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Comparative antifungal activities and biochemical effects of monoterpenes on plant pathogenic fungi

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ABSTRACT

The antifungal activity of twelve monoterpenes, camphene, (*R*)-camphor, (*R*)-carvone, 1,8-cineole, cuminaldehyde, (*S*)-fenchone, geraniol, (*S*)-limonene, (*R*)-linalool, (*1R,2S,5R*)-menthol, myrcene and thymol was evaluated against four plant pathogenic fungi *Rhizoctonia solani*, *Fusarium oxysporum*, *Penecillium digitatum* and *Asperigallus niger* by using mycelial growth inhibitory technique. (*S*)-limonene and thymol were examined for their inhibitory effects on pectin methyl esterase (PME), cellulase and polyphenol oxidase (PPO) of tested fungi. Thymol was the most potent antifungal compound against the four test fungi with EC_{50} values of 33.50, 50.35, 20.14 and 23.80 mg/L on *R. solani*, *F. oxysporum*, *P. digitatum* and *A. niger*, respectively. The antifungal activity of thymol was comparable to a reference fungicide, carbendazim. (*S*)-limonene and 1,8-cineole exhibited pronounced antifungal activity against the four tested fungi. The most effective antifungal compounds thymol and (*S*)-limonene showed strong inhibitory effect on the activity of PME and cellulase but revealed no inhibitory effect on PPO. The results showed that PME was more sensitive than cellulase to thymol and (*S*)-limonene. This is the first report on the inhibitory effects of monoterpenes thymol and (*S*)-limonene on PME, cellulase and PPO. The results indicated that monoterpenes may cause their antifungal activity by inhibiting PME and cellulase. The strong antifungal activity of thymol, (*S*)-limonene and 1,8-cineole reported in this study indicated that these compounds have a potential to be used as fungicides.

1. Introduction

For many years, synthetic fungicides have been used for control plant pathogenic fungi. However, the extensive used of these chemicals led to the development of resistance in many areas around the world [1]. In order to overcome this problem, higher concentrations of these chemicals were used, but this increases the risk of high-level toxic residues in the products. Thus, there has been a growing interest on the research of the possible use of plant secondary metabolites for pest and disease control in agriculture [2]. The plants have long been recognized to provide a potential source of different class of chemical compounds, known as phytochemicals, such as terpenoids, alkaloids, phenolics, glucosides, etc., which are effective products for pest control. Essential oils and their major constituents, monoterpenes, are among the most promising classes of natural products that can be used as safer pest and disease control agents.

Monoterpenes are the main constituents in the majority of plant essential oils and give plants their unique odoriferous properties because of their low boiling points. They are biosynthesized from geranyl pyrophosphate, the ubiquitous acyclic C_{10} intermediate of the isoprenoid pathway [3]. Monoterpenes can be classified into two major groups: monoterpene hydrocarbons and oxygenated monoterpenes. The latter group includes alcohols, aldehydes, ketones, ethers and acids [4]. The natural pesticidal properties of some monoterpenes make them useful as potential alternative pest control agents as well as good lead compounds for the development of safe, effective, and fully biodegradable pesticides. Monoterpenes possess many pesticidal activities, including insecticidal [5,6] herbicidal [7,8], fungicidal [9,10], bactericidal [11,12] properties.

As part of our ongoing search for new antifungal compounds, twelve monoterpenes camphene, (*R*)-camphor, (*R*)-carvone, 1,8-cineole, cuminaldehyde, (*S*)-fenchone, geraniol, (*S*)-limonene, (*R*)-linalool, (*1R,2S,5R*)-menthol, myrcene and thymol were evaluated for their antifungal activity against four plant pathogenic fungi *Rhizoctonia solani* and *Fusarium oxysporum*, *Penecillium digitatum* and *Asperigallus niger*, which cause damping-off, vascular mold, green mold and black mold, respectively. Some of these monoterpenes have been reported to possess antifungal activity against plant pathogenic fungi. For example, Garcia et al. [13] demonstrated that L-carvone strongly inhibited the growth of post-harvest fungi *Colletotrichum musae*, *Colletotrichum gloeosporioides* and *Fusarium*

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subglutinans f.sp. *ananas*. Similarly, geraniol showed fungistatic and fungicidal effect against *P. digitatum*, *Penicillium italicum* and *Geotrichum candidum* [9]. It has been reported that thymol completely inhibited mycelial growth of 17 phytopathogenic fungi, including *R. solani* and *F. oxysporum* [14]. Carvone had a potential to control potato sprout and it had promising antifungal activity against other potato storage diseases *Fusarium sulphureum, Phoma exigua* var. *foveata* and *Helminthosporium solani* [15]. Kordali et al. [16] evaluated the antifungal activities of some oxygenated monoterpenes, including camphor, carvone, 1,8-cineole, fenchone, geraniol, linalool, linalool and menthol against 31 plant pathogen fungi. They stated that some of monoterpenes had potent inhibitory effects against most of the tested fungal species.

In general, the inhibitory action of monoterpenes on microorganism cells involves cytoplasm granulation, cytoplasmic membrane rupturing and inactivation and/or synthesis inhibition of intracellular and extracellular enzymes [17]. However, there are no reports available in the literature on the inhibitory effects of monoterpenes on specific enzymes such as pectin methyl esterase (PME), cellulase and polyphenol oxidase (PPO). It is also noted that most of monoterpenes evaluated against fungi at single and/or double concentrations without calculating the toxicity factor (EC_{50} values). Therefore, the aim of this study was to determine the comparative antifungal activity of twelve monoterpenes against four plant pathogenic fungi and to evaluate the inhibitory effect of these compounds on PME, cellulase and PPO to explore their possible mode of action.

2. Materials and methods

2.1. Chemicals

Twelve monoterpenes, camphene (95%), (*R*)-camphor (98%), (*R*)-carvone (98%), 1,8-cineole (99%), cuminaldehyde (98%), (*S*)-fenchone (98%), geraniol (98%), (*S*)-limonene (96%), (*R*)-linalool (95%), (*1R,2S,5R*)-menthol (98%), myrcene (90%) and thymol (98%) were purchased from Sigma–Aldrich Chemical Co., Steinheim, Germany. Chemical structures of these monoterpenes are shown in Fig. 1. Carbendazim (95%) was supplied by Kafr El-Zayat Pesticides and Chemicals Co. and used as a reference fungicide. All chemicals were of highest grade commercially available.

2.2. Fungi

Four plant pathogenic fungi species used, *R. solani* (Kuhn, isolated from *Phaseolus vulgaris*), *F. oxysporum* (Schltdl., isolated from *Zea mays* seeds), *P. digitatum* (Pers., isolated from *Citrus sinensis*) and *A. niger* (Tiegh, isolated from *Solanum melogena*), were obtained from the Fungicide Bioassay Laboratory, Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University. The fungi were maintained during the course the experiments on potato dextrose agar medium (PDA: potato 200, dextrose 20 and agar 15 $g l^{-1}$ in distilled water) at 25 °C.

2.3. In vitro antifungal assay

The antifungal activity of the twelve monoterpenes was tested using radial growth technique [18,19]. Appropriate volumes of the stock solutions of the monoterpenes in dimethyl sulfoxide (DMSO) were added to PDA medium immediately before it was poured into the Petri dishes (9.0 cm diameter) at 40–45 °C to obtain a series of concentrations (2.5, 5, 10, 25, 50, 100, 200, 300, 400, 500 mg/L) Each concentration was tested in triplicate. Parallel controls were maintained with DMSO mixed with PDA. The discs of mycelial felt (0.5 cm diameter) of the plant pathogenic fungi, taken from 8-day-old cultures on PDA plates, were transferred aseptically to the centre of Petri dishes. Carbendazim was used as reference fungicide. The treatments were incubated at 27 °C in the dark. Colony growth diameter was measured after the fungal growth in the control treatments had completely covered the Petri dishes. Percentage of mycelial growth inhibition was calculated from the formula: Mycelial growth inhibition = [(DC-DT)/DC] × 100 [20], where DC and DT are average diameters of fungal colony of control and treatment, respectively. The concentration of the monoterpene that inhibiting the fungi mycelial growth by 50% (EC_{50}) was determined by a linear regression method [21].

2.4. Pectin methyl esterase (PME) activity assay

The tested fungi were grown on PDA medium supplied with 1% pectin (apple pectin apipecin 150 SAG). The pH of the medium was adjusted at 7.0 by using 0.05 N sodium hydroxide solution and autoclaved for 15 min. The autoclaved medium was readjusted to pH 7.0 with the sterile 0.05 N sodium hydroxide and then inoculated with fungi. After 8 days of incubation at 27 °C, the medium was filtrated through Whatman No.1 paper. The filtrate was used as a source of crude pectin methyl esterase enzyme. The activity of PME was measured according to the method described by Talboys and Busch [22] with some modifications. To 7 ml of reaction mixture (pectin (0.5 g), sodium chloride (0.58 g) bromothymol blue solution (0.05%) (2.5 ml), chloroform (4 ml) and distilled water up to 1000 ml, pH 7), 2.0 ml of crude enzyme and 1 ml of tested monoterpene were added. The monoterpenes were tested at final concentrations of 0.5, 1, 5, 10, 50, 100, 250 and 500 mg/L. The treatments were incubated at 30 °C for 24 h, and then titrated to pH 7.0 with sodium hydroxide (0.01 N). Treatment without monoterpene and treatment without crude enzyme were prepared and served as control and blank. Each treatment was replicated three times. The Inhibition percentage of the PME activity was calculated from the equation: $I\ (\%) = [(A - B)/A] \times 100$, where A is a volume (ml) of NaOH (0.01 N) in control treatment and B is a volume (ml) of NaOH (0.01 N) in treatment. I_{50} (concentration of monoterpene required to cause a 50% inhibition of enzymatic activity) values were determined by a linear regression method [21].

2.5. Cellulase activity assay

The crude cellulase enzyme was obtained from fungi cultures grown on PDA medium amended with 3% of carboxymethyl cellulose for 12 days at 27 °C. The crude enzyme (1 ml) was added to citrate buffer, pH 4.8 (2 ml) and the mixture was warmed in a water bath at 50 °C for 30 min. Then 1 ml of the tested monoterpenes was added and incubated at 28 °C for 24 h. The monoterpenes were tested at concentrations of 1, 5, 10, 50, 100, 250 and 500, 750 and 1000 mg/L. Then 3 ml of the reaction mixture (3,5-dinitrosalicylic acid (10 g), sodium hydroxide (10 g), phenol (20 ml), sodium sulfate (0.5 g) and distilled water up to 1000 ml) was added. Three replicates of each treatment, control and blank (without enzyme) were prepared. After incubation for 15 min at 50 °C in a water bath, the absorbance was measured at 575 nm. The inhibition percentage of cellulase activity was calculated from the equation: $I\ (\%) = [(Ac - At)/Ac] \times 100$, where Ac is the absorbance in control and At is the absorbance in treatment.

2.6. Polyphenol oxidase (PPO) activity assay

Determination of polyphenol oxidase activity was carried out according to the method described by Broesh [23]. The PDA medium containing the 10, 50, 100, 250, 500, 750 and 1000 mg/L of the tested monotepenes was prepared in 100 ml conical flasks. The discs of the tested fungi were placed on the surface of the

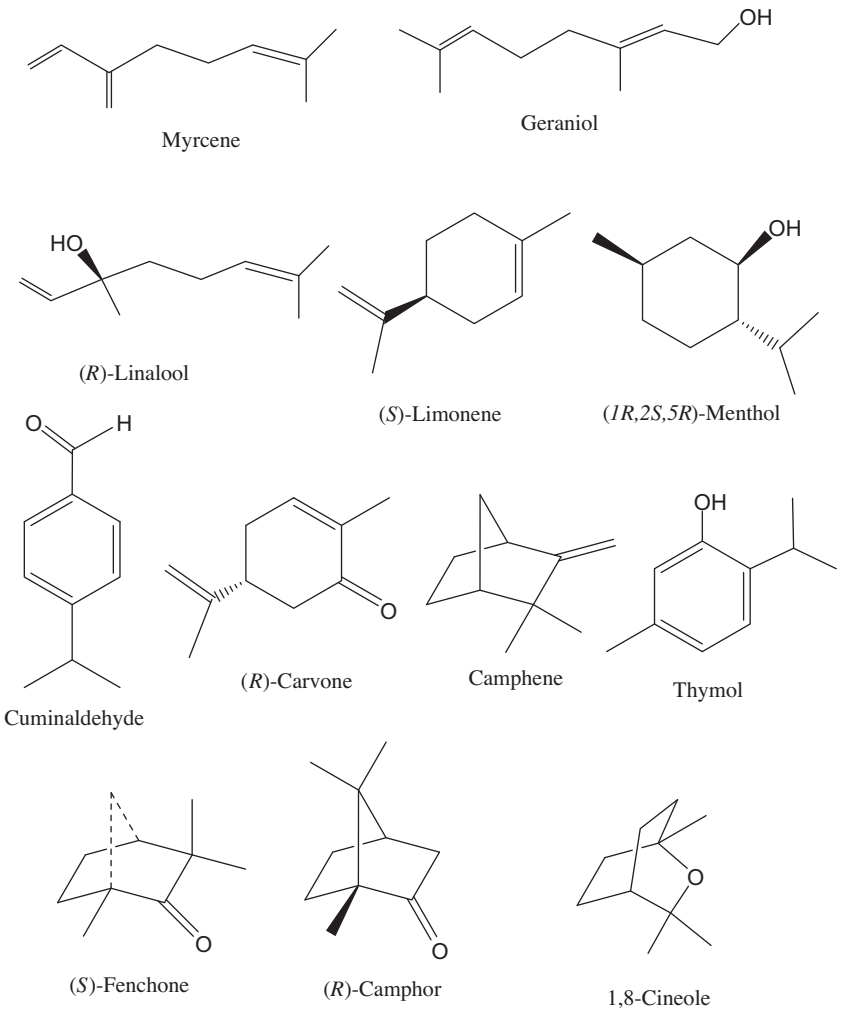

Fig. 1. The chemical structures of the tested monoterpenes.

medium and incubated until the hyphal growth in untreated flasks (control) was completed. Then the medium was filtrated under vacuum. The filtrate was centrifuged for 15 min at 4000 rpm. The supernatants (PPO source) (1 ml) were added to the reaction mixture (2.0 ml of borate buffer (pH 9.0), 1.0 ml of 1% p-aminobenzoic acid (alcoholic solution) and 2.0 ml of 1% catechol). Enzyme activity was measured as optical density at 575 nm after one-hour of incubation in water bath at 45 °C. The inhibition percentage of PPO activity was calculated from the equation: $I\ (\%) = [(Ac - At)/Ac] \times 100$, where *Ac* is the absorbance in control and *At* is the absorbance in treatment.

2.7. Statistical analysis

The concentration–response data were subjected to Probit analysis [21] to obtain the EC_{50} and IC_{50} values using the SPSS 12.0 software program (Statistical Package for Social Sciences, USA). The values of EC_{50} and IC_{50} were considered to be significantly different, if the 95% confidence limits did not overlap.

3. Results and discussion

3.1. Antifungal activity of monoterpenes against plant pathogenic fungi

The inhibitory effects of twelve monoterpenes, including three monoterpene hydrocarbons and nine oxygenated monoterpenes were evaluated against four plant pathogenic fungi, *R. solani*, *F. oxysporum*, *P. digitatum* and *A. niger*. The results show that the monoterpenes exhibited various mycelial growth inhibitions against the tested fungi depending on fungi species and tested compounds. Thymol revealed the highest inhibitory effect against *R. solani* with EC_{50} value of 33.50 mg/L followed by (*S*)-limonene, (*R*)-linalool and 1,8-cineole, while geraniol and (*S*)-fenchone were the less effective compounds (Table 1). In general, all of the tested compounds showed pronounced antifungal activity against *R. solani* as the EC_{50} values ranged between 33.50 and 357.0 mg/L. There was no significant difference between inhibitory effects of thymol and the reference fungicide, carbendazim. Similarly, thymol was the most potent inhibitor aganist *F. oxysporum* but (*R*)-carvone and (*1R,2S,5R*)-menthol were the least effective (Table 1). Among the tested monoterpenes, thymol, (*S*)-limonene and 1,8-cineole exhibited promising antifungal activity against *P. digitatum* and *A. niger* (Table 2). Although, all of the tested monoterpenes showed less inhibitory effects than carbendazim, no significant differences were observed between thymol and the fungicide against four tested fungi.

It has been reported that some monoterpenes possess antifungal potential against plant pathogenic fungi [9,13,14,16,24–28]. As can be seen from our results thymol, (*S*)-limonene and 1,8-cineole were the most potent antifungal compounds against the four tested fungi and the activity of thymol was parallel to the commercial carbendazim. These findings are in agreement with the results of previous studies stated that thymol was the

Table 1
Comparative antifungal activity of monoterpenes against *Rizoctonia solani* and *Fusarium oxysporium*.

Monoterpene	*Rizoctonia solani*				*Fusarium oxysporium*			
	EC_{50}[a] (mg/l)	95% Confidence limits		Slope ± S.E.[b]	EC_{50} (mg/l)	95% Confidence limits		Slope ± S.E.
		Lower	Upper			Lower	Upper	
Camphene	140.8	110.9	176.1	1.04 ± 0.08	314.2	252.3	397.8	1.07 ± 0.09
(*R*)-Camphor	83.49	42.36	138.8	0.97 ± 0.08	157.1	122.8	199.0	0.97 ± 0.08
(*R*)-Carvone	274.0	114.1	767.7	1.29 ± 0.10	432.5	356.5	536.4	1.28 ± 0.11
1,8-Cineole	77.73	26.30	154.8	0.99 ± 0.08	148.4	94.17	224.5	0.95 ± 0.08
Cuminaldehyde	79.47	27.46	158.8	1.04 ± 0.08	363.5	73.4	1087	1.63 ± 0.17
(*S*)-Fenchone	298.8	182.9	530.7	1.05 ± 0.08	275.9	202.3	388.8	0.72 ± 0.08
Geraniol	357.0	181.1	940.0	1.00 ± 0.09	168.3	101.7	269.2	1.04 ± 0.09
(*S*)-Limonene	72.98	17.53	165.1	0.95 ± 0.08	153.2	120.6	192.5	1.01 ± 0.08
(*R*)-Linalool	73.68	37.49	121.0	0.91 ± 0.08	192.7	154.6	239.5	1.08 ± 0.09
(*1R,2S,5R*)-Menthol	121.9	64.04	207.8	1.09 ± 0.08	394.4	311.5	515.3	1.01 ± 0.09
Myrcene	130.3	62.18	238.4	1.21 ± 0.09	336.9	198.2	658.1	1.03 ± 0.09
Thymol	33.50	9.34	66.68	1.13 ± 0.99	50.37	18.51	94.05	1.12 ± 0.09
Carbendazim	25.14	13.04	60.47	0.92 ± 0.09	37.98	27.73	55.59	0.94 ± 0.10

[a] The concentration causing 50% mycelial growth inhibition.
[b] Slope of the concentration-inhibition regression line.

Table 2
Comparative antifungal activity of monoterpenes against *Penecillium digitatum* and *Asperigallus niger*.

Monoterpene	*Penecillium digitatum*				*Asperigallus niger*			
	EC_{50}[a] (mg/l)	95% Confidence limits		Slope ± S.E.[b]	EC_{50} (mg/l)	95% Confidence limits		Slope ± S.E.
		Lower	Upper			Lower	Upper	
Camphene	244.2	197.8	302.8	1.12 ± 0.09	121.5	60.34	232.6	0.97 ± 0.09
(*R*)-Camphor	367.0	207.2	801.4	0.92 ± 0.09	231.0	184.5	290.6	1.04 ± 0.09
(*R*)-Carvone	418.0	243.7	890.7	1.14 ± 0.10	120.0	56.50	218.8	1.26 ± 0.09
1,8-Cineole	51.61	12.27	110.32	0.87 ± 0.08	36.40	22.90	51.96	0.79 ± 0.08
Cuminaldehyde	157.8	77.26	292.5	1.35 ± 0.09	193.2	94.58	384.5	1.20 ± 0.09
(*S*)-Fenchone	330.6	203.7	603.2	0.89 ± 0.08	193.8	98.15	389.3	0.71 ± 0.08
Geraniol	73.86	30.49	134.8	1.10 ± 0.09	128.7	77.77	199.0	0.96 ± 0.08
(*S*)-Limonene	26.83	7.30	54.03	0.92 ± 0.08	38.04	9.52	78.84	0.89 ± 0.08
(*R*)-Linalool	136.7	53.23	291.2	1.25 ± 0.10	266.6	211.7	339.7	1.00 ± 0.09
(*1R,2S,5R*)-Menthol	213.9	113.8	414.7	0.97 ± 0.08	204.6	76.71	543.9	1.18 ± 0.09
Myrcene	95.46	34.37	195.6	1.19 ± 0.09	98.50	37.26	196.6	1.06 ± 0.084
Thymol	20.14	0.06	54.06	1.21 ± 0.14	23.80	1.61	58.07	1.17 ± 0.12
Carbendazim	13.63	10.61	17.53	1.15 ± 0.10	18.61	13.92	25.38	0.95 ± 0.09

[a] The concentration causing 50% mycelial growth inhibition.
[b] Slope of the concentration-inhibition regression line.

most potent monoterpenes against plant pathogenic fungi [14,29–31].

3.2. Inhibitory effect of thymol and (S)-limonene on pectin methyl esterase, cellulase and polyphenol oxidase enzymes

The results of antifungal activity revealed that thymol and (*S*)-limonene were the most potent compounds against the four tested fungi. Therefore, their inhibitory effect on pectin methyl esterase (PME), cellulase and polyphenol oxidase (PPO) was evaluated to explore the possible mode of action of these compounds. The results showed that thymol had pronounced inhibitory effect on cellulase activity isolated from four tested fungi (Table 3). The highest inhibitory activity was observed with enzyme isolated from *P. digitatum* (Pers.) (IC_{50} = 6.08 mg/L). Also, thymol exhibited strong inhibition of PME isolated from the tested fungi, particularly the enzyme of *A. niger* with IC_{50} value of 1.28 mg/L. This compound had no inhibitory effect on PPO isolated from *R. solani, F. oxysporum* and *A. niger* and weak inhibitory effect against PPO of *P. digitatum*.

On the other hand, (*S*)-limonene caused remarkable inhibition of cellulase activity of *A. niger* and *P. digitatum* (Table 3). Similarly, it showed strong inhibitory effect on PME activity of the four tested fungi. However, the enzyme isolated from *P. digitatum* was the most sensitive (IC_{50} = 19.22 mg/L). (*S*)-Limonene revealed weak inhibitory effect on PPO of *P. digitatum* while it had no inhibitory effect on the enzyme of *R. solani, F. oxysporum* and *A. niger*. In general, PME was more sensitive than cellulase, while PPO was the least sensitive to thymol and (*S*)-limonene.

The mechanisms of antifungal action of monoterpenes are not fully understood. However, several studies concluded that, as lipophilic agents, they execute their action at the level of the membrane and membrane embedded enzymes [32,33]. It has been reported that these compounds caused their action due to a change in the fatty acid composition of cell membrane [34]. It is also stated that the activity of monterpemes was attributed to their interactions with cellular membranes. These interactions may result in changes such as inhibition of respiration and alteration in permeability [35]. Consistent with these findings, our results indicated that thymol and (*S*)-limonene are potent inhibitors of PME. This enzyme modifies the degree of methylesterification of pectins, which are major components of fungi cell walls. Such changes in pectin structure are associated with changes in cellular adhesion, plasticity, pH and ionic contents of the cell wall and influence fungi development, membrane integrity and permeability. To the best of our knowledge, this is the first report on the inhibitory effect of monoterpenes on PME. On the other hand, fungi produce cellulase to degrade cell walls during pathogenesis and inhibition of this enzyme ultimately affects the disease development [36]. It has been reported that the

Table 3
Inhibitory effects of thymol and (*S*)-limonene on PPO, PME and cellulase activities.

Enzyme	Fungus	Thymol				(*S*)-Limonene			
		I_{50}[a] (mg/l)	95% Confidence limits		Slope ± S.E.[b]	I_{50} (mg/l)	95% Confidence limits		Slope ± S.E.
			Lower	Upper			Lower	Upper	
Cellulase	*R. solani*	332.7	252.2	458.4	1.06 ± 0.10	228.4	163.5	334.2	0.81 ± 0.09
	F. oxysporium	535.8	338.8	1018	0.66 ± 0.09	666.7	472.3	1047	0.98 ± 0.11
	P. digitatum	6.08	2.74	10.29	0.95 ± 0.12	80.67	48.76	125.8	0.59 ± 0.08
	A. niger	44.56	30.37	61.39	0.90 ± 0.09	54.62	34.80	79.50	0.73 ± 0.09
PME	*R. solani*	17.18	9.66	26.51	0.84 ± 0.10	61.58	40.99	87.45	0.77 ± 0.09
	F. oxysporium	140.0	110.7	177.2	1.24 ± 0.10	112.2	75.24	165.7	0.69 ± 0.08
	P. digitatum	64.68	45.48	88.35	0.88 ± 0.09	19.22	10.53	29.69	0.78 ± 0.09
	A. niger	1.28	0.10	4.33	0.52 ± 0.10	126.99	94.93	169.8	0.96 ± 0.09
PPO	*R. solani*	>1000				>1000			
	F. oxysporium	>1000				>1000			
	P. digitatum	414.6	239.3	932.8	0.51 ± 0.08	389.0	259.5	657.7	0.71 ± 0.09
	A. niger	>1000				>1000			

[a] The concentration causing 50% enzyme inhibition.
[b] Slope of the concentration-inhibition regression line.

production and activity of cellulase inhibited by commercial fungicides [37–39]. In this study, thymol and (*S*)-limonene caused strong inhibitory effect of cellulase activity. It can be concluded that monoterpenes cause their antifungal activity through previously reported mechanisms of action and the inhibition of PME and cellulase. This also can be confirmed from the positive correlation between the antifungal activity in terms of EC_{50} values and the inhibitory effect of enzymes in terms of IC_{50}.

With regard to the structure-antifungal relationship, thymol (an aromatic alcohol) was the most potent compounds against the four tested fungi. Both (*S*)-limonene (a monocyclic monoterpene hydrocarbon) and 1,8-cineole (an ether-containing monoterpene) showed promising activity. In contrast, (*R*)-carvone and (*R*)-camphor (both ketones) were among the less effective compounds. Surprisingly, (*1R,2S,5R*)-menthol, a non-aromatic alcohol, showed a very weak antifungal activity. Potent antifungal properties of thymol against various plant pathogens were also previously documented [30,31].

In summary, the results of this study revealed that three monoterpenes (thymol, (*S*)-limonene and 1,8-cineole) are potent antifungal compounds and can be used for control plant pathogenic fungi. The strong inhibitory effect of thymol and (*S*)-limonene on PME and cellulase indicated that these two enzymes are targets for monoterpenes.

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