

Effect of some essential oils on mycelial growth of *Penicillium digitatum* Sacc.

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Abstract The antifungal action of four essential oils of *Foeniculum vulgare* (fennel), *Thymus vulgaris* (thyme), *Eugenia caryophyllata* (Clove) and *Salvia officinalis* (sage) was tested in vitro against *Penicillium digitatum* Sacc. Direct contact and vapour phase were used to test the antifungal activity of these essential oils against *P. digitatum* that is responsible for green mould rot of citrus fruits. The vapour phase and direct contact of clove and thyme essential oils exhibited the strongest toxicity and totally inhibited the mycelial growth of the test fungus. Thyme and clove essential oils completely inhibited *P. digitatum* growth either when added into the medium $600 \mu\text{l l}^{-1}$ or by their volatiles with $24 \mu\text{l}$ per 8 cm diameter Petri dish. In in vitro mycelial growth assay showed fungistatic and fungicidal activity by clove and thyme essential oils. Sage and fennel oils did not show any inhibitory activity on this fungus. Scanning electron microscopy (SEM) was done to study the mode of action of clove oil in *P. digitatum* and it was observed that treatment with the oil leads to large alterations in hyphal morphology.

Keywords Antifungal activity · Chemical analysis · Essential oils · *Penicillium digitatum* · Scanning electron microscopy

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Abbreviations

O _s O ₄	Osmium tetroxide
PDA	Potato dextrose agar
PBS	Phosphate-buffer saline (pH 7.4)
EO	Essential oil
GC	Gas chromatography
SEM	Scanning electron microscopy
GC-MS	Gas chromatography combined with mass spectrometry
ANOVA	Analysis of variance
LSD	Least significant difference
RI	Retention indices

Introduction

Postharvest diseases cause serious losses to fruits, vegetables and other plant products during transit and storage. Citrus fruits are produced in subtropical regions distant from consumer markets and often must be stored for the market economy as dictates. Months-long delay between harvest and consumption may result in postharvest losses due to pathological and physiological diseases (Scora and Scora 1998). Green mould (*Penicillium digitatum* Sacc.) is one of the predominant pathogens on citrus fruits worldwide (Eckert and Eaks 1989). In fact, the green mould is the most important source of postharvest rots in areas with little summer rainfall (Scora and Scora 1998). This wound-obligate pathogen has a relatively short disease cycle (3–5 days at 25°C) and, on a single fruit, can generate 1–2 billion conidia, which are efficiently dispersed via air currents (Holmes and Eckert 1999). A number of fungicides such as benzimidazoles, aromatic hydrocarbons, and sterol biosynthesis inhibitors are in use as postharvest treatments to

control the pathogen. A serious problem in the effective use of these chemicals is the development of resistance by *P. digitatum*. The application of higher concentrations of chemicals to control the resistant strains, if possible, increases the hazard of high levels of toxic residues in products (Daferera et al. 2000). Thus, in the past few years, research has focused on the use of natural preservatives.

Aromatic plants generate volatile C₁₀ and C₁₆ terpenes, which are derived from isoprene unit. These substances, which are known as essential oils (EO), can be isolated from plants by system distillation or other modified methods. A broad variety of terpene hydrocarbons, cyclic or non-cyclic, and their oxygenated isoprenoid compounds are present in EO as components (Daferera et al. 2000).

In the past few decades, several studies have investigated the antifungal properties of EO against postharvest pathogens (Dixit et al. 1995; Caccioni et al. 1998; Daferera et al. 2000; Venturini et al. 2002; Obagwu and Korsten 2003; Shahi et al. 2003). The EOs have two key advantages; they are organic natural substances which denotes both public health safety and environment friendliness, and there is low risk of resistance development by postharvest pathogens due to the mixture of oil components with apparently different antifungal activity mechanisms.

The aim of the present study is to determine the inhibitory effects of four EO on the growth of *P. digitatum*.

Materials and methods

Essential oils were provided from Zardband Pharmaceutical Co. (Tehran, Iran).

Oil analyses

Gas chromatography (GC) analysis: GC analysis was performed using a Shimadzu GC-9A gas chromatograph equipped with a DB-5 fused silica column (30 m × 0.25 mm, film thickness 0.25 μm). Oven temperature was maintained at 40°C for 5 min and then programmed to 220°C at a rate of 4°C/min. Injector and detector (FID) temperature was 240°C; helium was used as carrier gas with a linear velocity of 32 cm/s. Percentages were calculated by electronic integration of FID peak areas without the use of response factor correction.

Gas chromatography/mass spectrometry (GC-MS) analysis: The analysis was carried out using a Hewlett-Packard gas chromatography HP 5973 Series II equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm, film thickness 0.25 μm) and DB 5973 mass selective detector. Injector and detector temperatures were 250 and 260°C, respectively. The oven temperature was programmed from

60 to 220°C at a rate of 6°C/min. Helium gas was employed as the carrier gas at 1 ml/min flow rate; source 70 eV.

EO samples were diluted in normal hexane and 0.1 μl was injected into the oven.

Identification of components: The components of the oils were identified by comparison of their mass spectra with those of a computer library or with authentic compounds and confirmed by comparison of their retention indices either with those of authentic compounds or with data published in the literature (Stenhagen et al. 1974; Adams 2001). Mass spectra from the literature was also compared (Shibamoto 1987; Davies 1990).

Pathogen

Isolate 6B-1 of *Penicillium digitatum* that was most pathogenic out of ten isolates obtained from decaying orange fruits in the north of Iran was selected for experiments. A 7–14-day old culture of the isolate on potato dextrose agar (PDA) at 25°C was used as conidium source. Conidia were scrapped from the agar then suspended in distilled water with 0.001% Tween-80 (v/v) and its concentration was adjusted to 10⁶ conidia ml⁻¹ using a haemocytometer.

Antifungal activity of essential oils

The inhibitory effects of the four EO were tested in vitro on mycelial growth of *P. digitatum*. In effect, two methods were used to test the antifungal activity of these EO. The agar dilution method consisted of adding different amounts of pure EO dissolved in absolute ethyl alcohol, 5% Tween-20 (v/v), to PDA before pouring it into Petri dishes (8 cm diameter) when the medium was still warm (40–45°C), to obtain concentrations of 200, 400 or 600 μl l⁻¹. A 5 mm diameter disc of inoculum of the test fungus with a cork-borer, cut from the periphery of an actively growing culture on PDA plates, was placed at the center in each Petri plate. Controls consist of 200, 400 and 600 μl l⁻¹ of ethyl alcohol + 5% Tween-20 mixed with PDA. Then the colony radius was measured after 3 days at 25°C. The percentage of inhibition of mycelial growth was calculated from mean values of colony diameter in treated and control Petri dishes. Transfer onto fresh PDA Petri dishes confirmed the fungistatic or fungicidal activity of each EO, and mycelial growth was monitored for the next 7 days at 25°C.

The volatile activity of the EO was assayed by inoculating 10 μl of conidial (spore) suspension (10⁶ conidia ml⁻¹) at the center of a PDA Petri dish (8 cm diameter). Sterile filter paper discs (7 cm diameter) soaked in 3, 6, 12 or 24 μl pure EO were placed on the inner surface of the Petri dish lid. The dishes were sealed with parafilm and

incubated upside-down at 25°C. Colony radius (in cm) was measured after 7 days. When complete inhibition of mycelial growth occurred, the lids were removed and replaced with lids containing sterile distilled water-soaked filter papers, then observed for a further 7 days at 25°C.

In both types of experiments, three replicates per treatment were carried out and each experiment was repeated at least twice. Fungitoxicity was expressed as percentage inhibition of mycelial growth, according to the formula

$$MGI (\%) = [(C - T)/C] \times 100$$

where, *MGI* (%) is the percent mycelial growth inhibition, *C* the colony radius of the pathogen when growing without EO; and *T* the colony radius of the pathogen for each EO concentration.

Data analysis

Data on the inhibitory effects of EO on mycelial growth was analyzed by variance analysis (ANOVA) using SAS software. The separation of means was done by using the Least Significant Difference (LSD) test at $P \leq 0.01$.

Scanning electron microscopy

Three-day-old fungal cultures of *P. digitatum* on PDA treated with sterile filter paper discs (10 cm diameter) soaked in 150 µl of pure clove EO placed on the inner surface of the Petri dish lid. Then, the dishes were sealed with parafilm and incubated upside-down at 25°C, and negative control without oil was used for SEM to study the mode of action of the oil. The segments measuring 5–10 mm were cut at periphery of the colony from the cultures growing on the PDA plates and were promptly placed in vials containing 3% glutaraldehyde in phosphate buffer saline (PBS) (pH 7.4) at 4°C and fixed for 24 h, then were washed three times for 10 min with PBS to remove the glutaraldehyde and fixed with 1% (w/v) osmium tetroxide (O₃O₄) for 2 h at room temperature. After another three washes with PBS for 10 min each, the samples were dehydrated in an ethanol series (25%, 50%, 70%, and 95%) for 20 min in each alcohol dilution and finally with absolute ethanol for 30 min. The samples were dropped into liquid nitrogen (N₂) to freeze rapidly and then the frozen samples were transferred into freeze-drier, i.e. a special chamber designed to maintain the temperature lower than –80°C, and the apparatus was rapidly evacuated into the 10⁻¹ Pascal. Then the dried samples were placed on aluminum stubs before gold-palladium coating with a BAL-TEC S 100A sputter coater. Finally, the samples were packed in vacuum and observed under a Philips XL30

scanning electron microscope (SEM) at an accelerating voltage of 25 kV.

Results

The results of the oils analysis (only for thyme and clove oils that had highest inhibitory activity) are presented in Table 1. The oil of clove (*E. caryophyllata*) was particularly rich in eugenol (78.5%) and β-caryophyllene (13.8%). At the same time, the thyme (*Thymus vulgaris*) oil contained thymol (46%), *p*-cymene (17.6%) and δ-terpinene (14.8%).

The results obtained in assays of antifungal activity (direct contact and vapour phase methods) of the oils tested are shown in Figs. 1 and 2. From these data, it emerges that the most effective oils were those of clove and thyme, with 100% inhibition of *P. digitatum* using the two methods mentioned above.

Table 1 Essential oil composition of *Thymus vulgaris* and *Eugenia caryophyllata*

	Name of compound	RI ^a	Thyme	Clove
1	Tricyclene	922	1.2	–
2	α-Thujene	928	1.04	–
3	Camphene	942	0.66	–
4	Sabinene	969	1.81	–
5	β-Pinene	980	0.1	–
6	Myrcene	987	2.45	–
7	α-Phellandrene	1002	0.18	–
8	α-Terpinene	1012	2.11	–
9	<i>p</i> -Cymene	1018	17.69	–
10	Limonen	1025	1.21	–
11	1,8-Cineole	1027	0.39	–
12	δ-Terpinene	1051	14.87	–
13	<i>trans</i> Sabinene	1060	1.01	–
14	Linalool	1097	2.38	–
15	Borneol	1157	1.1	–
16	Berpinene-4-ol	1168	1.74	–
17	Methyl thymol	1227	0.79	–
18	Methyl carvacrol	1237	0.43	–
19	Thymol	1289	46.07	–
20	Carvacrol	1301	2.59	–
21	Eugenol	1356	–	78.53
22	α-Compaene	1376	–	0.21
23	β-Caryophyllene	1409	0.74	13.81
24	α-Humulene	1454	–	2.79
25	Eugenyl acetate	1524	–	4.37
26	Caryophyllen oxide	1580	–	0.26

^a RI, retention indices

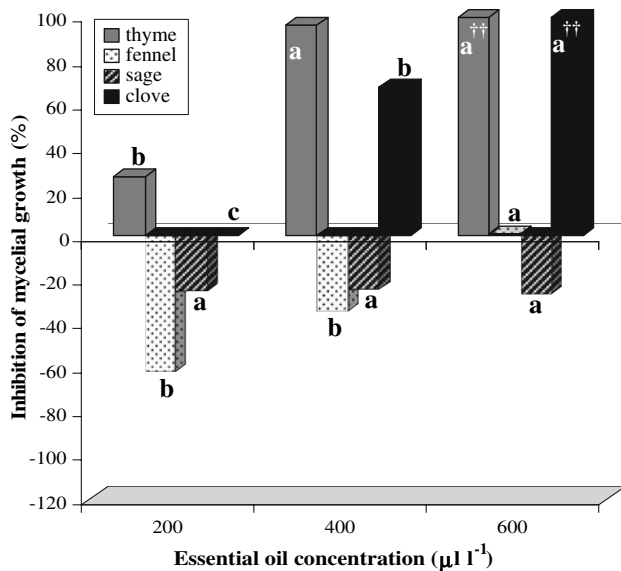


Fig. 1 Percentage inhibition of mycelial growth *Penicillium digitatum* in vitro by agar dilution method. Colony radius was measured after 3 days at 25°C. Different letters on columns in each EO indicate significant differences ($P \leq 0.01$) by least significant difference test. The columns in the negative region indicate positive effect of EO on mycelial growth. †† Fungistatic activity

The oils of clove and thyme were completely active against *P. digitatum* when added into the medium (direct contact) at 600 µl l⁻¹, but this inhibitory effect was fungistatic, not fungicidal. The suppressive effect of these EO on colony growth was gradually reduced with concentration. Sage and fennel oils had no inhibitory effect on the growth and even increased the growth of *P. digitatum*.

Clove and thyme oils in vapour phase showed the highest inhibitory activity against *P. digitatum* (Fig. 2). Inhibitory activity was highest at 24 µl for these two EO. The inhibitory activity of the clove and thyme oils of the volatile compounds were fungicidal, whereas in vapour phase, sage and fennel oils did not have inhibitory activity on *P. digitatum*, and were practically ineffective.

The results of SEM on the control showed a normal morphology and linearly shaped hyphae and the apical hyphae were tapered with a smooth surface (Fig. 3a, b). Treatment of clove oil on the *P. digitatum* caused irregular branching of hyphae in the apical part (Fig. 3c) and the loss of linearity (Fig. 3d). The effects of clove oil on the morphology of *P. digitatum* hyphae revealed alterations in the morphology of the hyphae, which appeared severely collapsed and squashed due to the lack of cytoplasm (Fig. 3e). A few small vesicles on the apical surface were observed and a layer of extruded material completely covered the older part of the mycelium (Fig. 3f, g). A massive increase in extruded amorphous-fibrillar material was marked in the innermost part of the mycelium (Fig. 3h).

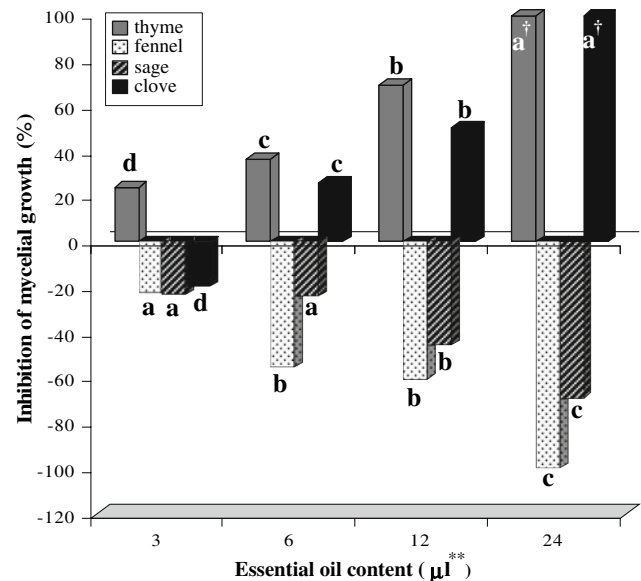
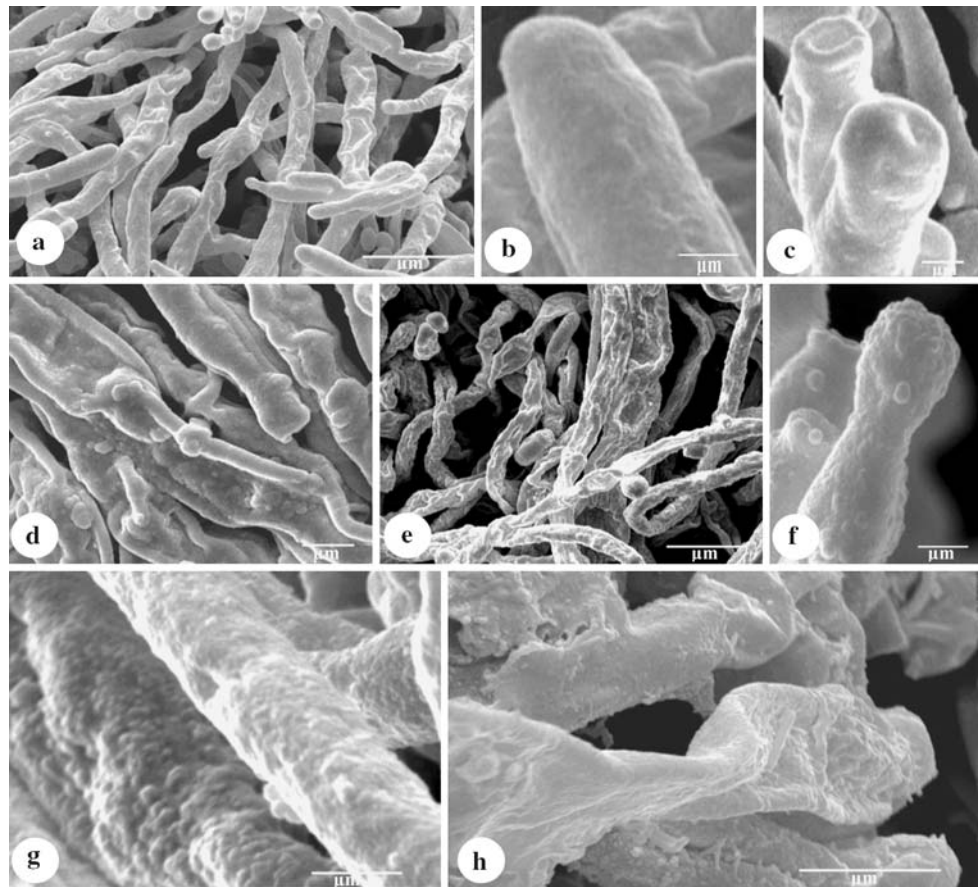


Fig. 2 Percentage inhibition of mycelial growth *Penicillium digitatum* in vitro by volatiles method. Colony radius was measured after 7 days at 25°C. Different letters on columns in each EO indicate significant differences ($P \leq 0.01$) by least significant difference test. The columns in the negative region indicate positive effect of EO on mycelial growth. † Fungicidal activity ** Microliters of crude essential oil per Petri dish

Discussion

The results of the present study indicate that clove and thyme EO have a significant effect on the growth of *P. digitatum* with Figs. 1 and 2 reporting data on antifungal activity of the oils against *P. digitatum*. Moreover, even at concentrations that caused lower than 100% mycelial growth inhibition, conidia have lost their pigmentation (became hyaline). According to Yigit et al. (2000) this effect might decrease virulence of the pathogen; hence a decrease in the incidence of the disease. The strong antifungal activity of thyme and clove EO could be due to their high content of phenolic compounds. Our results are in agreement with those reported by Faïd et al. (1996), in where it has been established that a relationship exists between the high activity of the oils and presence of phenolic components. According to Faïd et al. (1996) antimicrobial activity of major oil compounds is in the order: phenols (highest activity) > alcohols > aldehydes > ketones > ethers > hydrocarbons. Terpenoids that are structurally related to phenols like carvacrol, thymol, eugenol and isoeugenol exhibit powerful antiseptic properties, probably because of their lipophilic properties and specific functional groups such as free OH group. The presence of an aromatic ring with a phenolic hydroxylic group forms hydrogen bonds with active sites of target enzymes which is responsible for the fungitoxicity of the EO (Farag et al. 1989). This hypothesis fits well with the activity

Fig. 3 (a, b) Mycelium of untreated (control) *P. digitatum* with free and linearly shaped hyphae, (c, d) anomalous branching and notching of the terminal hyphae in *P. digitatum* treated, (e) severely collapsed and squashed mycelium, (f, g) warty surface of mycelium, (h) amorphous-fibrillar material in *P. digitatum* treated. Bars: a, e—10 μm ; h—5 μm ; d, g—2 μm ; b, c, f—1 μm



of EO containing high amounts of phenolic compounds such as the thyme and clove oils.

In this study, despite the fact that the thyme EO had lower content of phenolic components than that of clove, it showed higher antifungal activity in contrast. According to Farag et al. (1989) this effect is due to the relative position of the hydroxyl group that could be effective on the phenolic components activity, and by the same reason, thyme oil in spite of less phenolic components, has more inhibition effects on the *P. digitatum* compared with clove oil. Therefore, the importance of hydroxyl group in the phenolic structure is confirmed.

It is important to note that the clove and thyme oils showed the strongest antifungal activity against mycelial growth of *P. digitatum* in the vapour phase (Fig. 2) perhaps due to the high volatility of phenols.

The effects of clove oil on the morphology of *P. digitatum* hyphae examined by SEM revealed malformation in the morphology of the hyphae. It is well known that mycelial growth takes place at the apex of terminal hyphae (Sietma and Wessels 1994). As the result, the alterations observed at this place after treatment with clove oil can explain the reduced growth of the fungus. Changes in the outer wall of the hyphae suggest a change in the normal assembly of the wall components leading to their incorrect

arrangement at the apical dome (extruded material, Fig. 3 f–h). The loss of linear form and branching of terminal hyphae could be induced by a loss of directionality of the vesicles which causes them to discharge parietal materials at positions where exocytosis does not normally take place. Such modifications induced by EO may be related to the interference of EO components with enzymatic reactions of wall synthesis, which affects fungal morphogenesis and growth (Romagnoli et al. 2005). Our observations are also supported by the findings of the surface modifications in SEM study as observed by Romagnoli et al. (2005) who used *Thymus patula* EO against *Botrytis cinerea*.

Detailed in vitro studies on EO of *Thymus vulgaris* and *Eugenia caryophyllata* indicate their potentiality as ideal antifungal compounds against postharvest citrus spoilage fungi. We propose further research in order to test the efficiency of botanical pesticides in the prevention of citrus fruit rotting (due to green mould).

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