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ORIGINAL ARTICLE

Antifungal activity of thymol and carvacrol against postharvest pathogens *Botrytis cinerea*

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Abstract *Botrytis cinerea* is a primary pathogen causing stem and fruit rot during pre- and post-harvest. In the present study, the main purpose was to inquire into the antifungal activity and potential mechanisms of thymol and carvacrol against *B. cinerea*. During the experiment, the effects of thymol and carvacrol on physical and biochemical parameters of *B. cinerea* were evaluated. Results indicated that thymol and carvacrol exhibited strong antifungal activity against the targeted pathogen, with minimum inhibitory concentration and minimum fungicidal concentration of 65 mg/L and 100 mg/L for thymol, and 120 μL/L and 140 μL/L for carvacrol. Thymol and carvacrol changed obviously the morphology of *B. cinerea* hyphae by disrupting and distorting the mycelia through scanning electron microscopy. The membrane permeability of *B. cinerea* hyphae was prompted with the increment of two chemical agents' concentration, as evidenced by extracellular conductivity increase, the release of cell constituent, and the decrease of extracellular pH. Furthermore, a marked decline in total lipid content of *B. cinerea* cells was induced by the two chemical agents, suggesting that the cell membrane structures were destructed. Therefore, present results indicated that thymol and carvacrol may be used as a good alternative to conventional fungicides against *B. cinerea* in controlling grey molds in horticultural products.

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Keywords *Botrytis cinerea* · Antifungal activity · Membrane permeability · Cell constituent release · Extracellular conductivity

Introduction

Botrytis cinerea seriously damaged many important economic ornamental crops, fruits and vegetables including pre- and post-harvest (Qin et al. 2010). Numerous researches have reported that numerous essential oils extracted from various plants have antifungal activity against postharvest pathogens.

By means of adding 250 μL L^{-1} thyme and oregano oils to PDA medium, spore production and mycelia growth in *Penicillium digitatum* were repressed completely (Daferera et al. 2000). When added to the medium at 1 mL L^{-1} other essential oils, such as cinnamon and clove essential oils, also repressed completely *P. italicum* and *P. digitatum* (Plaza et al. 2004). Thymol (TH) and carvacrol (CAR) are two main components of thyme and oregano essential oils (EOs), and are generally recognized as healthy with no risk to the consumers by the European Commission, Food and Drug Administration (FDA, USA) (Burt et al. 2005).

The essential oil of *O. compactum*, which contains carvacrol (58.1%), *p*-cymene (11.4%) and thymol (9.0%) as major components, carvacrol and thymol were more toxic against *B. cinerea* (Bouchra et al. 2003). Both thymol and carvacrol had a significant antifungal activity through inhibiting the growth of postharvest pathogen such as *P. digitatum*, *P. italicum*, *Fusarium* spp, and *Aspergillus* spp, with the predominant efficacy by thymol (Pérez-Alfonso et al. 2012). Thyme oil vapor fumigation could effectively control gray mold of sweet cherries (Serrano et al. 2005) and brown rot of apricot (Liu and Chu 2002), and

suppressed the mycelium growth of *C. gloeosporioides* originated from avocado in vivo and in vitro (Sellamuthu et al. 2013).

R-(-)-carvone was one of the very efficient volatile components from *Lippia scaberrima* essential oil on repressing the mycelia growth of *B. cinerea* isolated from table grapes (Martínez-Romero et al. 2007), *Alternaria* sp. isolated from mango (Regnier et al. 2008), *P. digitatum* isolated from oranges (Du Plooy et al. 2009), and *Colletotrichum gloeosporioides* isolated from avocado fruit (Combrinck et al. 2011).

Although cell wall and membrane were disrupted with morphological collapse, deformation and deterioration of the hyphae and/or conidia by thymol or carvacrol acts, the antifungal mechanism of action is not well understood (Neri et al. 2006). The anti-fungal activity of thymol and carvacrol, and their synergistic potential with antibiotics, has been depicted in many researches (Langeveld et al. 2014). However, information on the antifungal activity of thymol and carvacrol against *B. cinerea* is fairly limited.

The oil, carvacrol, thymol and commercial herbicide, 2,4-D, isooctyl ester showed potent hytotoxic effects against all plant species tested (Kordali et al. 2008). The use of thymol in drugs is limited by its moderate cytotoxicity that was shown both in in vitro on human cells (Suzuki et al. 1987). Moreover, carvacrol and thymol are a volatile compound which easily evaporates and/or decomposes (oxidation) during food processing owing to direct exposure to light or oxygen (Martínez-Hernández et al. 2017).

Accordingly, the purpose of the present research is to measure the antifungal efficacy of thyme and carvacrol against the mycelia growth of *B. cinerea*. The anti-fungal action mechanism will be evaluated by measuring (1) the membrane integrity and morphology of membrane surfaces with fluorescence microscope and scanning electron microscopy (SEM), (2) the extracellular conductivity and extracellular pH, (3) the release of 260 nm absorbing intracellular constituents, and (4) Potassium (K^+) efflux and the total lipid content.

Materials and methods

Fungal species

The *B. cinerea* strain was generously provided by Dr. Taotao Wang from Huazhong Agricultural University, Wuhan, China. The strain was initially isolated from the decaying tomato fruits, and preserved at present in the Department of Biological and Food Engineering, Xiangtan University, Hunan. The fungus was cultured and purified at 28 ± 2 °C on PDA medium. The fungal suspensions were adjusted to approximately 5×10^5 CFU/mL with a hamacytometer, and diluted with sterile potato dextrose buffer (PDB).

Chemicals

Thymol (99%) and carvacrol (98%) were bought from TCI Company (Shanghai, China). The two chemicals were analytical reagents.

Antifungal activity of thymol and carvacrol

Effects of thymol and carvacrol on the mycelia growth of *B. cinerea* were tested by agar dilution method in vitro (Yahyazadeh et al. 2008). Thymol was dissolved in dimethyl sulfoxide (DMSO) solution. A certain amount of thymol and carvacrol was added to PDA mediums (0.05% Tween-80) to reach the desired concentrations of 0, 20, 40, 60, 80, and 100 mg/L, 0, 20, 40, 60, 80, 100, 120, 140 and 160 μL/L, respectively. And then, 20 mL of PDA solution was poured into sterile Petri plates (90 mm diameter). A disc of inocula (6 mm diameter) was removed from 7-day-old cultures on PDA plates with a punching bear. Soon afterwards, the disc of mycelia was placed centrally upside down on another new Petri plate. The Petri plates were cultured for 48 h at 28 ± 2 °C, and each treatment was conducted in triplicates. The percentage of inhibition of mycelial growth was calculated according to the following formula (Yahyazadeh et al. 2008).

$$\text{MGI}\,(\%) = [(\text{dc} - \text{dt})/\text{dc}] \times 100$$

where dc (cm) is the mean colony diameter for the control sets and dt (cm) is the mean colony diameter for the treatment sets. The minimum concentration that repressed completely fungus growth was deemed to the minimum inhibitory concentration (MIC). The minimum fungicidal concentration (MFC) was considered as the lowest concentration that guarded against pathogen growth after a following 72 h of incubation in a fresh PDA plate at 28 ± 2 °C, which indicated in excess of 99.5% killing of the initial inoculums (Talibi et al. 2012).

Scanning electron microscopy (SEM)

Fungal mycelia cultured for 6 days on PDA medium, which were handled with thymol and carvacrol at different dosages (0, MIC and MFC), were observed by utilizing SEM (Helal et al. 2007; Yahyazadeh et al. 2008). About 5 × 10 mm segments were removed from culture medium growing on PDA plates and quickly placed in vials containing glutaraldehyde (3%, v/v) in phosphate buffer (0.05 M, pH 6.8) at 4 °C. The samples were placed in the above solution for 48 h for fixation and then washed three

times for 20 min each with distilled water. They were dehydrated in a graded series of ethanol (30%, 50%, 70%, and 95%, v/v) for 20 min, respectively, and treated for 45 min with absolute ethanol. Samples were then dried in liquid carbon dioxide at critical point. Fungal segments were stored in desiccators until further utilization. After drying, samples prepared were installed on standard 1/2-inch SEM stubs with double-stick adhesive tabs and coated with gold–palladium electroplating (60 s, 1.8 mA, 2.4 kV) in a Polaron SEM Coating System sputter coater. All samples were observed in JEOL JSM-6360 LV SEM (JEOL, Tokyo, Japan) operating at 2000 × levels of magnification and 25 kV. All the tests were performed in triplicate.

Release of cellular constituents

The cellular constituents in the mycelia were measured on the basis of the method of Paul et al. (2011), with minor modifications. In brief, 6-day-old *B. cinerea* mycelia from 100 mL PDB culture were gained through centrifugating for 20 min at 4000×*g*, rinsed three times, and re-suspended in 100 mL of pH 7.0 phosphate buffered saline. The above suspensions were handled with thymol and carvacrol at different concentrations (0, MIC and MFC) and incubated at 28 ± 2 °C with continuous agitation in an environmental incubator shaker (200 rpm) for 0, 30, 60 and 120 min, respectively. Subsequently, these samples (2 mL) were collected by centrifuging for 2 min at 12,000×*g*. In order to measure the concentration of the released components, the supernatant (1 mL) was used for determining the absorbance with the UV-2450 UV/Vis Spectrophotometer at 260 nm. Three repetitions of three independent experiments were considered.

Determination of extracellular pH and extracellular conductivity

The extracellular pH in *B. cinerea* cells was measured by a Delta 320 pH meter according to the instructions. The extracellular conductivity in *B. cinerea* cells was determined with a DDS-W conductivity meter (Shao et al. 2013). Originally, 100 µL of fungal suspensions (10^5 CFU/mL) was poured into 20 mL PDB medium and cultured in a moist chamber for 2 d at 28 ± 2 °C. The solutions were centrifuged for 20 min at 4000×*g* and the residual precipitation was collected, rinsed for 2–3 times with sterilized water, and re-suspended with 20 mL of sterilized water. After the two chemicals at MIC or MFC were added, the extracellular pH and extracellular conductivity of *B. cinerea* cells were measured at 0, 30, 60 and 120 min of treatment, respectively. These flasks with no the two chemicals were served as control. The results were expressed as pH in the growth media in each interval of incubation and amount of extracellular conductivity (µs/cm).

Measurement of lipid content

The total lipid content in *B. cinerea* cells with the two chemicals at three concentrations (0, MIC and MFC) were measured by means of the phosphor-vanillin method (Helal et al. 2007). 6-day-old mycelia were collected from 50 mL PDB medium after centrifuging for 10 min at 4000×*g*. And then, the samples were dried for 4 h with a vacuum freeze drier. Dry mycelia (0.1 g) were ground with liquid nitrogen and extracted with 4.0 mL of methanol-chloroform-water mixture (2:1:0.8, v/v/v) in a new test tube with violent shaking for 30 min. The solutions were centrifuged for 10 min at 4000×*g*. The lower phase contained lipids, which were collected. They were mixed thoroughly with 0.2 mL of saline solution and centrifuged for 10 min at 4000×*g*. Then, an aliquot of chloroform (0.2 mL) and lipid mixture (0.2 mL) was transferred to another new tube, and 0.5 mL H_2SO_4 was added, heated for 10 min in a boiling water bath. After that, 3 mL phosphovanillin was added and shaken vigorously, and then incubated at room temperature for 10 min. The absorbance at 520 nm was utilized to calculate total lipid content from the standard calibration curve with cholesterol as a standard.

Membrane fatty acid extraction and analysis

The extraction of cell membrane fatty acid was carried out owing to the method described by Folch et al. (1957) with slight modifications. The pellets (1.0 g, dry weight) were ground into homogenate by adding chloroform/methanol (1:1, v:v). The mixtures were blended by agitating for 15 min, and then rinsed with 2 mL of sterile water. The mixture was centrifuged for 15 min at 3000 rpm, after vortexing some seconds. The lipid contained in the lower chloroform phase is recovered and evaporated with a rotary vacuum evaporator. The fatty acid contents were measured by a GC Agilent 7820A equipped with a flame ionization detector and capillary column HP-5 using pure nitrogen as carrier gas at 1 mL/min in a split mode (20:1), working with the following temperature program: 150 °C for 2 min, ramp of 10 °C/min to 170 °C, and 170 °C for 0.5 min. Subsequently, increased at 5 °C/min to 200 °C and kept at 200 °C for 1 min. increased at 2 °C/min to 260 °C and kept at 260 °C for 2 min. Moreover, the molecular weights of all the fatty acid methyl esters were identified by GC–MS (QP2010 Ultra, SHIMADZU). Identification of the constituents was based on comparing their retention times with those of the authentic samples (Sigma, USA), and

quantified by gas chromatography. The results were formulated as mg/g of fatty acid each, which was figured out as the peak area ratio of test samples to standard samples.

Statistical analysis

All the numerical values were described as average value ± standard deviation (SD). These experiments were conducted in three independent replicates. All data were subjected to analyses of variance (ANOVA one-way) using SPSS 15.0 software (SPSS, Chicago, IL, USA). The level of significance was performed by Duncan's multiple range tests at $p < 0.05$.

Results

Antifungal assay

In our present study, all series concentration of thymol and carvacrol could effect the growth of *B. cinerea*. The fungicidal activity of thymol and carvacrol was investigated and the results were presented in Table 1. The effect of thymol and carvacrol on the mycelia growth of *B. cinerea* in vitro was determined. Thymol and carvacrol inhibited the mycelial growth of *B. cinerea* in a dose dependent manner. Therefore, we concluded that the MIC and MFC values of thymol were 65 mg/L and 100 mg/L, respectively. The MIC and MFC values of carvacrol were 120 μL/L and 140 μL/L, respectively.

SEM

The scanning electron microscope (SEM) image (Fig. 1) clearly showed the difference between the treated and untreated *B. cinerea* hyphae with thymol and carvacrol. Carvacrol and thymol exhibited a prominent antifungal activity through inhibiting the mycelia growth of *B. cinerea*. Fungus in control groups had regular, normal, homogenous and tubular hyphae. After 2 d of treatment with thymol and carvacrol at MIC or MFC, all the fungal mycelia revealed considerable changes on hyphal morphology. The mycelia were squashed and distorted with varying degrees, which were treated with carvacrol at MIC. This phenomenon was more severely observed in these hyphae after thymol treatment. Furthermore, after treatment with thymol and carvacrol at MFC, distorted and shrunken mycelia were observed, even some of these mycelia appeared disrupted after thymol treatment. These observations were well explained that the fungicidal activity of thymol had better than that of carvacrol.

Effect of thymol and carvacrol on plasma membrane integrity of *B. cinerea*

Thymol and carvacrol caused the plasma membranes of *B. cinerea* severe damage. The membrane integrity in *B. cinerea* spores decreased with the extension of incubating time or amount of antifungal agents in PDB containing thymol and carvacrol. However, control preserved intact plasma membranes (Fig. 2).

Release of cell constituents

Botrytis cinerea mycelia were treated with thymol and carvacrol for 0, 30, 60, and 120 min at various concentrations (0, MIC, and MFC), respectively. The results about the release of cell constituents were shown in Fig. 3A and B. When the fungi were treated with thymol and carvacrol at MFC, an immediately significant raise in the release of

Table 1 Inhibitory effect of thymol and carvacrol on the mycelial growth of *B. cinerea*

Thymol concentration (μL/L)	Mycelial growth (mm)	Growth inhibition (%)*	Carvacrol concentration (μL/L)	Mycelial growth (mm)	Growth inhibition (%)*
0	10.2 ± 0.56^{b}	0 ± 2.8^{b}	0	10.2 ± 0.56^{b}	0 ± 2.8^{b}
10	10.9 ± 1.24^{a}	$-6.9 \pm 3.6^{**a}$	20	11.2 ± 0.95^{a}	$-9.8 \pm 2.1^{**a}$
20	9.5 ± 0.45^{c}	14.9 ± 1.4^{c}	40	9.1 ± 0.64^{c}	23.4 ± 1.7^{c}
30	8.7 ± 0.47^{d}	31.9 ± 2.1^{d}	60	8.6 ± 0.78^{d}	34.0 ± 3.2^{d}
40	7.4 ± 0.75^{e}	59.6 ± 3.2^{e}	80	8.2 ± 0.42^{e}	42.6 ± 2.5^{e}
50	6.8 ± 0.85^{f}	72.3 ± 2.7^{f}	100	7.6 ± 1.24^{f}	55.3 ± 1.6^{f}
65	5.5 ± 0^{g}	100 ± 0^{g}	120	5.5 ± 0^{g}	100 ± 0^{g}
100	5.5 ± 0^{g}	100 ± 0^{g}	140	5.5 ± 0^{g}	100 ± 0^{g}

[a–f]Significant differences at $p < 0.05$ level according to Duncan's multiple range test

*Values are average ± SD (n = 6)

**Additive concentration can promote the growth of mycelial

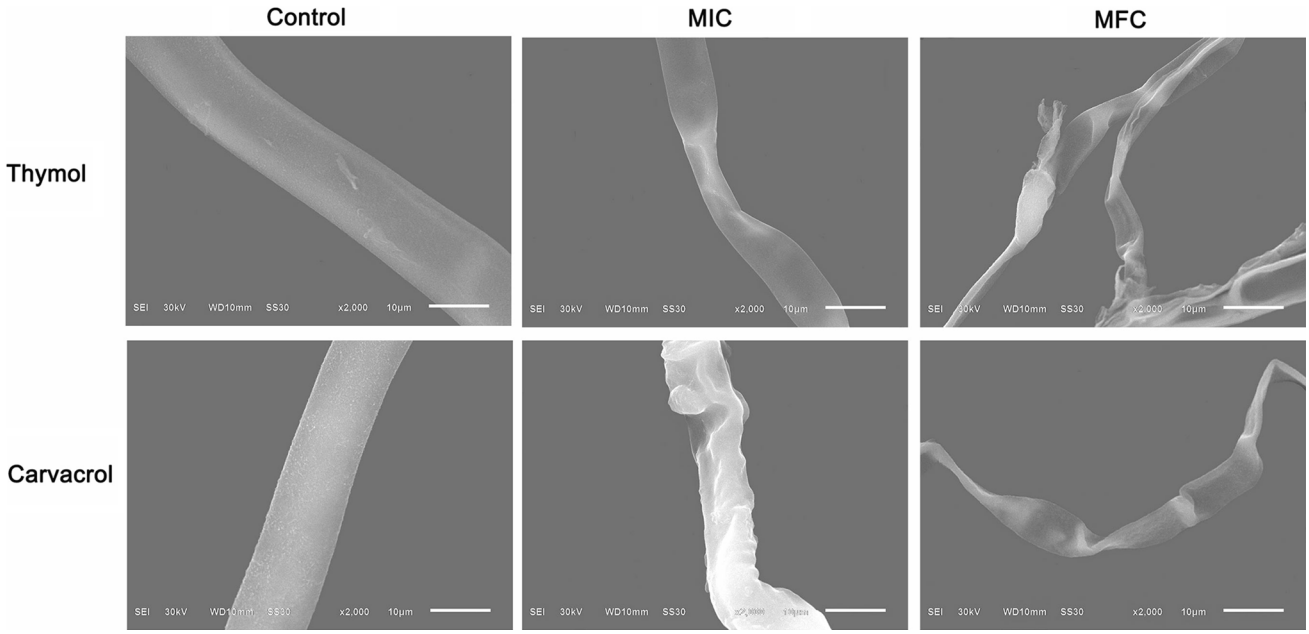

Fig. 1 Scanning electron microphotography of *B. cinerea*: untreated; treated with 65 mg/L, 100 mg/L thymol, respectively; treated with 120 μL/L, 140 μL/L carvacrol, respectively

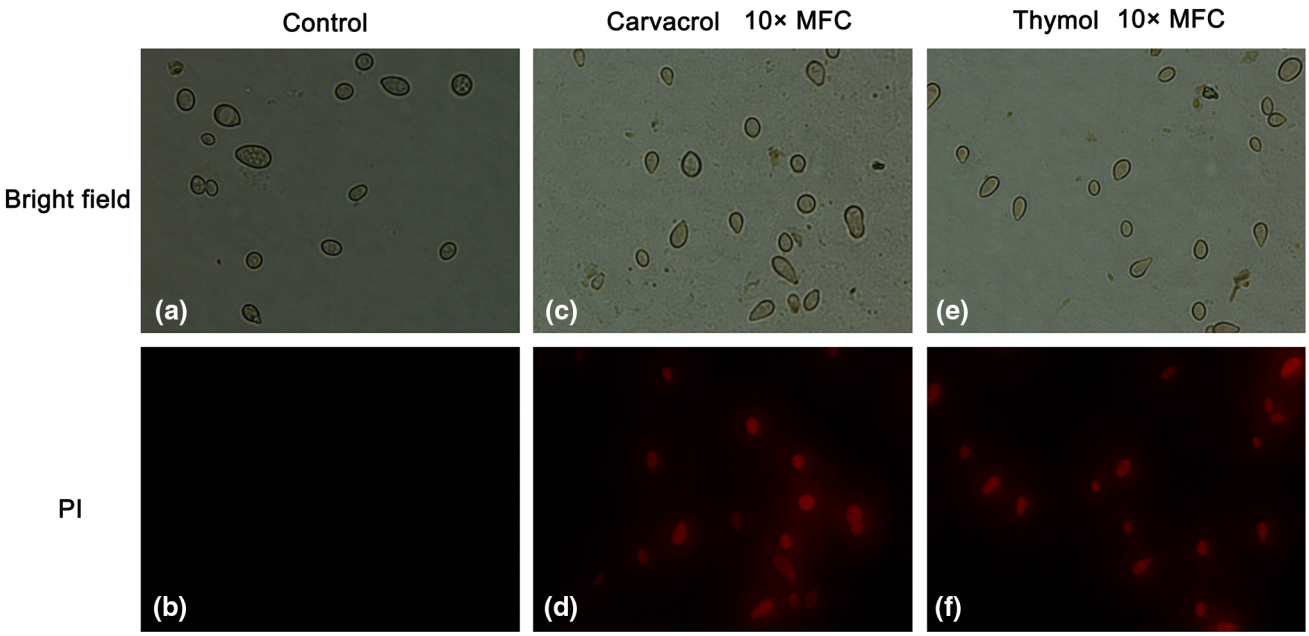

Fig. 2 Effect of 10 × MFC carvacrol and thymol on the membrane integrity of *B. cinerea* spores. Spore were cultured at 28 + 2 °C in PDB medium supplement with 0 (**a** bright field; **b** PI) or 10 × MFC carvacrol (**c** bright field; **d** PI) and 10 × MFC thyme (**e** bright field; **f** PI) for 2 h of incubation, spore were stained with the PI and observed with a fluorescence microscope

intracellular substances was found. Meanwhile, OD_{260nm} values increased with the extension of processing time. The OD_{260} value in the *B. cinerea* suspensions exposure to thymol at MIC for 120 min reached 0.701, which was obviously higher than that in control (0.442) and carvacrol (0.479). However, during the entire treatment process, the changes in the OD_{260} value at MIC were not significant, compared with thymol (Fig. 3A and B). The OD_{260} values in *B. cinerea* suspensions exposure to thymol at MIC and MFC remained a sharp upward trend, whereas the OD_{260} values in *B. cinerea* suspensions exposure to carvacrol at MIC or MFC kept a relatively stable upward trend.

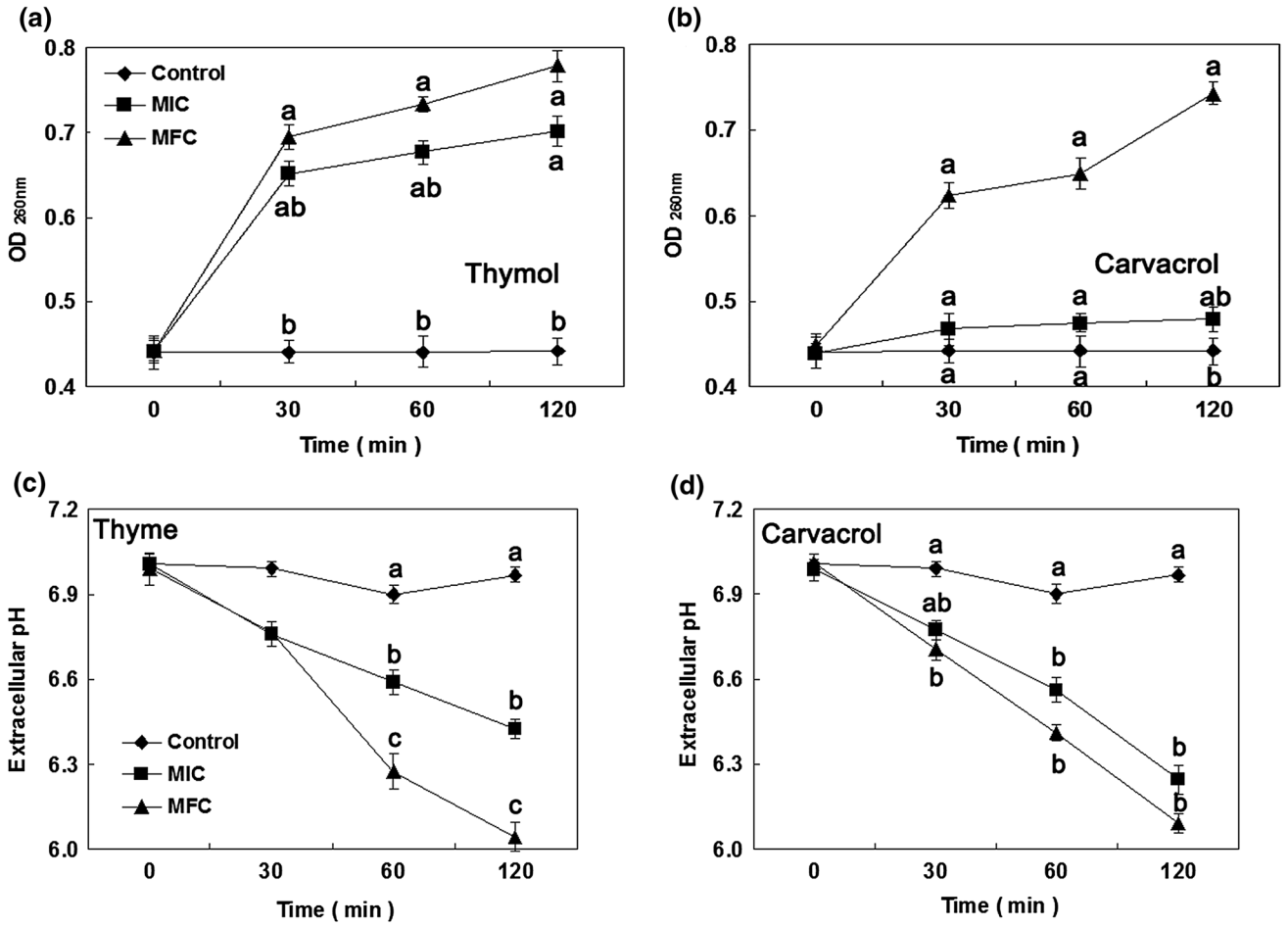

Fig. 3 Effect of thymol and carvacrol on the 260 nm absorbing material release (**a**, **b**) and extracellular pH (**c**, **d**) of *B. cinerea* [(diamond): control; (square): MIC; (circle): MFC]. Data presented are the means of pooled data. Error bars indicate the SDs of the means (n = 3). Means with different letters in the same column were significantly different at the level of $p < 0.05$

Extracellular pH

The extracellular pH in *B. cinerea* mycelia exposed to thymol and carvacrol was showed in Fig. 3C and D. The extracellular pH sharply decreased after incubation with thymol and carvacrol at MIC or MFC. Conversely, the extracellular pH in *B. cinerea* suspensions in control slowly decreased during the original 60 min of exposure period, and increased slightly at 120 min of treatment. The values of extracellular pH in *B. cinerea* suspensions treated with thymol and carvacrol at MFC for 120 min were 6.05 and 6.09, respectively, which were remarkably below control (6.97).

Potassium ion efflux

The leakage of potassium ions (K^+) from *B. cinerea* mycelia incubated with thymol and carvacrol (Fig. 4A and B). The release of K^+ was significantly induced by thymol at MFC during the original 30 min of exposure period. After 30 min of treatment by thymol at MFC, the K^+ concentration in *B. cinerea* mycelia was 13.6 μg/mL. By contrast, after 30 min of treatment with carvacrol at MFC, K^+ concentration in *B. cinerea* mycelia was 9.9 μg/mL, which was significantly less than that of thymol at MFC. After 120 min of treatment with thymol at MFC, K^+ concentration presented a moderate ascending trend, and reached to 16.0 μg/mL.

Extracellular conductivity

The extracellular conductivity of *B. cinerea* mycelia treated with thymol and carvacrol at MIC or MFC for a period of 0–120 min were presented in Fig. 4C and D. After 30 min of treatment with thymol at MIC and MFC, the extracellular conductivity was 122.3 and 138.7 μs/cm, respectively, and was obviously above control (91.0 μs/cm), carvacrol at MIC (101.6 μs/cm) and MFC (105.6 μs/cm), respectively. After 120 min of treatment with thymol and carvacrol at MIC and MFC, the extracellular

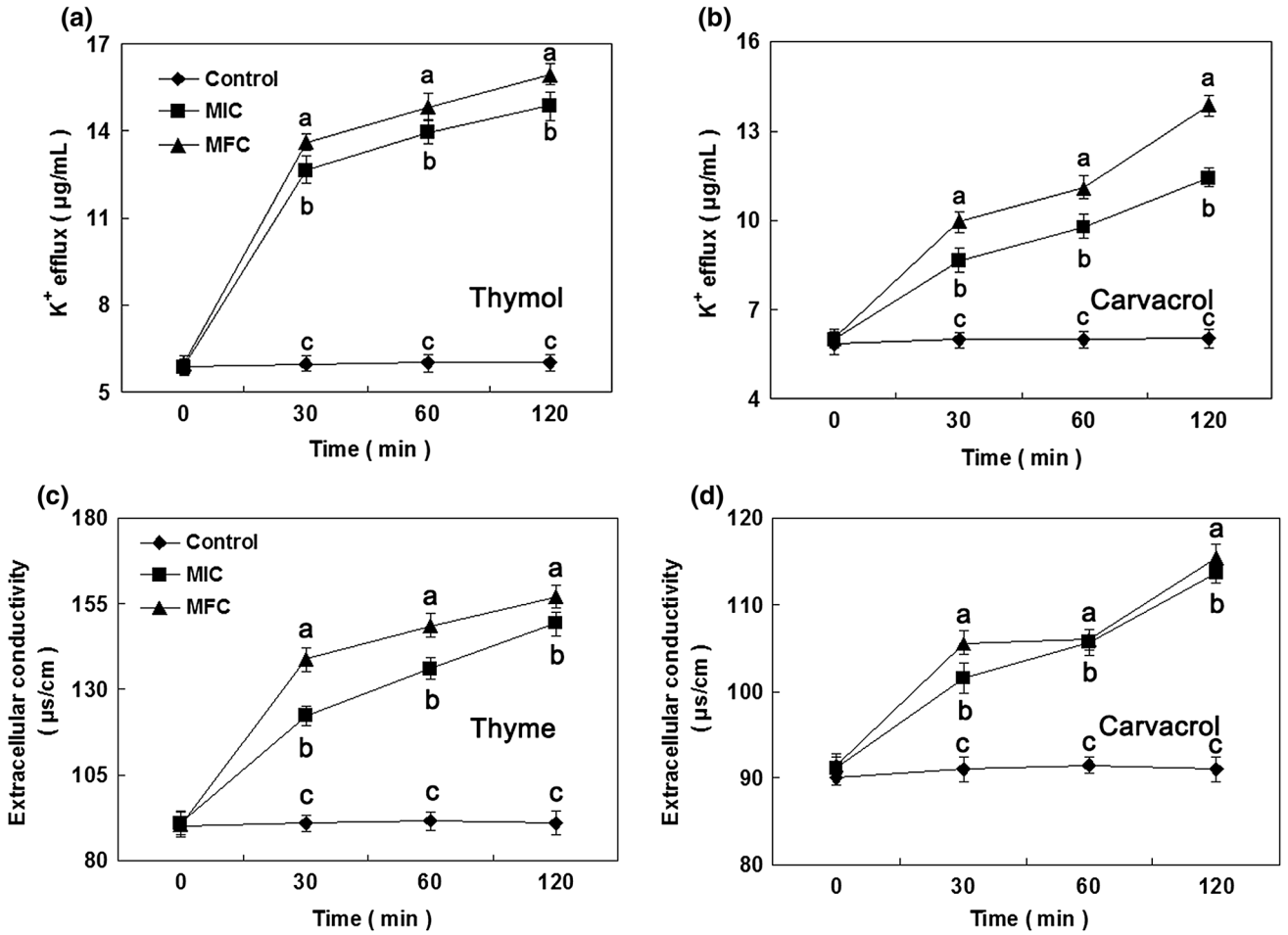

Fig. 4 Effect of thymol and carvacrol on the K^+ efflux (**a**, **b**) and extracellular conductivity (**c**, **d**) of *B. cinerea* [(diamond): control; (square): MIC; (circle): MFC]. Data presented are the means of pooled data. Error bars indicate the SDs of the means (n = 3). Means with different letters in the same column were significantly different at the level of $p < 0.05$

conductivity in *B. cinerea* suspensions significantly increased, and reached to 149.2 and 113.7 μs/cm for MIC, and 157.1 and 115.4 μs/cm for MFC, respectively.

Total lipid content

Total lipid content in *B. cinerea* mycelia treated with thymol and carvacrol was provided in Fig. 5. During the original 30 min of exposure, total lipid contents in *B. cinerea* mycelia obviously declined as the increasing concentration of thymol and carvacrol. After 30 min of exposure to thymol at MIC and MFC, the total lipid contents in *B. cinerea* cells were 66.8 ± 4.0 and 52.8 ± 3.2 mg/g DW, respectively, which were remarkably below control (101.0 ± 3.3 mg/g DW). Whereas the total lipid contents in *B. cinerea* cells treated with carvacrol at MIC and MFC for 30 min were 75.0 ± 3.6 and 58.1 ± 5.0 mg/g DW, respectively. Moreover, the total lipid contents in *B. cinerea* cells with thymol and carvacrol continuously decreased thereafter.

Fatty acid analysis

The fatty acid composition of *B. cinerea* mycelia treated with thymol and carvacrol at various concentrations (0, MIC and MFC) were presented in Table 2. The main compositions of fatty acids detected in control were $C_{16:0}$, $C_{17:0}$, $C_{19:0}$, $C_{18:2\omega6,\ 9}$, $C_{19:2\omega6,\ 9}$ and $C_{21:2\omega7,\ 9}$, respectively. When the chemical stresses were applied, and especially at MFC, brought about a dramatical increase of $C_{16:0}$ and $C_{17:0}$. Moreover, the supplementation with thymol and carvacrol decreased some fatty acid contents, such as $C_{19:0}$ and $C_{19:2\omega6,\ 9}$. As the concentration of treatment reagents increased, the fatty acids contents detected were also promoted.

Discussion

In this study, thymol and carvacrol could effectively repress *B. cinerea* growth in vitro, and their inhibitory effect was a concentration-dependent manner. The results

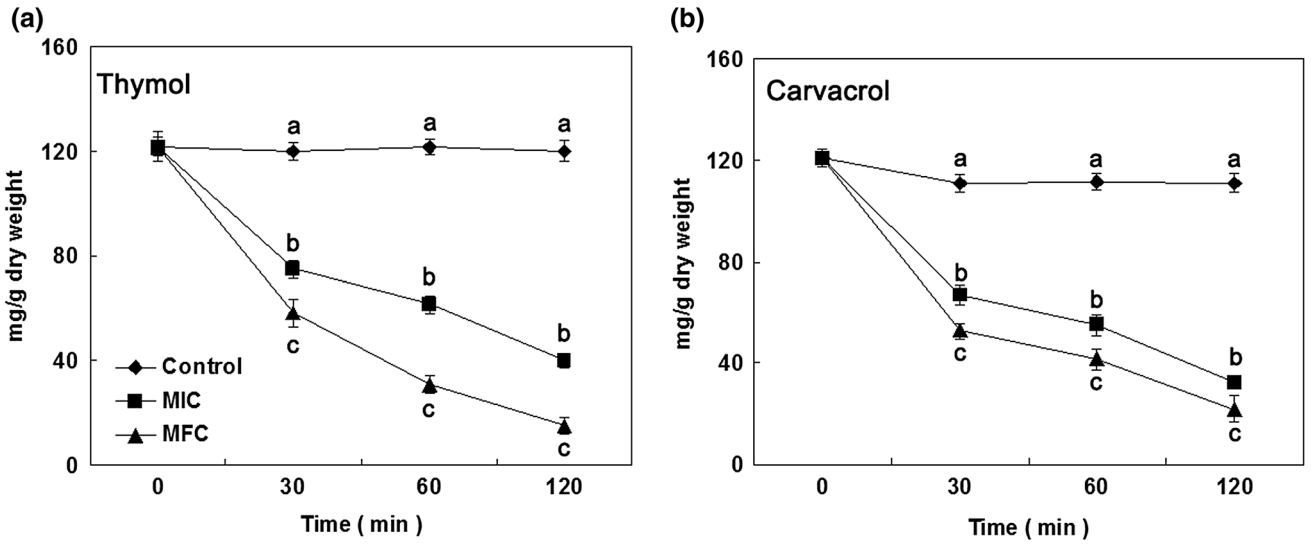

Fig. 5 Total lipid content of B. cinerea cells in the presence of thymol (**a**) and carvacrol (**b**) [(diamond): control; (square): MIC; (circle): MFC]. Data presented are the means of pooled data. Error bars indicate the SDs of the means (n = 3). Means with different letters in the same column were significantly different at the level of $p < 0.05$

Table 2 Fatty acids composition evaluated by GC–MS in *B. cinerea* mycelia treated with thymol and carvacrol at different concentrations (0, MIC and MFC)

Fatty acids	Total composition (mg/g fresh weight) in *B. cinerea* mycelia treated with thymol and carvacrol				
	Control	Thymol		Carvacrol	
		MIC	MFC	MIC	MFC
$C_{17:0}$	0.27 ± 0.02^{b}	0.23 ± 0.03^{b}	0.60 ± 0.04^{a}	0.21 ± 0.05^{b}	0.59 ± 0.08^{a}
$C_{19:2\omega6,9}$	0.79 ± 0.03^{a}	0.32 ± 0.06^{b}	0.49 ± 0.03^{b}	0.31 ± 0.07^{b}	0.61 ± 0.02^{ab}
$C_{19:0}$	0.03 ± 0.01^{a}	0.04 ± 0.01^{a}	0.07 ± 0.02^{a}	0.05 ± 0.02^{a}	0.12 ± 0.04^{a}
$C_{16:0}$	0.10 ± 0.01^{a}	0.19 ± 0.04^{a}	0.28 ± 0.03^{a}	–	–
$C_{18:2\omega6,9}$	0.31 ± 0.04	–	–	–	–
$C_{21:2\omega7,9}$	0.02 ± 0.01^{a}	0.01 ± 0.01^{a}	0.04 ± 0.01^{a}	–	–
$C_{23:0}$	–	–	–	0.10 ± 0.01^{a}	0.14 ± 0.02^{a}

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the same row were significantly different at the level of $p < 0.05$

were accordance with previous reports described these chemicals against pathogens (Pérez-Alfonso et al. 2012; Jesus et al. 2015; Ochoa-Velasco et al. 2017; Kanchana et al. 2017). No fungal mycelium was observed with 250 µL L^{-1} and more of thymol or 500 µL L^{-1} of carvacrol on the lemon surface (Pérez-Alfonso et al. 2012). Moreover, thymol had higher antifungal effect than that of carvacrol on the basis of previous reports (Numpaque et al. 2011). This finding suggested that thymol showed the more effective anti-fungal effect, and followed by carvacrol.

The potential antimicrobial activity mechanisms of volatile compounds are because they can pierce through cell membrane, where these substances disturb intracellular metabolism (Marino et al. 2001), and modify the efflux pumps activities (Langeveld et al. 2014). Furthermore, the lipophilicity of volatile compounds increased membrane permeability and fluidity, inhibited respiration, disturbed membrane-embedded proteins, induced ions leakage and other intracellular contents, and altered transport processes of ion in fungi (Burt 2004; Fadli et al. 2012; Khan et al. 2010).

SEM examinations could reveal that rough surface and shrinkage of cells were apparent in the treated hyphae (Tao et al. 2014). SEM observations also confirmed the damage that the treated hypha demonstrated bending, entwining, shriveling, collapse and disorganization, and the breakage of the hypha was clearly visible (Jiang et al. 2015). In the present experiment, *B. cinerea* mycelia were treated with

thymol and carvacrol, and were used for SEM observations. SEM images revealed that the two chemicals could influence the morphology of *B. cinerea* hyphae, and disrupt membrane integrity, as reported by damaging cell membrane of *R. stolonifer* (Jiang et al. 2015). Consequently, the antifungal mechanisms of thymol and carvacrol against *B. cinerea* were because of their actions on cellular membrane and disrupting the cytoplasm of hyphae, eventually resulting in mycelia death.

Membrane permeability parameters included potassium ions leakage, 260 nm absorbing materials loss, and change in extracellular pH. These parameters are usually adopted for indicating gross and irreversible damage happened on plasma membranes (Paul et al. 2011; Shao et al. 2013). In the present study, the release of cell constituents and extracellular conductivity were dramatically increased in a dose-dependent manner, compared with control. The intracellular proton leakages were distinctly induced by thymol and carvacrol after 30 min of treatment, as evidenced by extracellular pH decrease. The irreversible damage occurred on the cytoplasmic membranes of *B. cinerea*, which lead to intracellular ions leak and an imbalance in osmotic pressure between extra- and intracellular membrane occur. The small substances would be the leakage of intracellular contents of the cells caused by the change of cellular membrane permeability and destruction of microbiological protective barriers (Zhao et al. 2017). In addition, the antifungal effect of thymol was higher than that of carvacrol, according to previous reports (Numpaque et al. 2011; Pérez-Alfonso et al. 2012). These reports supported our current results.

Lipids on cell membrane play an important role in adjusting membrane liquidity, raising membrane stability, and decreasing water-soluble materials permeability (Helal et al. 2007). Volatile substances like terpenes reportedly could penetrate or disrupt these fungal lipid structures (Prashar et al. 2003). The total lipid content in *Aspergillus flavus* and *Aspergillus niger* cells remarkedly reduced from non-fumigated cells to fumigated cells with 1 μL/mL of *Cymbopogon citratus* essential oil (Helal et al. 2007). In this research, the treatment with thymol and carvacrol obviously reduced fungal total lipid content, which indicated that the two chemicals disrupted plasma membrane structure and integrity.

Conclusion

This research has revealed that thymol and carvacrol can obviously repress the mycelial growth of *B. cinerea*, which are promising anti-fungal compounds. The two chemicals can come into being obvious cytotoxic effect on *B. cinerea* cells, and the anti-fungal activity can be derived from cell membrane integrity damage and cell components leakage. These results indicated that thymol and carvacrol can be used as an alternative to traditional fungicides by fumigation to control post-harvest tomato pathogens.

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