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In vitro antifungal activity and mechanism of action of carvacrol against *Sclerotinia sclerotiorum* (Lib.) de Bary

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ABSTRACT: This study aimed to examine the antifungal effects of nine monoterpenes on *Sclerotinia sclerotiorum* via the mycelial growth rate method. The effects of carvacrol on the morphological structures of hyphae were investigated by scanning electron microscopy (SEM). The oxalic acid (OA), total protein contents, and the activity of cell wall-degrading enzymes, including chitinase, cellulase and β-1,3-glucanase, were assessed. The results showed that the antifungal rates of carvacrol, thymol and eugenol reached 100% at a concentration of 400 mg/L, and the EC_{50} values of carvacrol, thymol, and eugenol were 43.40, 56.22, and 86.63 mg/L, respectively. The treatment of *S. sclerotiorum* with carvacrol had no significant effect on sclerotia formation, but the mycelial surface was shrivelled, uneven and broken, with cytoplasm flowing out. The OA content of *S. sclerotiorum* was significantly reduced to 133.78 μg/mL after treatment with carvacrol. Additionally, the total protein content of *S. sclerotiorum* mycelia in the carvacrol treatment group was significantly reduced to 15.67 μg/mL compared with that of the control group, and the activity of cellulase in the carvacrol treatment group was significantly higher than that in the control group.

Keywords: monoterpenes; morphological structures of hyphae; oxalic acid; total protein content; cellulase

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic pathogen with a worldwide distribution that causes disease in more than 450 plant species, such as oilseed rape, sunflower, tobacco, a range of vegetables, and numerous flower crops (Ding et al. 2020). *S. sclerotiorum* causes *sclerotinia* stem rot (SSR), *sclerotinia* head rot, white mould, stalk rot or wilt in oilseed rape and leads to serious economic losses worldwide (Cheng et al. 2019). *S. sclerotiorum* has a high overwintering ability, as sclerotia can persist in soil or crop debris and exhibit strong stress resistance. Due to specialised resistant survival structures and a lack of resistance or immunity hybridisation, controlling *S. sclerotiorum* is difficult (Mao et al. 2018). Chemical fungicide application is effective, but its long-term application leads to environmental contamination, increased farming costs, ecological imbalance, and fungicide resistance of *S. sclerotiorum*. Therefore, an environmentally friendly and effective approach is needed to control *S. sclerotiorum*.

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Many essential oils and plant extracts have recently been used against fungal pathogens (Xie et al. 2017). Essential oils can effectively destroy the permeability of the fungal cell membrane and inhibit the growth of pathogenic fungi (Elshsfie et al. 2015). Soylu et al. (2010) reported that essential oils (EOs) obtained from the Lamiaceae family lead to the morphological degeneration of fungal hyphae *in vitro* assays, and the main manifestations are cytoplasmic coagulation, vacuolations, hyphal shrivelling, protoplast leakage and loss of conidiation. The plant cell wall is the physical barrier protecting the cells from infringement, but phytopathogenic fungi can overcome this physical barrier by producing an array of cell wall-degrading enzymes (CWDEs) (Quoc et al. 2017). EOs and plant extracts can also reduce the virulence of pathogenic fungi by inhibiting the enzymes produced by phytopathogenic fungi. Tian et al. (2012) reported that *Anethum graveolens* L. EOs inhibit the mitochondrial ATP enzymes and hydrogenase activity of *Aspergillus flavus*, and these effects result in a significant decrease in ATP content and a hindered normal energy metabolism in the cells. Therefore, EOs and plant extracts affect the energy metabolism of fungal cells.

Previous studies have shown that monoterpenes, which are plant metabolites, including esters, ketones, and phenols, can control fungal diseases. Man and Jiang (2002) found that the treatment of *A. flavus* with citral *in vitro* yielded damaged mitochondria, as revealed by a surface consisting of wrinkled pleats, an irregular morphology, a chaotic structure, and a non-slippery surface, and decreased the activity of malate dehydrogenase and succinate. Due to their lower costs and environmental friendliness, monoterpenes possess good volatility (Zhou et al. 2019); therefore, monoterpenes have great potential in preventing and controlling pathogens and protecting fruits and vegetables.

The present study verified that monoterpenes have potential as natural fungicidal agents. However, few studies have been devoted to the activities of EOs and plant extracts against *S. sclerotiorum* (Soylu et al. 2010), and we investigated the effects of monoterpenes against *S. sclerotiorum in vitro*. The objectives of our study were to *(i)* evaluate the activity of nine monoterpenes against *S. sclerotiorum*; *(ii)* study the effects of carvacrol on the morphological structures of *S. sclerotiorum* hyphae and the formation of sclerotia; *(iii)* explore the effect of carvacrol on the oxalic acid (OA) content of *S. sclerotiorum*; and *(iv)* explore the effect of carvacrol on the total protein content and the activity of CWDEs, including chitinase, cellulase and β-1,3-glucanase. Our results can be used to further develop environmentally friendly and ecologically safe fungicides against *S. sclerotiorum*.

MATERIAL AND METHODS

Chemicals. Bornyl acetate, carvacrol, neryl acetate, citronellyl acetate, terpinyl acetate, eugenol, geranyl acetate, terpinolene, thymol and carbendazim were obtained from commercial sources and stored at –20 °C. All chemical structures and purity metrics are shown in Table 1.

Fungi. The *S. sclerotiorum* (Lib.) de Bary strain used in this experiment was provided by the Key Laboratory of Biology and Sustainable Management of Plant Diseases and Pests of Anhui Higher Education Institutes, College of Plant Protection, Anhui Agricultural University, Hefei, China.

Antifungal assays. The inhibitory effects of nine monoterpenes on *S. sclerotiorum in vitro* were investigated using the mycelial growth rate method (Zhang et al. 2020). First, inoculation mycelial plugs (5 mm in diameter) were placed on potato dextrose agar (PDA) plates with one monoterpene at a concentration of 400 mg/L. PDA plates without any monoterpenes served as controls. The antifungal assays were conducted three times for each monoterpene, and each isolate was incubated at 23 ± 1 °C. The mean values for the two perpendicular diameters of each isolate were obtained, and the growth inhibition (I) (%) was calculated using the following formula:

$$I = \frac{C - T}{C} \times 100 \quad (1)$$

where: I – the percentage of mycelia growth inhibition by monoterpenes; C – the zone diameter in the control plate (cm); T – the zone diameter in the experimental plate (cm).

Afterwards, monoterpenes with an inhibitory rate of 100% were selected, and their median effective concentration (EC_{50}) was measured on a series of PDA plates with different concentrations of the test compound. The percentage of the inhibition (I) obtained with each concentration of the test compound was calculated using the above-described

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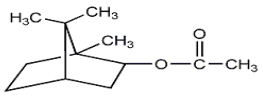
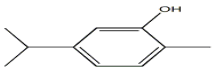
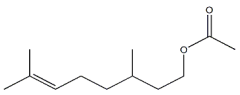
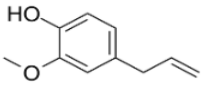
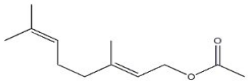
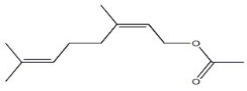
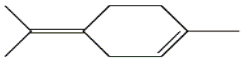
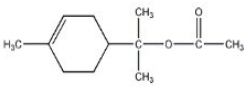
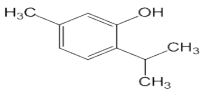
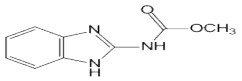
Table 1. Structural formula and purity indices of ten test compounds

No.	Compounds	Purity (%)	Structure	Supplier Information
1	bornyl acetate	70		TCI (Shanghai, China)
2	carvacrol	98		TCI (Shanghai, China)
3	citronellyl acetate	95		Aladdin (Shanghai, China)
4	eugenol	98		Sigma-Aldrich (Shanghai, China)
5	geranyl acetate	96		Macklin (Shanghai, China)
6	neryl acetate	95		TCI (Shanghai, China)
7	terpinolene	95		J&K (Beijing, China)
8	terpinyl acetate	85		Aladdin (Shanghai, China)
9	thymol	99		J&K (Beijing, China)
10	carbendazim	98		J&K (Beijing, China)

method. The EC_{50} values based on the percentage of growth inhibition for the monoterpene concentration were analysed by regression (Kuang et al. 2011).

Effect of carvacrol on sclerotium formation in *S. sclerotiorum*. Fresh mycelial plugs (5 mm) were placed in PDA plates containing the EC_{50} concen-

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tration of carvacrol. Plates without carvacrol were used as controls. Each isolate was incubated in the growth chamber (23 ± 1 °C) for ten days, and three replicates were included in the experiment. The formation of sclerotia in each colony was observed, and the number of sclerotia was counted.

Effect of carvacrol on the mycelial morphology of *S. sclerotiorum*. Fresh mycelial plugs were placed in PDA plates with the EC_{50} value of carvacrol. Plates without carvacrol were used as controls. Each isolate was incubated in the growth chamber (23 ± 1 °C) for two days, and three replicates were included in the experiment. The mycelial plugs were cut from the margin of the PDA plates (a thickness of approximately 1 mm and an area of approximately 4–5 mm^2), fixed with 2.5% glutaraldehyde (4 °C, 12 h), and washed three times with 0.1 M phosphate buffer (pH 7.2) for 20 min at room temperature. After sample fixation, dehydration was conducted via a gradient of ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 100%), and each concentration was tested for approximately 20 min. The *S. sclerotiorum* mycelium was rinsed with acetone (100%) for 20 min, and two replicates were included in the experiment. The mycelia were dried with a vacuum dryer, embedded, sprayed, and observed by scanning electron microscopy (SEM) (Hitachi S-4800, Tokyo, Japan).

Effect of carvacrol on the OA content of *S. sclerotiorum*. According to Duan's method, the OA contents of *S. sclerotiorum* in the carvacrol treatment group and the control group without carvacrol were determined (Duan et al. 2013). A standard curve for oxalate was prepared in a 10 mL microtube using 0.4 mL of $FeCl_3$ solution (0.5 mg/mL), 4 mL of HCl–KCl buffer solution (KCl 50 mmol/L, pH 2), and 0.24 mL of sulfosalicylic acid solution (5 mg/mL). A series of volumes (0 µL, 20 µL, 40 µL, 80 µL and 100 µL) of sodium oxalate solution (2 mg/mL) were then added, and the total volume was increased to 5 mL with double-distilled water. The mixture was blended with a vortex mixer (10 s) and incubated (30 min, 25 °C). The absorbance of the solution at 510 nm was measured with a spectrophotometer. The standard curve was generated by plotting the relationship between the absorbance and the concentration of sodium oxalate.

To determine the OA content, six mycelial plugs (5 mm in diameter) were placed into a triangular flask containing 100 mL of PD liquid medium containing carvacrol with an EC_{50} value of 43.40 mg/L and incubated in a constant-temperature incubator (23 °C, 175 r/min) for 3 days. Treatments without carvacrol were used as controls. After three days, the contents were centrifuged (1 500 rpm, 10 min), and the absorbance at 510 nm was measured according to the standard curve method. The experiment was performed three times.

Effect of carvacrol on the total protein content and chitinase, *β*-1,3-glucanase, and cellulase activities of *S. sclerotiorum* mycelia. Six *S. sclerotiorum* plugs (5 mm in diameter) were placed into a triangular flask containing 100 mL of PD liquid medium and incubated in a constant-temperature incubator (23 °C, 175 r/min) for two days. Carvacrol was added to the treatment group to obtain a final concentration in line with the EC_{50} value (43.40 mg/L), and the samples were then incubated in a constant-temperature incubator (23 °C, 175 r/min) for another day. The same volume of sterile water was added to the control group. The flasks were shaken for two days, and the mycelia were filtered, washed three times with sterile water and then ground with 0.05 mol/L Tris-HCl buffer (pH 7.5) in an ice mortar. After centrifugation (15 000 r/min, 20 min, 4 °C), the supernatant was stored at –20 °C. Chitinase, β-1,3-glucanase and cellulase activities were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

Statistical analyses. All data are presented as the means ± standard errors (SEs). The significance of the differences was analysed by independent-sample t-test using the SPSS software package (version 19.0). P values < 0.05 were considered to indicate significance.

RESULTS AND DISCUSSION

Antifungal assays of monoterpenes. *S. sclerotiorum* is a typical necrotrophic fungus, and fungicides are widely used to control SSR caused by *S. sclerotiorum* (Wang et al. 2014). However, the new strategy for *S. sclerotiorum* management has forced the exploration of new control methods due to the poor control effect obtained with traditional methods and pesticide residues. To use biodegradable, low-toxicity, natural products for the control of plant diseases and insect pests, plant extracts and EOs have been directly applied (*in vitro*) as antifungal agents.

The antifungal activities of nine monoterpenes were investigated against *S. sclerotiorum* at a con-

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Table 2. Antifungal activities of nine monoterpenes against *Sclerotinia sclerotiorum*

Compounds	Inhibition[a] (%, mean ± SE)
Bornyl acetate	54.47 ± 0.79
Carvacrol	100.00 ± 0.00
Citronellyl acetate	35.38 ± 1.39
Eugenol	100.00 ± 0.00
Geranyl acetate	48.74 ± 0.48
Neryl acetate	48.98 ± 2.40
Terpinolene	7.90 ± 1.14
Terpinyl acetate	49.50 ± 4.56
Thymol	100.00 ± 0.00

[a]nine monoterpenes at a concentration of 400 mg/mL

centration of 400 mg/L. According to the inhibition of mycelial growth on PDA, nine monoterpenes showed inhibitory activity against *S. sclerotiorum* (Table 2). Carvacrol, thymol and eugenol exhibited the highest activity against *S. sclerotiorum*, with antifungal indices reaching 100%, whereas the antifungal indices of the other compounds were less than 60%. Additionally, the results from the analysis of the effects of carvacrol, thymol and eugenol are summarised in Table 3. According to the EC_{50} values, carvacrol, thymol and eugenol were ranked as follows based on their antifungal activities against *S. sclerotiorum*: carvacrol (43.40 mg/L) > thymol (56.22 mg/L) > eugenol (86.63 mg/L). Dias et al. (2019) reported that limonene exhibits high inhibitory activity on the mycelial growth of *S. sclerotiorum*, and the *in vitro* inhibition rate obtained with a dose of 200 µL was 100%. Valadares et al. (2018) found that *Piper aduncum* inflorescences at doses above 30 µL induced 100% inhibition of *S. sclerotiorum* mycelial growth, and 50 µL of *P. aduncum* leaves induced 98.74% inhibition of *in vitro* antifungal activity. Some plant monoterpenes have been confirmed to show very strong toxicity to fungi and bacteria in several studies (Xie et al. 2017; Zhang et al. 2018; Zhou et al. 2019).

Table 3. Inhibitory effects of 4 compounds on *Sclerotinia sclerotiorum* against *S. sclerotiorum*

Compounds	EC_{50}/(mg/L) (95%CL)
Carvacrol	43.400 (39.303–48.060)
Eugenol	86.630 (79.640–94.470)
Thymol	56.220 (50.600–62.780)
Carbendazim	0.361 (0.326–0.406)

Effects of carvacrol on sclerotia formation and of monoterpenes on the mycelial morphology of *S. sclerotiorum*. After approximately seven days of incubation at 23 ± 1 °C in a humidified incubator, carvacrol exerted a certain inhibitory effect on the formation of *S. sclerotiorum* sclerotia. Few sclerotia formed in the plates treated with carvacrol, but no significant differences were found between the carvacrol-treated and control groups ($P > 0.05$). The sclerotia that formed in the carvacrol-treated and control plates were 60.67 ± 4.16 and 72.67 ± 1.53, respectively, and the inhibition rate was 16.53%. In this study, carvacrol exerted a certain inhibitory effect against *S. sclerotiorum* and the formation of *S. sclerotiorum* sclerotia. Edris and Farrag (2003) confirmed that peppermint and sweet basil EOs and menthol (their major constituents) exert a very strong inhibitory effect on the growth of fungi dose-dependent, indicating that they exhibit antifungal activity *in vitro*.

Carvacrol is a main component of EOs produced by numerous aromatics (Bueno-Durán et al. 2014) and is widely used for the inhibition of fungi and bacteria. Wang et al. (2018) reported that carvacrol exhibits good to excellent *in vitro* antifungal activity against *Botrytis cinerea*. In a previous study, Carvacrol was demonstrated to be an effective antibacterial agent against both gram-positive and gram-negative bacteria (Kachur & Suntres 2020). In addition, carvacrol significantly inhibits the mycelial growth of several plant pathogenic fungi (Wang et al. 2018). Moreover, carvacrol could effectively inhibit the growth of *Rhizopus stolonifer* and exhibit *in vitro* antifungal activity, which disrupts the cell membranes and the hyphal wall, causing leakage of the cytoplasm and breaking the hyphae (Zhou et al. 2018).

The effect of carvacrol on the mycelial growth of *S. sclerotiorum* was observed by scanning electron microscopy (Figure 1). The mycelium of the control group was uniform, full and smooth (Figure 1A). Treatment with carvacrol at an EC_{50} value of 43.40 mg/L caused the mycelial surface to shrivel and shrink (Figure 1B), and some mycelium broke, which caused the content to flow out and the cells to be ablated. The results showed that the target of carvacrol on *S. sclerotiorum* may be located in the cell wall, and carvacrol thus firstly destroys the morphology and function of the cell wall and further causes loss of the normal physiological functions of intracellular organelles, which results in the inhibition of fungi. A similar result was found

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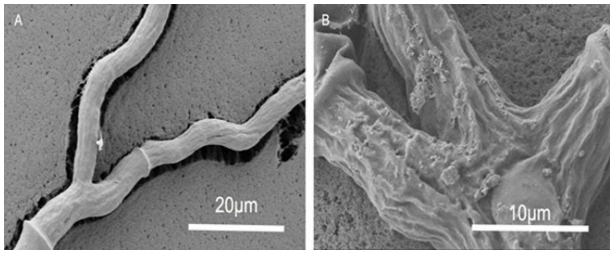

Figure 1. Scanning electron microphotograph of hyphal morphology of *Sclerotinia sclerotiorum* treated with carvacrol
A – control; B – treated with carvacrol

by Ma et al. (2015), who reported that treatment with Dill seed essential oil at 1.00 μL/mL caused the hyphal aggregates of *S. sclerotiorum* to become shrivelled and induced the collapse of hyphae (contact phase). The changes in the sclerotia morphology were significant. Therefore, EOs degrade the integrity of the cell wall, which causes cytoplasmic membrane leakage, cell lysis and, eventually, cell death (Jing et al. 2014).

Effects on the OA content. OA is a secondary metabolite of *S. sclerotiorum* that plays an important role in the pathogenic process by making the pathogen more susceptible to host infection. As shown in Figure 2, carvacrol treatment significantly decreased the OA content of *S. sclerotiorum* compared with that of the control group ($P < 0.05$). The OA contents of *S. sclerotiorum* treated with carvacrol and the control group were 133.78 μg/mL and 176.01 μg/mL, respectively. *S. sclerotiorum* is a highly destructive plant phytopathogenic fungus with a broad host range and is considered an aggressive pathogen by OA that rapidly kills host cells and tissues. It uses CWDEs to destroy plant tissues further during infection. OA plays an indispensable role in the pathogenicity of the dicot pathogen *S. sclerotiorum*, and higher OA production is associated with increased aggressiveness and vice versa (Gill et al. 2021). Wang et al. (2017) found that propamidine exhibits strong antifungal activity against *S. sclerotiorum in vitro*, which causes significant decreases in sclerotia production, cell membrane permeability and OA content. In this research, treatment with carvacrol at the EC_{50} significantly decreased the OA content compared to the control. Therefore, carvacrol has potential applications in the control of *S. sclerotiorum*.

Effect on the total protein content and CWDEs. He et al. (2018) reported that the integrity of the *Colletotrichum acutatum* cell membrane is broken by cinnamon EOs, which causes the outflow of proteins, sugars and nucleic acids, and these EOs could be used to control *Rhizopus* rot on strawberry and peach fruits. Zhang et al. (2022) found that *Cymbopogon flexuosus* EOs and their main components can cause a loss of protein from *Fusarium avenaceum* mycelia *in vitro*. In this study, treatment with carvacrol at the EC_{50} significantly decreased the protein content of *S. sclerotiorum* mycelia compared with that of the control. The same results were reported by Abdelhamid and Yousef (2021), who demonstrated that carvacrol induces leakage of intracellular potassium ions, proteins and nucleic acids. As shown in Figure 3, the total protein content of *Sclerotinia sclerotiorum* mycelia in the carvacrol treatment group was significantly less than that in the control group ($P < 0.05$). The total protein contents of *S. sclerotiorum* in the carvacrol and control groups were 15.67 μg/mL and 24.88 μg/mL, respectively.

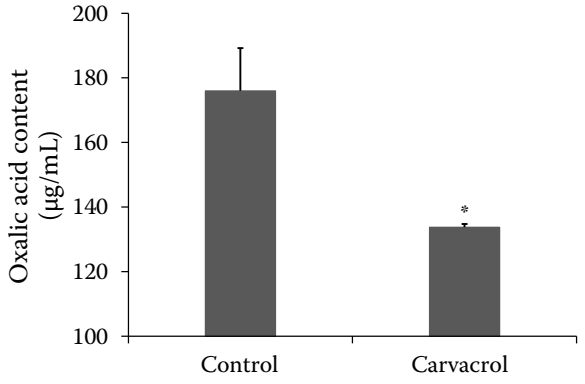

Figure 2. Effects of carvacrol on the oxalic acid content of *Sclerotinia sclerotiorummorphology* of *S. sclerotiorum* treated with carvacrol
*$P < 0.05$

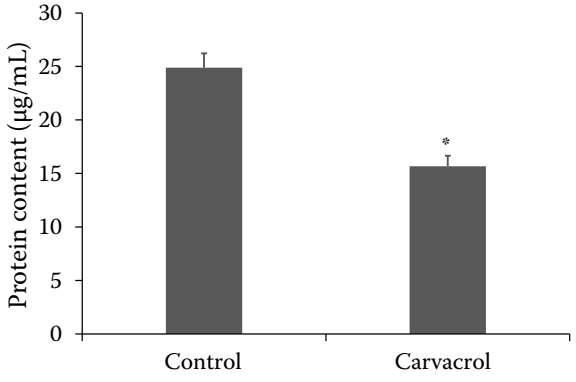

Figure 3. Effects of carvacrol on the protein content of *Sclerotinia sclerotiorum*
*$P < 0.05$

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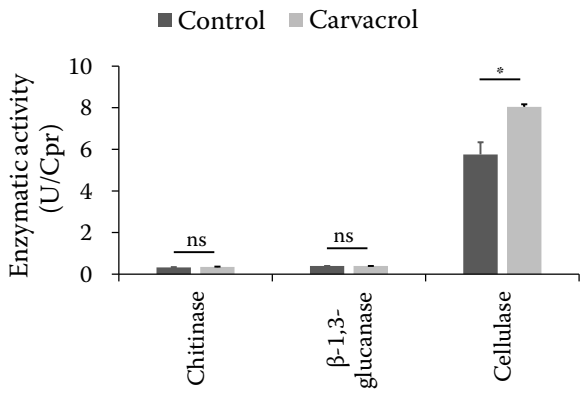

Figure 4. Effects of carvacrol on the activities of chitinase, β-1,3-glucanase and cellulose of *Sclerotinia sclerotiorum*
*$P < 0.05$

Most pathogenic fungi can degrade host cell walls by secreting various CWDEs, including chitinase β-1,3-glucanase, cellulase and other hydrolases, to obtain nutrients for growth and reproduction. After treatment with carvacrol for 24 h, the activities of chitinase, β-1,3-glucanase and cellulase of *S. sclerotiorum* were evaluated, and the results are shown in Figure 4. The activities of chitinase and β-1,3-glucanase did not significantly differ between the carvacrol-treated and control groups ($P > 0.05$). Compared with that of the control group, cellulase activity in the carvacrol treatment group was significantly increased ($P < 0.05$).

CWDEs play an important role in promoting pathogenesis and causing disease symptoms, which cause the degradation of wax, cuticles, and cell walls, tissue invasion and pathogen dissemination (Liu et al. 2005). Phytopathogenic fungi produce enzymes that focus on the deconstruction of plant cell wall components, i.e., cellulase, hemicellulase, and pectin, and these enzymes include chitinase, β-1,3-glucanase and cellulase (Su et al. 2021). The EOs of *Mentha piperita* and *Bunium persicum* have a greater effect on reducing the cellulase activity of *Rhizoctonia solani* and *Macrophomina phaseolina* (Khaledi et al. 2015). The ability to produce chitinases and β-1,3-glucanases is critical for the mycoparasitic process (Viterbo et al. 2002). In addition, cellulase may aid the penetration of the fungus into host cells (Bateman et al. 1964). Therefore, these enzymes are very important in biological control, reducing or weakening the harm induced by the fungus to host plants by controlling the activity of these enzymes. In this study, cellulase activity in the carvacrol treatment group was significantly higher than in the control group; however, the activities of chitinase and β-1,3-glucanase were not significantly different. Enzymes and OA suppress host defences during the pathogenic phase; therefore, inhibiting the activity of CWDEs is an effective method against the virulence of these fungi. However, the cellulase activity of *S. sclerotiorum* was enhanced by treatment with carvacrol in this study. We speculated that the growth of *S. sclerotiorum* decreases due to the action of EOs, which produces more nutrients for growth by increasing their activity due to a reduction in the amount of OA. Therefore, based on our results, further tests are needed to investigate the cause.

CONCLUSION

Our study investigated in vitro antifungal activity of nine monoterpenes against *S. sclerotiorum*. Our results found that carvacrol showed very strong toxicity to *S. sclerotiorum.* The mycelial surface of *S. sclerotiorum* treated with carvacrol was shrivelled, uneven and broken, with cytoplasm flowing out. Then, the OA and total protein contents, as well as the activity of cell wall-degrading enzymes, were assessed. In short, carvacrol could effectively inhibit *S. sclerotiorum*, and have the potential to be developed as a botanical fungicide.

Declaration of competing interest

The authors declare that they have no conflicts of interest or personal relationships that could have appeared to influence the work reported in this paper.

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