysporum*. Different lowercase letters indicate statistically significant differences between treatments ($p < 0.05$).

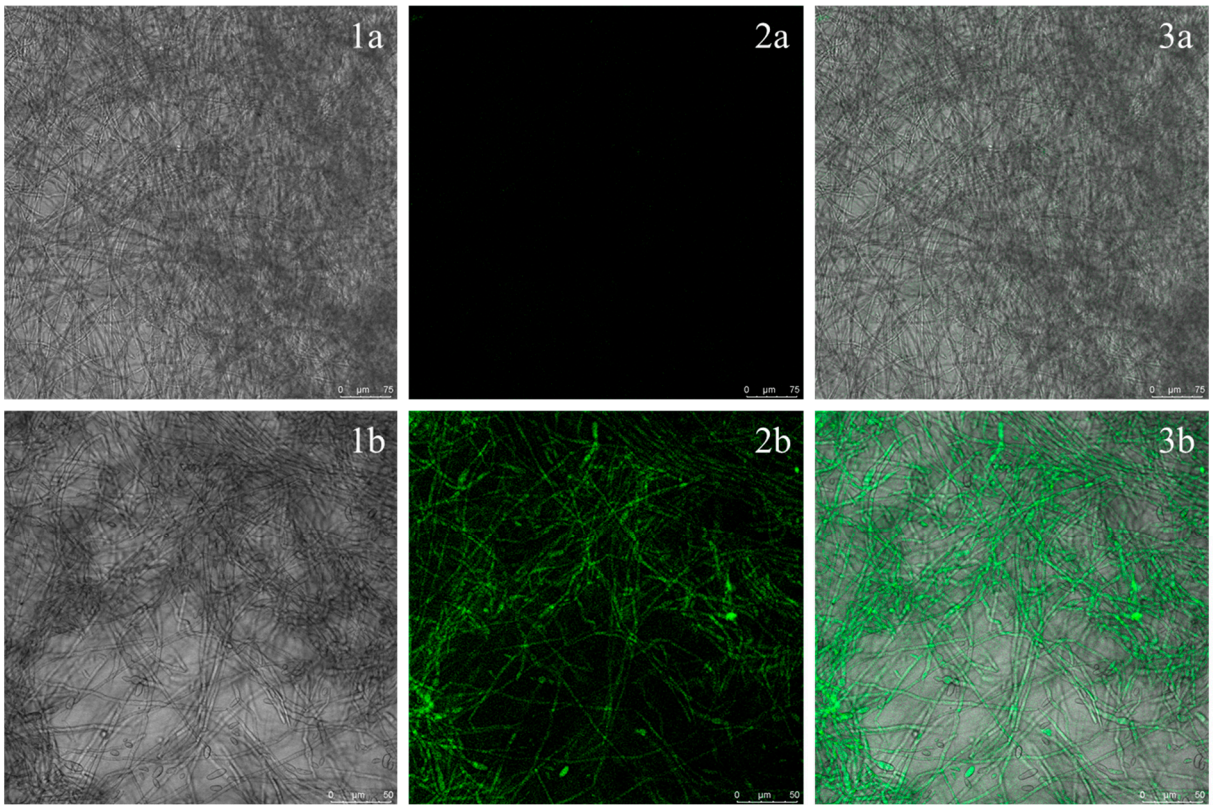

Figure 8. The effect of the *O. vulgare* EO on the production of reactive oxygen species. (**a**) Control and (**b**) 1/2 MIC *O. vulgare* EO treatment; (**1**) bright field, (**2**) DCFH-DA, and (**3**) merge.

3.9. Effects of Exogenous ROS Scavengers on the Activity of O. vulgare EO

The effect of exogenous ROS scavengers on the fungal inhibitory effect of the *O. vulgare* EO is shown in Figure 9A. The inhibition rate of the *O. vulgare* EO against *F. oxysporum* was 79.05%, while the inhibition rate was only 16.83%, 48.09%, and 50.17% with the addition of GSH, NAC, and MAN, respectively (Figure 9B).

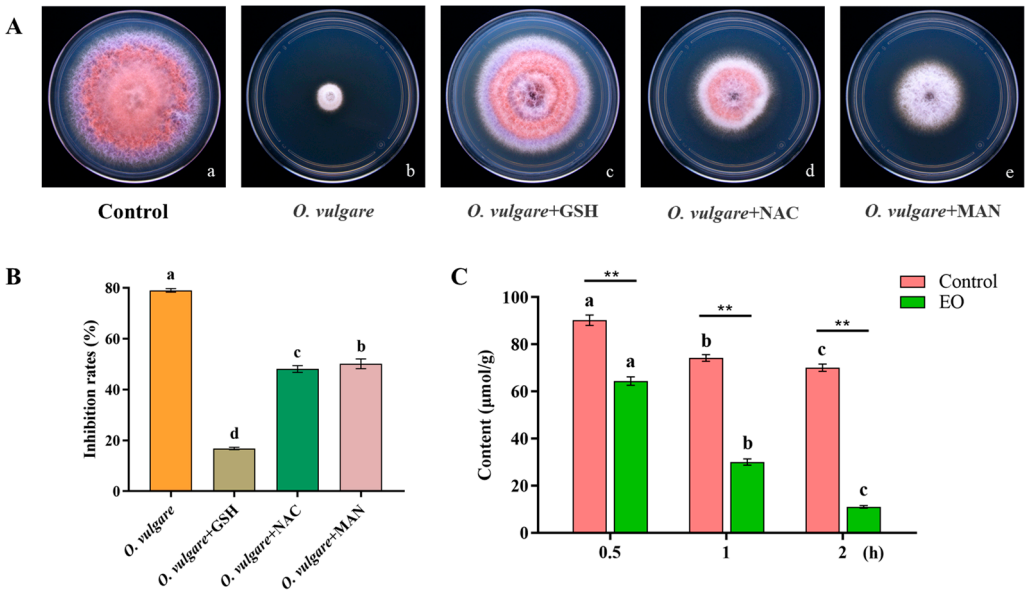

Figure 9. The effect of exogenous ROS scavengers on the antifungal effect of the *O. vulgare EO* (**A**). Inhibition rates for different treatments (**B**). Intracellular GSH detection (**C**). Different lowercase letters for the same treatment indicate significant differences across time ($p < 0.05$). ** represents highly significant differences ($p < 0.001$).

3.10. Intracellular GSH Content

The intracellular GSH content of mycelium was measured (Figure 9C). The results showed that when treated with 1/2 MIC *O. vulgare* EO, the intracellular GSH of the mycelium was significantly reduced compared to that of the control group. When treated with the *O. vulgare* EO for 2 h, the intracellular GSH content of the mycelium was only 15.64% of that of the control.

4. Discussion

Atractylodes chinensis is a perennial plant of the genus *Atractylodes* of the family Asteraceae [42]. In recent years, spurred by the increasing demand for *A. chinensis*, its artificial cultivation has expanded across many areas. However, its intensive cultivation has led to an increasing incidence of root rot, which seriously impacts its quality and yields [16]. Chemical pesticide usage is a typical strategy utilized for controlling root rot in *A. chinensis*. Chemical pesticides are effective for the control of pathogenic fungi; however, they also cause serious harm to ecosystems, non-target organisms, and human health [43]. The long-term use of chemical pesticides can also induce the resistance of pathogenic fungi, thus, reducing their effectiveness [44]. Plant EOs have garnered great interest from researchers due to their potent antifungal effects, easy decomposition, and environmental compatibility [45,46]. Labiatae is a large plant family with worldwide distribution, and its members are typically rich in EOs [47,48]. Labiatae EOs contain many aliphatic compounds, aromatic compounds, and terpene derivatives [47]. Previous studies have shown that the EOs of Labiatae plants can inhibit a wide range of bacteria and fungi [30–32]. In this study, we evaluated the inhibitory effects of *O. vulgare*, *T. mongolicus*, and *P. cablin* EOs on the root rot pathogen of *A. chinensis*. Since the *O. vulgare* EO exhibited the strongest inhibitory effect, the inhibitory mechanism of the *O. vulgare* EO against the dominant strain of root rot (*F. oxysporum*) was further investigated. This study provided a theoretical basis for the development of natural and efficient fungicides for the control of root rot in *A. chinensis*.

The results revealed that the three EOs had inhibitory effects against *F. oxysporum*, *F. solani*, and *F. redolens*. Among them, the *O. vulgare* and *T. mongolicus* EOs exhibited significantly high antifungal activities and completely inhibited the mycelial growth of the three pathogenic fungi. EOs possess broad-spectrum antimicrobial activities, which can inhibit the growth of mycelium via diffusion, and plants naturally use these chemicals

to resist pests and diseases [26]. The fungicidal effects of volatiles of the *O. vulgare* and *T. mongolicus* EOs were further investigated, and results showed that the volatiles of the *O. vulgare* EO had strong inhibitory effects on the three pathogenic fungi, with an inhibition rate of 100%. Parikh et al. also reported that the volatiles of *O. vulgare* EO had excellent fungicidal activities and completely inhibited the mycelial growth of pathogenic fungi [26]. However, the inhibitory effects of the volatiles of *T. mongolicus EO* on pathogenic fungi were lower than that of direct contact. This difference was also reported in a previous study [49], which was related to the concentration, polarity, and volatility of the EO [50].

Determining the composition of an EO is important for exploring its fungicidal mechanism and selective inhibitory properties [40]. This study revealed that the main components of the *O. vulgare* EO were carvacrol and thymol, and those of the *T. mongolicus* EO were γ-pinene, o-cymene, and thymol. Wu et al. analyzed the chemical composition of the *O. vulgare* EO using GC-MS and showed that the contents of carvacrol and thymol in it were 72.69% and 6.02%, respectively [40]. Shin and Kim studied the composition of *T. mongolicus* EO and showed that the three most abundant components were thymol, γ-pinene, and o-cymene [51]. Our findings were generally consistent with these previous studies, with some variations in content. Variations in the compositions and contents of the EOs of plants may be correlated with climatic conditions, altitude, geographic location, soil nutrients, extraction methods, plant age, harvesting time, and fertilizer and pesticide application [52].

The antifungal activities of EOs may be derived from their principal components [53]. In this study, the antifungal effects of carvacrol, thymol, γ-pinene, and o-cymene were determined with Oxford cups. Carvacrol and thymol exhibited strong inhibitory effects against the three pathogenic fungi under study, while γ-pinene and o-cymene showed weaker inhibitory effects. Notably, the inhibitory effects of carvacrol and thymol against *F. solani* and *F. redolens* were stronger than those of chemical pesticides. Carvacrol and thymol are constitutional isomers that belong to the phenolic group; certain phenolic compounds have been shown to have inhibitory activity against some species of fungi and bacteria [54–56]. Phenolic compounds contain hydroxyl groups that eliminate pathogenic fungi by binding to and deactivating their enzymatically active centers through the formation of hydrogen bonds [57]. A preceding study indicated that carvacrol and thymol inhibited *Xanthomonas campestris* pv. campestris (Xcc) to control the black rot of cabbage [58]. Zhao et al. found that carvacrol and thymol inhibited *Botrytis cinerea*'s mycelial growth [59]. Due to their excellent fungicidal activities and environmental compatibility, carvacrol and thymol can be utilized as novel pesticides for the control of plant diseases in the future. In this study, the *O. vulgare* EO exhibited very strong direct and indirect inhibitory effects against the three pathogenic fungi, which was likely due to its high level of phenolics [60]. Although less inhibitory than phenolics, o-cymene and γ-pinene may synergistically influence the antimicrobial effect of EOs.

The MIC value refers to the minimum EO concentration required to completely inhibit the growth of pathogenic fungi [61]. Our results indicated that the two EOs and two phenols showed strong inhibitory effects on the root rot pathogens of *A. chinensis*. Carvacrol and thymol had lower MIC (mg/mL) values compared to the EOs. Furthermore, an earlier study reported that monomers had more potent fungicidal activities than EOs [62]. Carbendazim is a synthetic fungicide that is widely used in agricultural production and has high fungicidal activities. The MICs of carbendazim against *F. oxysporum*, *F. solani*, and *F. redolens* ranged from 0.05–0.07 mg/mL. However, the half-life of carbendazim in nature is very long, and very low doses can produce serious "teratogenic, mutagenic and carcinogenic" effects in mammals [63,64]. Therefore, certain components of plant EOs can serve as new natural fungicides with great potential in the future.

Light microscopy showed that following the *O. vulgare* EO treatment, cavities appeared on the surface of *F. oxysporum* mycelium, which became locally deformed or was lysed. Scanning electron microscopy results showed that the *O. vulgare* EO altered the cell membrane structure of *F. oxysporum*. The results of transmission electron microscopy (TEM) revealed that the *O. vulgare* EO could damage the cell wall structures of *F. oxysporum*,

resulting in barrier disruption, which translated to induced abnormalities in the fungal plasma membrane structure and ultimately led to cell death. The fungal cell wall is a cellular structure unique to fungi, which consists of polysaccharides that wrap around the cytoplasm [65]. Fungal cell walls mainly play a protective role in maintaining the inherent cellular morphology and integrity, in addition to sustaining normal cellular metabolism, ion exchange, and osmotic pressure [66,67]. The absence of cell wall structures leads to the rupture of the plasma membrane and cell lysis; thus, the integrity of the cell wall is essential for the survival of the fungus [68]. A previous study showed that the *L. verbena* EO disrupted the cell wall of *Pseudosciaena D4*, resulting in increased cell membrane permeability and leakage of intracellular contents, ultimately leading to cell death [69]. A further study disclosed that the *M. haplocalyx* EO penetrated the cell wall and dissolved the internal organelles to inhibit the growth of *F. oxysporum* [37]. This study suggests that the cell wall may be a viable target for the *O. vulgare* EO in *F. oxysporum*.

Proteins and nucleic acids are critical biomolecules within cytoplasms and nuclei of cells [70,71]. The leakage of DNA and proteins reflects the integrity of the cell membrane [72]. This study showed a significant leakage of DNA and proteins within the mycelium of *F. oxysporum* after treatment with the *O. vulgare* EO, which indicated that the plasma membrane was severely damaged. This was consistent with the morphological and ultrastructural results. The hydrophobic characteristics of EOs enable them to enter the cell membranes of pathogenic fungi, alter their structures, and increase their permeability [73].

Reactive oxygen species (ROS) is a general term for a class of oxidatively active molecules that are produced by cellular energy metabolism under aerobic conditions, which play an important role in the physiological and pathological processes of organisms [74]. However, excess ROS induce oxidative stress, which alters the structures of biomolecules (DNA, proteins, and lipids), and ultimately leads to cell death [75]. For this study, DCFH-DA fluorescent probes were used to determine the intracellular ROS content of mycelium [76]. The results revealed that the mycelium of *F. oxysporum* emitted significant fluorescence following the *O. vulgare* EO treatment, suggesting that it could exert inhibitory effects by inducing the generation of ROS. Wu et al. reported that the *O. vulgare* EO inhibited the growth of *Rhizoctonia solani* by inducing the production of ROS [40]. Reactive oxygen scavenger is a collective term for a class of substances with reactive oxygen scavenging properties (e.g., GSH, NAC, MAN, etc.). In this study, all three ROS scavengers (GSH, NAC, and MAN) alleviated the inhibitory effects of the *O. vulgare* EO on *F. oxysporum*, with GSH having the strongest impact. Thus, the inhibitory activities of the *O. vulgare* EO against *F. oxysporum* were positively correlated with the accumulation of ROS in mycelial cells.

Glutathione (GSH) is a naturally reactive peptide composed of glutamic acid, cysteine, and glycine and is widely found in living organisms [77,78]. GSH possesses antioxidant and integrative detoxification activities that help cells to maintain normal immune system functions [79,80]. In this study, the content of GSH in the *O. vulgare* EO-treated mycelium was significantly reduced over a short timeline (0.5–2 h) compared to the control. This result indicated that the *O. vulgare* EO depleted intracellular GSH, resulting in a large accumulation of ROS in the mycelium.

Plant EOs have potent inhibitory activities against pathogenic fungi and can be used as natural and safe pesticides. However, in recent years, some studies have shown that high concentrations of EOs or extracts can inhibit seed germination [81]. Thus, appropriate concentrations of EOs should be selected to control plant diseases to avoid their negative effects on seeds. In addition, this study was conducted under sealed conditions in the laboratory, and the effect of volatility on the results was not taken into account. EOs and their main ingredients are highly volatile, so their stability is low. Volatility has become a major limitation in the application of EOs, which may cause the actual application effectiveness to be lower than that observed in the experimental results. Currently, researchers have developed methods to improve the stability and persistence of EOs, such as microcap-

sules, microemulsions, and nanoemulsions [82,83]. In the future, avenues to combine these methods to increase the effectiveness of EOs should be further investigated.

5. Conclusions

In this study, the inhibitory effect of three EOs of Labiatae plants on the root rot pathogens of *A. chinensis* was investigated. The *O. vulgare* EO and its main components exhibited excellent inhibitory activities against *F. oxysporum*, *F. solani*, and *F. redolens*. The results of in vivo experiments showed that the *O. vulgare* EO was effective in reducing the damage caused by *F. oxysporum* in *A. chinensis*. The inhibitory mechanism of the *O. vulgare* EO was summarized as follows: 1. The *O. vulgare* EO could cause severe damage to cell walls and plasma membranes, increasing the permeability of cell membranes and leading to a massive leakage of intracellular contents. 2. The *O. vulgare* EO inhibited pathogenic fungi by inducing ROS production and reducing the amount of intracellular GSH, leading to a large accumulation of ROS. Collectively, the *O. vulgare* EO and its main components can be developed as environmentally friendly fungicides to replace synthetic pesticides for the control of root rot of *A. chinensis*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9101136/s1. Table S1: Chemical composition of the EO from *O. vulgare*; Table S2: Chemical composition of the EO from *T. mongolicus*; Figure S1. GC-MS chromatogram of the *Origanum vulgare* essential oil.; Figure S2. GC-MS chromatogram of the *Thymus mongolicus* essential oil.

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Data Availability Statement: Date is available upon request from Z.W.

Conflicts of Interest: The authors declare no conflict of interest.

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