Effects of *Origanum vulgare* essential oil and its two main components, carvacrol and thymol, on the plant pathogen *Botrytis cinerea*

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Abstract

Background: *Botrytis cinerea* is a necrotrophic plant pathogenic fungus that can cause gray mold in many plants. This pathogen has exhibited resistance to many fungicides. Thus, it has become necessary to look for new safe yet effective compounds against *B. cinerea*.

Methods: Essential oils from 17 plant species were assayed against *B. cinerea*, of which *Origanum vulgare* essential oil ~~with~~ showed strong inhibitory activity ~~against *B. cinerea* was screened from 17 kinds of plant essential oil~~, and ~~the~~ accordingly its main components were detected by GC/MS. Further study was conducted on ~~T~~the effects of *O. vulgare* essential oil, carvacrol and thymol *in vitro* ~~to~~ on mycelium growth and spore germination, mycelium morphology, leakages of cytoplasmic contents, mitochondrial injury and accumulation of reactive oxygen species (ROS) of *B. cinerea* ~~were studied *in vitro*~~. The control effects of oregano essential oil, carvacrol and thymol on tomato grey mould were evaluated *in vivo*.

Results: Of all the 17 plant essential oils tested, *Cinnamomum* ~~*C.*~~ *cassia*, *Litsea* ~~*L. cubeba*~~ and *O. vulgare* essential oils had the best inhibitory effect on *B. cinerea*, with 0.5 mg/mL completely inhibiting the mycelium growth of *B. cinerea*. Twenty-one different compounds of the essential oil of *O. vulgare* were identified by gas

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chromatography–mass spectrometry, and the main chemical components were carvacrol (89.98%), β-caryophyllene (3.34%), thymol (2.39%), α-humulene (1.38%) and 1-methyl-2-propan-2-ylbenzene isopropyl benzene (1.36%). *In vitro* experiment showed EC_{50} values of *O. vulgare* essential oil, carvacrol and thymol were 140.04, 9.09 and 21.32 μg/mL, respectively. Carvacrol and thymol completely inhibited the spore germination of *B. cinerea* at the concentration of 300 μg/mL while the inhibition rate of *O. vulgare* essential oil was 80.03%. EC_{50} of carvacrol and thymol can significantly ($P<0.05$) reduce the fresh and dry weight of mycelia. The collapse and damage on *B. cinerea* mycelia treated with 40 μg/mL of carvacrol and thymol was examined by scanning electron microscope (SEM). Through extracellular conductivity test and fluorescence microscope observation, it was found that carvacrol and thymol led to increase the permeability of target cells, the destruction of mitochondrial membrane and ROS accumulation. *In vivo* conditions, 1000 μg/mL carvacrol had the best protective and therapeutic effects on tomato gray mold (77.98% and 28.04%, respectively), and the protective effect was significantly higher than that of 400μg/mL pyrimethanil (43.15%).While the therapeutic and protective effects of 1000 μg/mL *O. vulgare* essential and thymol were comparable to agent control.

Conclusions: The essential oil of *O. vulgare*, which had strong inhibitory activity on *B. cinerea*, was screened from 17 species of plant essential oils in order to control the pathogen. Therefore, *O. vulgare* essential oil as well as its main components carvacrol and thymol has great application potential as natural fungicides or lead compounds for commercial fungicides in preventing and controlling plant diseases caused by *B. cinerea*.

Key words *Origanum vulgare*, carvacrol, thymol, *Botrytis cinerea*, antifungal activity, botanical fungicide.

Introduction

Botrytis cinerea, a necrotrophic plant pathogenic fungus with broad hosts, can cause gray mold of many plants. The infected plants include many important agricultural, economic and horticultural crops, with a total of more than 1400 species such as tomato, grape, strawberry, cucumber, orchid and other fruits, vegetables and flowers (Elad et al. 2016). It not only infects field crops, but also causes huge losses to the crops after harvesting ~~the post-harvest plants~~ (Gianfranco Romanazzi 2014). At present, the main fungicides for the prevention and treatment of gray mold are benzimidazoles, dicarboximides, carbamates and antibiotics, ~~and~~ where resistance of *B. cinerea* to these fungicides has been widely reported (Leroux et al. 2002; Bardas et al. 2010; Liu et al. 2019). Most~~ly~~ field investigations ha~~ve~~s demonstrated that *B. cinerea* has developed resistance to carbendazim, procymidone and diethofencarb ~~have developed resistance to *B. cinerea* of~~ in tomato and blueberry, and the point mutations have been identified (Adnan et al. 2018; Sautua et al. 2019). Therefore, the control of gray mold is still facing great challenges. It is of great significance to explore new antifungal components for the integrated management of gray mold.

An important ~~E~~essential oil ~~of *Origanum vulgare* is an aromatic volatile substance~~ extracted from *O. vulgare* Linn. (Lamiacea)~~,~~ is known to have strong antifungal and antioxidant activities, and has been widely used in the food and medicine (Rodriguez-Garcia et al. 2015; Lu et al. 2018). A ~~P~~previous study found that a volatile vapour of ~~o~~*Origanum* oil at 0.2 μg/mL completely inhibited the growth of *B. cinerea in vitro* (Soylu et al. 2010). Essential oil of *O. compactum* Benth. ~~essential oil~~ could completely inhibit the mycelial growth of *Fusarium* species and *Bipolaris oryzae* at the concentration of 300 μg/mL *in vitro* (Santamarina et al. 2015). Essential oil of *O. heracleoticum* had inhibitory effects on mycelial growth of *B. cinerea*, *Penicillium expansum*, *Aspergillus niger* and *Monilinia fructicola* at 1000 ppm *in vitro* (Della Pepa et al. 2019). So far, there is no evidence that oregano essential oil and its main components are harmful to humans and animals and have high phytotoxicity (Llana-Ruiz-Cabello et al. 2017; Elshafie et al. 2017),

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To date, researches on *Origanum* essential oil and its main components against plant pathogens mainly focus on bioactivities while the mechanism of antifungal action is still unclear and the actual application is also necessary to be evaluated. This study was conducted to determine: (i) the antifungal activity of 17 plant essential oils against *B. cinerea*, (ii) the main components of *O. vulgare* essential oil by GC–MS , (iii) the antifungal activities and mechanism of *O. vulgare* essential oil, carvacrol and thymol, on *B. cinerea in vitro*, (iiii) *in vivo* control effects of *O. vulgare* essential oil on *B. cinerea*, providing a foundation for the development and its utilization ~~of *O. vulgare*~~ as a ~~botanical~~ plant fungicide.

Materials and methods

Inhibitory activity of different plant essential oils on mycelium growth of *B. cinerea*

Seventeen plant essential oils were involved in the current study, namely *Origanum vulgare*, *Cinnamomum cassia*, *Perilla frutescens*, *Saussurea costus*, *Mentha spicata*, *Litsea cubeba* var. *formosana*, *Asarum sieboldii*, *Illicium verum*, *Foeniculum vulgare*, *Angelica dahurica*, *Curcuma zedoaria*, *Mentha haplocalyx*, *Artemisia argyi*, *Eucalyptus globulus*, *Syzygium aromaticum*, *Acorus tatarinowii* and~~,~~ turpentine. All the oils were purchased from Cedar pharmaceutical Co., LTD, Jiangxi, China. Isolates of *B. cinerea* ~~was isolated~~ were prepared from tomato fruits (Luoyang, China) and deposited at the Key Laboratory of Creation & Application of Novel Pesticides, Henan. The preliminary antifungal activities of these essential oils against *B. cinerea* were determined according to the method described by ~~(~~Farzaneh et al. (2015). Emulsions of the essential oils were prepared by sterile water with 0.5% (v/v) acetone and Tween 80, blended with PDA (200 g extract of boiled potatoes, 20 g dextrose and 20 g agar powder in 1000 mL distilled water) at 40-45℃ to obtain final concentrations of 0 (control), 0.5 and 2 mg/mL. A 5 mm mycelial disc from young cultures of target fungi (3d) was placed in treated sterile plates (D=7 cm) after ~~this~~ medium solidification. The petri dishes were sealed with sealing film and cultured upside-down in dark at 26.5℃.

The mycelial growth was measured by a nonius using decussation way at 2 ~~d~~, 4 ~~d~~ and 6 d, respectively. Three replicates per treatment were ~~carried out~~used in each experiment and the experiments were performed three times. Growth inhibition of each essential oil was computed by the formula (1):

$$\text{Inhibition}(\%) = (D_c - D_t)/(D_c - 0.5) \times 100 \quad (1)$$

Where D_c is the colony diameter of the control group; and D_t is the colony diameter of the treatment groups with essential oil.

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GC-MS analysis

The chemical compositions of *O. vulgare* essential oil was analyzed by GC-MS (Agilent-7890B, Agilent technology co., LTD, USA) according to the method reported by ~~(p~~Pyrimethanils et al. (2018). Chemicals in *O. vulgare* essential oil were separated with HP-5 MS capillary column (50 m×0.25 mm×0.25 μm), high-purity Helium (99.999%) as the carrier gas. The helium carrier gas rate ~~of~~ was 1.0 mL/min at a split ratio of 20.4:1, and the injection volume was 1.0 μL. The initial temperature was set at 60℃ for 25 mins, then gradually increased to 150℃ at a rate of 1.2℃·min^{-1}. Electron ionization was used as the ion source, and the ionization energy was set at 70 eV. The quadrupole temperature was 150℃, and the ion source temperature was 230℃. The sector mass analyzer was set to a range from 33 to 900 amu. Diluted samples (1/100, in acetone) at 1 μL each were injected and mass spectra were compared with the standard mass spectra from the NIST 2.2 database provided by the software of GC-MS system.

In vitro antifungal activities of *O. vulgare* essential oil, carvacrol and thymol against *B. cinerea*

O. vulgare essential oil, carvacrol (purity 99%) and thymol (purity 98.5%, Sigma Aldrich, St. Louis, MO, USA) were prepared into 1000 μg/mL mother liquor with sterile water containing 0.1% (V/V) acetone and Tween 80 (this concentration of mother liquor shall be used for dilution below unless otherwise specified). The fungus were grown on a PDA plate for 5 days, and then harvested using a 5 mm sterilized

puncher along the edges of the colonies, and the fungal blocks were placed in the center of PDA plates containing 2.5, 5, 10, 15, 40 μg/mL carvacrol , 10, 20, 40, 60, 80 μg/mL thymol and 50, 100, 150, 250, 400μg/mL *O. vulgare* essential oil. Cultures with 0.005% (0.008%, 0.04%) acetone and Tween 80 were used as the control, respectively. Incubation upside-down at 26.5℃. ~~Observe the~~The fungistatic effect was observed, and the colony diameter was measured and recorded ~~the colony diameter~~ when the diameter of the control was ~~in~~ exceeded~~ss~~ ~~of~~ 7.0 cm. Then, calculat~~ed~~ions were made for ~~the~~ *B. cinerea* mycelium inhibition ability of *O. vulgare* essential oil, carvacrol and thymol ~~of~~ ~~*B. cinerea*~~ and regression equation and EC_{50} values by probability value analysis. Three replicates per treatment were carried out in each experiment and the experiments were performed three times.

Antifungal activity of *O. vulgare* essential oil, carvacrol and thymol on spore germination of *B. cinerea*

~~The test~~ *B. cinerea* isolates ~~was~~ were cultured on PDA plates at 20℃ for 14 days. Then, the plates were injected with 5 ml sterile water and gently scraped with a sterile transfer pipette to obtain spore suspension (Jing et al. 2018). The spore concentration was adjusted to ~~(~~1×10^6 spores^{-1}~~)~~. *O. vulgare* essential oil, carvacrol and thymol were blended with acetone and Tween 80 and sterile water, respectively, and the concentration was adjusted to 100, 200, 300, 400, 500, 600 μg/mL at the same time~~,~~ A ~~an~~ 0.5 mL aliquot ~~(0.5 mL)~~ of conidium suspension of *B. cinerea* ~~and~~ plus 0.5 mL liquid were added to a tube. Then, 60 μL was transferred onto a double concave glass plate with pipette gun and placed in a wet sterile culture dish. Cultures with 0.03% acetone and Tween 80 ~~was~~ were used as the control. The ~~above~~ cultures were incubated at 28℃. After incubating for 8 h, spores of *B. cinerea* were observed with a microscope (Nikon Eclipse Ti-S, Tokyo, Japan) at a 200× magnification. The spore germination percentage was calculated by counting germinated spores among 200 spores. Three replicates per treatment were conducted in each experiment and the experiments were performed three times.

Effects of carvacrol and thymol on fresh and dry mycelial weight of *B. cinerea*

The fresh and dry mycelial weight of the tested pathogen treated with carvacrol and thymol were measured (Chen et al. 2014). According to the EC_{50} and EC_{90} of carvacrol and thymol, two components were dissolved and diluted in sterile water with 0.1% acetone and Tween 80, and transferred into Erlenmeyer flasks with 25 mL of PDB medium (200 g extract of boiled potatoes, 20 g dextrose in 1000 mL distilled water) to obtain carvacrol concentrations of 0 (control), EC_{50} (9.38 μg/mL) and EC_{90} (43.49 μg/mL) and thymol concentrations of 0 (control), EC_{50} (21.32 μg/mL) and EC_{90} (65.13 μg/mL), respectively. The pathogen was harvested along the edge of the colony (3d) with a 5mm sterilized punch, then inoculated into each flask and the flasks were incubated at 120 r/min shaking and 26.5℃. The mycelia groups were harvested on 3 days and strained off excess culture. ~~Remove the~~The agar blocks were removed with tweezers~~,~~and ~~–~~each mycelium groups were washed ~~each mycelium groups~~ with sterile water at least three times, and then filtered for 30 min to remove excess water on the surface. The fresh mycelia were weighed and ~~. T~~the dry mycelia ~~were weighed~~as well after drying at 60℃ for 12 h. Each treatment consisted of three replicates and the experiments were performed three times.

Effect of carvacrol and thymol on the relative leakage of *B. cinerea*

The permeability of the membrane was expressed in terms of relative extracellular conductivity and tested according to the method of ~~(~~Zhou et al. (2018) by using a Seven ~~E~~excellence multiparameter tester (METTLER TOLEDO Instrument Co., Ltd., Shanghai, China) with some modifications. Carvacrol and thymol mother liquor were prepared with sterile water and 10% dimethyl sulfoxide, and the final concentrations were 10, 200, 500 μg/mL and 70, 200, 500 μg/mL, respectively. Vinclozolin (500 μg/mL) was prepared with sterile water and 10% dimethyl sulfoxide as solvent. Fungal samples were collected 3 days after inoculation on PDA medium. Then the collected mycelium

was inoculated in PDB and shaken at 120 rpm and 26.5℃ for 108 hours. Remove the agar blocks with tweezers, wash each mycelium groups with sterile water at least three times, and filter for 30 min to remove excess water on the surface. The mycelia (0.1 g) was separately put into the medicine solution with the above series concentrations into 10 mL glass tubes of~~, 10 mL, and~~ 10% dimethyl sulfoxide sterile distilled water was the blank control, vinclozolin was the positive control. Extracellular conductivity was measured at 0, 10, 40, 140, 220, 360 and 400 min. Finally, the electrical conductivity was measured again after dead treatment. Three replicates per treatment were carried out in each experiment and the experiments were performed three times. The relative leakage was determined according to the formula (2):

$$\text{Relative leakage}(\%) = (C_t - C_0)/C_d \times 100 \quad (2)$$

Where C_t is the conductivity of current time samples, C_0 is the conductivity of initial time (0 min) samples, and C_d is the conductivity of dead treatment conductivity samples.

Scanning electron microscopy and fluorescent microscopy

Carvacrol and thymol were dissolved and diluted in sterile water with acetone and Tween 80. 1 mL of mixture and 9 mL PDA medium were mixed and poured into a 9 mm diameter Petri dishes. The final concentrations of 0 (CK), 25 and 40 μg/mL of Carvacrol and thymol were obtained, respectively. Medium containing 0.004% acetone and tween-80 was used as solvent control. The blocks of *B. cinerea* were inoculated on the medicated medium and cultured upside down at 26.5℃ for 5 days. The effects of carvacrol and thymol on hyphal morphology were analysed using the modified method of ~~(~~Li et al. (2017). For SEM observation, PDA blocks about 5×7 mm were cut from the edge of mycelium~~, m~~Mycelium exposed to different treatments was fixed with 25% glutaraldehyde for 48h at low temperature. Samples were washed for 3 h (10 min/ time) in 0.1 molL^{-1} phosphate buffer (pH 7.2). After being fixed with 1% osmium for 2h, the samples were washed with distilled water for 3 times (3 min/ time) and dehydrated in a graded series of ethanol concentrations (30, 50, 70, 80, 90% and twice at 100%) for 15 min at each stage. Samples were dried by CO_2 critical point drier (CPD 030, Leica,

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Wetzlar, Germany), then gold coated using a sputter coating machine (MC1000, Hitachi, Tokyo, Japan). All samples were viewed in a SEM (Model SU8010-3400N, Hitachi) operating at 10 kV at 2.5k× magnification.

The fluorescence intensity of H2DCFDA dye under specific excitation wave is related to the content of ROS, and the accumulation of ROS can be judged by the fluorescence intensity (Li et al. 2017). SYTOX Green is a fluorescent dye commonly used to study membrane integrity. It can pass through damaged membrane cells but cannot penetrate the complete cell membrane and emit Green fluorescence. Rhodamine-123 is a cationic dye that can penetrate the cell membrane and is an indicator of mitochondrial transmembrane potential. Mitochondrial matrix fluorescence intensity weakened or disappeared in normal cells, and strong yellow-green fluorescence was released when mitochondrial membrane was destroyed (Tian et al. 2012).

Mycelium was observed by Nikon Eclipse Ti-S inverted fluorescence microscope (Eclipse Ti-S, Tokyo, Japan) and slightly modified (Jing et al. 2018). The fungal blocks were inoculated into 50 mL erlenmeyer flasks with PDB culture medium and the flasks were incubated at 120 r/min and 26.5℃ for 2 days. Then the samples were incubated in PDB with the carvacrol and thymol (100 and 200 μg/mL) for 12 h, 0.1% acetone and Tween 80, ~~with~~ where sterile water was used as the blank control. The collected mycelia were stained with 1 μg/mL H2DCFDA, SYTOX Green or Rhodamine 123 for 30 min at 4℃ in darkness. After the dye~~e~~ing, the mycelia were washed with phosphate buffered saline to remove the residual dye and then observed by fluorescence microscope. The views were randomly selected from each group, and all experiments were repeated six times and the experiments were performed three times.

Protective and therapeutic effects of *O. vulgare* essential oil，carvacrol and thymol on tomato gray mold caused by *B. cinerea*

Protective effect experiment: tomato fruits with same size and weight were selected and put ~~them~~ in sterile plastic boxes (nine tomatoes in each box) with gauze at the bottom ~~. There were nine tomatoes in each box and pour~~ wetted with sterile water ~~into~~

~~the bottom of the box~~ to keep moisture. Tomatoes were ~~S~~sprayed with *O. vulgare* essential oil, carvacrol ~~and~~ or thymol at 500 and 1000 μg/mL, respectively. P~~p~~yrimethanil at 400 μg/mL was used as the standard control, and 0.1% acetone ~~and~~ plus Tween 80 with sterile water was used as the solvent control. Sterile water was used as the blank control. Each box ha~~s~~d one treatment, 9 repetitions for each treatment, and 3 fungal blocks for each repetition. After the liquid was dried, fungal blocks were placed on each tomato by acupuncture method and placed in the artificial climate box with humidity over 85% and temperature at 26.5°C. After 48h, the diameter of the spot was measured and the control effect was calculated. Therapeutic effect experiment: Similar to protection, the above mentioned tomato fruits were inoculated with fungal blocks and cultured under the above conditions. After 24 hours, the above mentioned concentration solution was taken out and sprayed on the tomato fruits for further culture. After 48 hours, the size of diseased spots was measured. The p~~P~~rotective and therapeutic effects ~~is~~ was calculated by the following formula (3):

$$\text{Control efficacy } (\%) = (D_c - D_t)/D_c \times 100 \qquad (3)$$

Where D_c is the diameter of disease spots in the control group; and D_t is the spot diameter in the treatment group.

Statistical analysis

The statistical software SPSS (V 20.0; Chicago, IL, USA) was used for all data analyses. Data were analysed by Duncan's multiple range test at the level $P < 0.05$.

Results

Inhibitory activities of 17 plant essential oils on mycelium growth of *B. cinerea*

The antifungal activities of 17 plant essential oils against *B. cinerea* ~~were~~ are shown in ~~t~~Table 1. *C. cassia*, *L. cubeba* and *O. vulgare* essential oils completely inhibited the mycelial growth at the concentration of 0.5 mg/mL. In addition, the inhibition rate of *M. spicata* and *S. aromaticum* oils reached 91.70% and 87.55%, respectively. At the concentration of 2 mg/mL, the inhibition rate of *M. haplocalyx*, star anise and *A.*

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sieboldii oils was 99.56% and 82.53%, respectively.

GC-MS analysis of *O. vulgare* essential oil

In total, 21 different chemical components were identified from the *O. vulgare* essential oil (Table 1). Carvacrol (89.98%) was found to be the major component, followed by β-caryophyllene (3.34%), thymol (2.39%), α-humulene (1.38%), and 1-methyl-2-propan-2-ylbenzene isopropyl benzene (1.36%). In addition, the isomers carvacrol and thymol had adjacent peaks ~~and were isomers of each other~~.

Antifungal activities of the *O. vulgare* essential oil, carvacrol and thymol against mycelial growth of *B. cinerea in vitro*

In vitro results (Table 3) showed that *O. vulgare* essential oil, carvacrol and thymol could significantly ~~influence~~ inhibit the mycelial growth of *B. cinerea*, ~~and~~ where the ~~inhibition~~ effect increased with increasing~~ed~~ the concentration. The EC_{50} of carvacrol and thymol were 9.09 and 21.32 μg/mL, respectively, lower than that of *O. vulgare* essential oil (140.04 μg/mL). Carvacrol and thymol as main antifungal components have been demonstrated, and were chosen for further study.

Antifungal activities of *O. vulgare* essential oil, carvacrol and thymol on spore germination of *B. cinerea*

Carvacrol and thymol at all tested concentrations (50-300 μg/mL) resulted in significantly lower germination of *B. cinerea* spores compared with the untreated ones (Table 4). Thymol at 250 μg/mL and carvol at 300 μg/mL can completely inhibit spore germination. However, the low concentration of *O. vulgare* essential oil had no significant effect on the spore germination rate of *B. cinerea*. The spore germination was 80.03% at the concentration of 300 μg/mL.

Effects of carvacrol and thymol on the mycelial morphology of *B. cinerea*

The mycelial morphology of *B. cinerea* treated with carvacrol and thymol at the concentration of 40 μg/mL was observed by SEM (Fig. 1). There were regular, uniform and complete mycelia with smooth surfaces in the control group, while the mycelia

treated with carvacrol and thymol showed great morphological changes, including irregular growth of mycelium, formation of verrucous surface, shrinkage, collapse and hollowing of hyphae.

Effects of carvacrol and thymol on ~~wet~~ fresh and dry mycelium weight of *B. cinereal*

The mycelial growth of *B. cinerea* in PDB medium containing carvol and thymol for 3 days was significantly inhibited (Fig. 2). The ~~wet~~ fresh ~~weight~~ and dry weights of the mycelia treated with carvacrol ~~EC_{50} (9.09 μg/mL) and EC_{90} (40.62 μg/mL)~~ were 21.93 and 5.83 mg, 8.30 and 4.13 mg, respectively and had EC_{50} of 9.09 μg/mL and EC_{90} of 40.62 μg/mL. The inhibition rates of ~~wet~~ fresh ~~weight~~ and dry weights were 86.88%, 96.51% and 78.53%, 89.31%, respectively. After being treated with thymol ~~EC_{50} (21.32 μg/mL), EC_{90} (65.13 μg/mL)~~, the mycelial inhibitions of ~~wet~~ fresh ~~weight~~ and dry weights were 78.06% and 98.23%, 69.94% and 95.97%, respectively where the EC_{50} reached 21.32 μg/mL and EC_{90} reached 65.13 μg/mL.

Effects of carvacrol and thymol on cell leakage of *B. cinerea*

A linear relative conductivity response with the increasing concentration of three reagents were found in all treatments (Fig. 3). Compared with the blank control, the two treatments with carvacrol and thymol maintained a higher level of relative conductivity. When carvacrol and thymol were at the lowest concentrations of 10 μg/mL and 70 μg/mL, at the treatment time points of 10, 40, 140, 220, 360 and 400 min, the relative conductivity were 2.98%, 10.26%, 32.48%, 37.97%, 47.68%, 51.51% and 10.87%, 15.41%, 23.58%, 28.81%, 37.87%, 39.83%, respectively. The relative conductivity was the same as of the positive control (vinclozolin) group at the same time. There was a more cell leakage when carvacrol and thymol increased to 200 μg/mL and 500 μg/mL, compared with that of the vinclozolin.

Effects of carvacrol and thymol on reactive oxygen species accumulation, fungal membrane integrity and mitochondrial injury

The results of ROS accumulation, fungal membrane integrity and mitochondria

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damage experiments ~~were~~ are presented in ~~following pictures~~ Figs 4-6. Compared with the untreated hyphae, the treated hyphae gave off strong green fluorescence, indicating there was a large amount of ROS accumulation in the mycelium (Fig. 4). SYTOX Green penetrated the mycelium treated with carvacrol and thymol, indicating that the integrity of the mycelial membrane was damaged (Fig. 5). Compared with normal mitochondria, ~~R~~rhodamine 123, which entered the mitochondria from the outside, was re-released after the membrane was damaged, emitting yellow-green fluorescence（Fig. 6）.

Application of fungicides and disease assessment *in vivo*

The results of *in vivo* conditions showed that *O. vulgare* essential oil, carvacrol and thymol have different protective and therapeutic effects on tomato gray mold caused by *B. cinerea* (Table 5). At the concentration of 500 μg/mL, the protective and therapeutic effects of *O. vulgare* essential oil were significantly lower than pyrimethanil at 400 μg/mL. Although ~~T~~the protective effect of carvacrol was comparable to pyrimethanil, ~~but the~~ its therapeutic effect was lower ~~than that of pyrimethanil~~. The therapeutic and protective effects of thymol were lower than that of the agent control treatment group. At the concentration of 1000 μg/mL, *O. vulgare* essential oil and thymol had the same protective and therapeutic effects as 400 μg/mL pyrimethanil. The protective effect of carvacrol was significantly higher than pyrimethanil while the therapeutic effect was similar to that of the agent control. Among ~~of~~ all the treatment groups, the protective effect of carvacrol (77.98%) was the best.

Discussion

It has become a trend in recent years to find biosafe fungicides to reduce environmental pollution and to cope with the resistance of traditional chemical agents. Many essential oils and their components exhibit antifungal properties, but their high production cost and low concentration of active ingredients limit their direct use in the control of plant and animal fungal diseases. Nevertheless, the use of plant essential oils to control agricultural fungal diseases has been a hot research topic with great application potential in controlling important fungal diseases of crops. It has been found

that α-pinene and β-caryophyllene from *Cupressus sempervirens* essential oil had high antimicrobial activity on *B. cinerea*, and the MIC was 125 μg/mL when used in combination with it (Rguez et al. 2018). A ~~P~~previous study also found that 500 μL/L peppermint oil and its volatile vapor at 25℃ could significantly inhibit the germination of conidia and the occurrence of disease *in vivo* (Xueuan et al. 2017). *Eucalyptus staigeriana* oil had the highest inhibitory activity against *B. cinerea* spores and hyphae at 0.5μL/mL (Pedrotti et al. 2019). In this study, through testing the biological activity of 17 plant essential oils against *B. cinerea*, it was found that 0.5 mg/mL *Origanum vulgare* (Soylu et al. 2010), *Litsea cubeba* and *Cinnamomum cassia* (Wang et al. 2014) and 2 mg/mL *Mentha haplocalyx* oils (Xueuan et al. 2017) could completely inhibit the growth of mycelia. Inhibition of 0.5 mg/mL *Mentha spicata*, *Syzygium aromaticum* oils (Gago et al. 2019) and 2 mg/mL *Illicium verum* (Lee et al. 2007) and *Foeniculum vulgare* oils (Lopez-Reyes et al. 2013) were all higher than 82.53%. The inhibitory effects of *Litsea cubeba* oil, *Mentha spicata* oil and *Asarum sieboldii* oil on *B. cinerea* have not been reported.

In vitro activity test showed that EC_{50} of *O. vulgare* essential oil on the mycelia growth of *B. cinerea* was 140.04 μg/mL, and the spore germination was 80.03% treated with 300 μg/mL *O. vulgare* essential oil. But the EC_{50} of carvacrol and thymol on the mycelial growth were 9.09 μg/mL and 21.32 μg/mL, respectively. In addition, the spore germination was completely inhibited by thymol at 250 μg/mL and carvacrol at 300 μg/mL. In general, the activities of carvacrol and thymol were significantly higher than that of *O. vulgare* essential oil. After growing for 3 days in PDB culture solution with EC_{90} (40.62 μg/mL) carvacrol and EC_{90} (65.13 μg/mL) thymol, the mycelial biomass was significantly inhibited above 89.31%. *In vivo* test results showed that 1000 μg/mL carvacrol had the best protective and therapeutic effects on tomato gray mold (77.98% and 28.04%, respectively), and the protective effect was significantly higher than that of the agent control. 1000 μg/mL *O. vulgare* essential oil has a better protective and therapeutic effect. The therapeutic and protective effects of 1000 μg/mL thymol were comparable to pyrimethanil. It can be seen that carvacrol and thymol are the main

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fungicidal components of *O. vulgare* essential oil.

Protoplasmic membrane system, as a protective screen of cells, is necessary for the survival of fungus (Zhou et al. 2018). In this experiment, the destruction of cell membrane was confirmed by SEM and SYTOX Green fluorescence staining. Consistent with previous expectations and recent reports (Zhang et al. 2019), the results of SEM showed that the normal form of *B. cinerea* mycelia ~~were~~ was damaged. After carvacrol and thymol treatment, the hypha appeared ~~shrinkage~~shrunk, collapsed and ~~disorganization~~ disorganized compared with that in the control. In subsequent experiments, the fluorescent microscopy observation also confirmed that carvacrol and thymol destroyed mycelial membranes of *B. cinerea* in this study.

Previous studies have shown that essential oil could lead to the rupture and damage of fungal cell membrane (Yu et al. 2015) and abnormal transport function, nucleic acid and intracellular protein leakage, and abnormal metabolism, which is the possible mechanism of antifungal action of essential oils (Chen et al. 2014; Jing et al. 2018). ~~.~~The relative leakage test showed that 200 μg /mL carvacrol and thymol could significantly increase the relative permeability of cells and 10 mg/L carvacrol and 70 mg/L thymol at the treatment time points of (0~400 min) ~~was~~ were equivalent to that of vinclozolin. It was speculated that carvacrol and thymol as well as vinclozolin with known mechanism of action can destroy the function of cell membrane (Choi et al. 1996; ~~M. J. C. S.~~ Cabral 2011) and cause leakage of ~~entocyte~~enterocyte.

ROS (highly potent oxidants containing oxygen) encompasses oxygen free radicals and nonradical oxidants, can be interconverted from one to another by enzymatic and nonenzymatic mechanisms. A large amount of ROS can lead to oxidative damage of DNA base in cells and affect various redox reactions intra- and extracellular processes (Zorov et al. 2014), and play an important role in cell apoptosis (Wu et al. 2018). By using fluorescent dye DCFH-DA and flow cytometer, there was increased ROS intensity in *Aspergillus flavus* after the treatment of *Anethum graveolens* L. essential oil at different doses (Tian et al. 2012). Tea tree oil could lead to decreased activity of enzymes related to the tricarboxylic acid cycle and mitochondrial dysfunction as well

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as sharply enhanced levels of ROS in *B. cinerea* (Li et al. 2017). In our experiment, the H2DCFDA staining experiment showed that carvacrol and thymol significantly increased the content of ROS in mycelia. This may indirectly lead to the death of fungal cells.

Mitochondria are important organelles that produce~~s~~ energy and biochemical reaction sites in cells. Mitochondrial damage results in disruption of the respiratory chain and the tricarboxylic acid (TCA) cycle pathway (Fernie et al. 2004), induces ROS accumulation and decreases intracellular productivity (Zorov et al. 2014). Hu found that *Curcuma longa* essential oil affected the mitochondrial ATPase and dehydrogenases activity (malate dehydrogenase, succinate dehydrogenase) in *A. flavus* (Hu et al. 2017). Through RNA-seq, it was found that the expression levels of most genes in *Fusarium oxysporum* after treatment with thymol, including glycolipid biosynthesis and glycolipid metabolism, were down-regulated while genes involved in antioxidant activity, chitin biosynthesis, and cell wall modification were up-regulated (Zhang et al. 2018). In Rhodamine-123 staining experiment~~al~~, the hyphae emitted strong green fluorescence after treatment with 200 μg/mL carvacrol and thymol. It was confirmed that mitochondria were damaged. There is no doubt that mitochondrial damage accelerates the rate of fungal cell apoptosis and leads to further accumulation of ROS. However, it was not clear whether the accumulation of ROS was caused by carvacrol and thymol or indirectly by the damage of mitochondria~~l~~.

Conclusion

In conclusion, this study proved that a variety of plant essential oils have fungicidal potential ~~of~~ against *B. cinerea*, and found that carvacrol and thymol, as the main components of *O. vulgare* essential oil, can destroy the mycelium morphology, increase cell membrane permeability, cause mitochondrial damage and ROS accumulation in *B. cinerea*. It was proved that carvacrol and thymol have ~~important~~ high potential in the control of gray mold caused by *B. cinerea*~~l~~.

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Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Animal studies and human participants

This article does not contain any studies with human participants or animal performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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