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Essential Oils Inhibit Mycelial Growth of *Rhizopus stolonifer*

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Summary

Rhizopus rot caused by *Rhizopus stolonifer*, is considered as a major destructive postharvest disease of stone fruits such as peach that severely decreases the shelf life of stored fruits. The aim of this research was to determine and monitor the antifungal activity of essential oils from three medicinal plants, to control *Rhizopus stolonifer* on peach fruits and enhance fruit shelf life and storage ability. Essential oils extracted from sage (*Salvia officinalis*), savory (*Satureja hortensis*) and zataria or Shiraz thyme (*Zataria multiflora*). These essential oils were added to Petri dishes containing PDA (potato dextrose agar) by two methods, direct contact and fumigation. Results obviously showed that fumigation with zataria and savory essential oils completely inhibited *R. stolonifer* growth by volatiles with 24 and 48 µl concentrations. In the case of direct contact, zataria at 360 µl l^{-1} had maximum inhibition (96 %) upon mycelial growth of *R. stolonifer*. The main components of zataria oil were carvacrol (38.50 %), thymol (25.50 %) and p-cymene (13.35 %), and savory oil were carvacrol (38.00 %), *p*-cymene (32.20 %) and γ-terpinene (21.65 %).

Key words. Essential oil – *Rhizopus stolonifer* – postharvest – *Salvia officinalis* – *Satureja hortensis* – *Zataria multiflora*

Introduction

Postharvest diseases limit the storage period and marketing life of peaches. Rhizopus rot, caused by *Rhizopus stolonifer* (Ehrenb. Fr) Vuill., is one of the most destructive postharvest diseases of stone fruits. The spores of *R. stolonifer* are very common in the atmosphere and this infection of fruits occurs mainly at wound sites during harvest or packing (Ogawa et al. 1963; Luepschen et al. 1971). Disease control is achieved mainly through the use of the fungicides benomyl and iprodione were available for postharvest treatment to reduce decay and extend the shelf-life of stone fruits (Fan et al. 2000). Elucidating non-chemical control methods to reduce postharvest decay is becoming increasingly important. The use of fungicides is becoming more restricted due to health concerns (Ragsdale and Sisler 1994). On the other hand, the application of fungicides may lead to the development of fungicide- resistant strains of postharvest pathogens (Spotts and Cervantes 1986; Holmes and Ekert 1999). It is therefore necessary to develop alternatives to synthetic chemical control to reduce environmental risks and raise consumer confidence. Thus, biological control has emerged as an effective strategy to combat major postharvest fruits decays (Janisiewicz and Korsten 2002). Postharvest biocontrol is especially feasible because harvested fruits are readily accessible to treatment with antagonists and many postharvest pathogens of fruits infect through wounds after harvest (Janisiewicz and Jeffers 1997; Nunes et al. 2001).

In the past few decades several studies have investigated the antifungal properties of essential oils against postharvest pathogens (Giamperi et al. 2002). The advantage of essential oils is their bioactivity in the vapour phase, a characteristic that makes them attractive as possible fumigants for stored product protection. These essential oils are thought to play a role in plant defence mechanisms against phytopathogenic micro-organisms (Mihaliak et al. 1991). They are biodegradable in nature, non-pollutant and possess no residual or phytotoxic properties, these natural products have the potential to be safe fungicides to replace the synthetic ones (Nychas 1995). Most of the essential oils have been reported to inhibit postharvest fungi under *in vitro* conditions (Bishop and Reagan 1998; Singh and Tripathi 1999; Bellerbeck et al. 2001; Hidalgo et al. 2002). Some of the essential oils have been reported to protect stored commodities from biodeterioration. There are also some reports on essential oils in enhancing storage life of fruit and vegetables by controlling their fungal rotting.

Zataria multiflora belongs to the *Laminaceae* family that geographically grows only in Iran, Pakistan and Afghanistan (Ali et al. 2000). This plant has the local name of 'Avishan Shirazi' (in Iran) and traditional uses such as antiseptic, anaesthetic and antispasmodic (Hosseinzadeh et al. 2000). The main constituents of the essential oil of this plant are phenolic compounds such as carvacrol, thymol and eugenol (Shaffiee and Javidnia 1997).

The fungitoxic potency of the essential oils may be due to synergism between their components. Thus, there would be negligible chance of development of resistant races of fungi after application of essential oils to fruit and vegetables. The enhancement of fungitoxic potential of mixtures of the oils may be due to the joint action of

two or more substances present in the oils (Scardavi 1966). This synergism would be beneficial in postharvest protection because the pathogen would not easily become resistant against the components. However, more work on the synergistic action of plant products under *in vitro* and *in vivo* conditions is required. The essential oils, their major individual aroma constituents and blends of the major individual constituents at different ratios inhibited the growth of the fungi in a dose-dependent manner. The aim of the present research was to determine the inhibitory effects of three essential oils on the growth of *R. stolonifer*.

Materials and Methods

Essential oil preparation

The essential oils of sage (*Salvia officinalis*), savory (*Satureja hortensis*) and Iranian thyme (zataria) (*Zataria multiflora* Bioss.) were extracted by herb steam distillation.

Oil analyses

Gas chromatography. 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 μ m). Oven temperature was maintained at 40 °C for 5 min and then programmed to 220 °C at a rate of 4 °C min^{-1}. Injector and detector (FID) temperature was 240 °C; helium was used as carrier gas with a linear velocity of 32 cm s^{-1}. Percentages of essential oils components were calculated by electronic integration of FID peak areas without the use of response factor correction.

Gas chromatography/mass spectrometry. The GC-MS 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 μ m) and DB5973 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^{-1}. Helium gas was employed as the carrier gas at 1 ml min^{-1} flow rate; source 70 eV. Essential oils samples were diluted in normal hexane and 0.1 μl l^{-1} was injected into the oven.

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 were also compared (Shibamoto 1987; Davies 1990).

Fungal spore suspension and inoculums preparation

The plate of the *R. stolonifer* was sub-cultured by streaking the spores onto fresh potato dextrose agar (PDA) plates and incubated for 7 d at 25 °C. The seven days old spores cultures of the fungus was suspended in sterile distilled water containing 0.001 ml L^{-1} of Tween 80. The spore suspensions were then filtered with 6 layers of cheesecloth to remove debris such as mycelia and condensed agar fragments, and its concentration was adjusted to 10^6 fungal spores ml^{-1} suspensions using a haemocytometer.

Antifungal activity of essential oils

The inhibitory effects of the three essential oils were tested *in vitro* on mycelial growth of *R. stolonifer*. Two methods were used to test the antifungal activity of essential oils. The agar dilution method consisted of adding different amounts of pure essential oils dissolved in absolute ethyl alcohol, 5 % Tween 80 (v/v), to PDA before pouring it into Petri dishes (8 cm diameter) while the medium was still warm (40–45 °C), to obtain concentrations of 120, 240 or 360 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 centre in each Petri plate. Controls consist of 120, 240 and 360 μl l^{-1} of ethyl alcohol + 5 % Tween-20 mixed with PDA. Then the colony radius was measured after 3 d 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 essential oil, and mycelial growth was monitored for the next 7 d at 25 °C.

The volatile activity of the essential oils was assayed by inoculating spore suspension (10^6 conidia ml^{-1}) at the center of a PDA Petri dish (8 cm diameter). Sterile filter paper discs (7 cm diameter) soaked in 1, 3, 6, 12, 24 or 48 μl pure essential oils 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 3 d. 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 d at 25 °C. In both types of experiments, three replicates per treatment were carried out.

The percentage inhibition of the fungal growth was determined on the growth in test plates compared to the respective control plates (Juliano et al. 2000) according to the equation:

$$\text{Inhibition \%} = 100\ (C - T) / C$$

Where C is the diameter of fungal growth on the control and T is the diameter of the fungal growth on the test plate.

Data analysis

Data on inhibitory effect of essential oils on mycelia growth were subjected to analysis of variance (ANOVA) using SPSS and MSTATC software. The means comparisons were made using Duncan's Multiple range tests at $P \leq 0.01$.

Results

Water distilled essential oil from aerial parts of *Z. multiflora Boiss*, *S. hortensis* and *S. officinalis* was analyzed by GC and GC-MS. The components identified are given in

Table 1 with their relative percentages. Sage oil was particularly rich in Thujone (24.70 %) and Camphor (26.40 %), and the savory oil had particularly *p*-cymene (32.20 %) and carvacrol (38.00 %). The zataria oil contained carvacrol (38.50 %), thymol (25.50 %) and *p*-cymene (13.35 %) (Table 1). In addition, phenols were the most abundant compound group of the oil (78.27 %). Possible modes of action of phenolic and terpene compounds previously have been reviewed (Davidson 2001).

The results obtained in assays of antifungal activity of the oils tested in agar dilution method, showed that zataria oil in 360 and 240 µl l^{-1} indicated 96.12 and 91.85 % growth inhibition of fungi mycelium respectively. The best inhibitory effect of savory oils was at 240 µl l^{-1} (86.12 %) concentration, although sage oil didn't show acceptable inhibitory effect on the *R. stolonifer* (Fig. 1). The oils of zataria and savory were most acceptable inhibitor against *R. stolonifer* when added into the medium (direct contact) at 360 µl l^{-1}, but this inhibitory effect was fungistatic at 120 µl l^{-1} for both zataria and savory, and fungicidal at 240 and 360 µl l^{-1} (data not shown).

In vapour phase, means comparisons showed significant differences of growth inhibition at different essential oils concentrations ($P \leq 0.01$). Sage oil had not acceptable inhibitory effect on the *R. stolonifer* at low concentrations. Although, the effect of all three oil treatments showed inhibitory effect at 24 and 48 µl concentrations. In the lowest concentration (1 µl), only zataria essential oil could inhibit mycelial growth of *R. stolonifer* (Fig. 2). Inhibitory activity of the zataria, sage and savory oils of the volatile compounds were fungicidal (data not shown). Results as shown in Fig. 1 (agar dilution method) and Fig. 2 (fumigation method) indicated that the most effective oils were zataria and savory with 100 % inhibition of *R. stolonifer*.

Discussion and Conclusion

The obtained results indicated that zataria, sage and savory essential oils have a significant inhibitory effect on the growth of *R. stolonifer* (Figs. 1 and 2). As mentioned, the main components of applied essential oils in this research were carvacrol, thymol and *p*-cymene. The antifungal activities of applied compounds in the present research have been reported previously by other researchers (Burt 2004; Nguefack et al. 2004). However, the mechanisms of action of these compounds previously have not been completely elucidated. Lis-Balchin and Deans (1997) reported that strong antimicrobial activity could be correlated with essential oils containing high percentage of monoterpenes, thymol, eugenol and cinnamic aldehyde. These results are in agreement with those published by Omidbeygi et al. (2007) and Suhr and

Table 1. Composition of essential oils from savory (*Satureja hortensis*), zataria (*Zataria multiflora*) and sage (*Salvia officinalis*).

Compound	Savory (%)	Zataria (%)	Sage (%)	RI	Compound	Savory (%)	Zataria (%)	Sage (%)	RI
Salvene	–	–	0.3	865.0	Myrtanol	–	–	1.0	1648.0
Tricyclene	–	–	0.2	922.0	Thymol	–	25.5	0.2	1292.8
α-Pinene	0.80	3.6	3.7	940.3	Bornyl acetat	–	–	1.3	1283.0
Comphene	–	0.5	6.6	956.2	Eugenol	–	–	0.1	1356.0
Verbenene	–	–	1.6	1204.0	β-Caryophyllene	–	–	0.7	1409.0
Sabinene	–	0.6	0.2	976.0	α-Humulene	–	–	0.9	1473.2
β-Pinene	0.80	1.5	0.4	984.9	Caryophyllene oxide	–	–	0.2	1585.0
Myrcene	1.50	0.3	0.9	989.2	Guaiol	–	–	3.3	1596.0
α-Terpinene	2.20	5.2	0.2	1020.8	Humulene oxide	–	–	0.4	1587.0
p-Cymene	32.20	13.4	0.2	1028.6	14-Hydroxy-9-epi-β-Caryophyllene	–	–	0.6	1669.0
Limonene	–	0.9	2.4	1033.2	Lyral	–	–	0.1	1418.0
1,8-Cineol	–	–	9.6	1037.1	α-Santalol	–	–	0.3	1668.0
γ-Terpinene	21.65	–	0.1	1061.5	Δ-3-Carene	–	0.1	–	1014.0
p-Cymenene	–	–	0.2	1018.0	Linalool	0.08	2.2	–	1100.0
Terpinolene	0.06	–	0.2	1064.0	Carvacrol	38.00	38.5	–	1310.8
β-Thujone	–	–	6.4	1103.0	E-Caryophyllene	–	2.2	–	1412.0
α-Thujone	0.80	–	24.7	930.2	β-Bisabolene	0.20	0.1	–	1503.0
Camphor	–	–	26.4	1143.0	α-Phellandrene	0.20	–	–	1004.0
Isopulegol	–	–	0.2	2014.0	(*E*)-β-Ocimene	0.50	–	–	1113.0
Pinocamphone	–	–	1.1	1168.0	Terpinene-4-ol	0.09	–	–	1189.0
Borneol	–	1.4	4.2	1157.0	Carvacrol acetat	0.03	–	–	1373.2
Terpinene-u-ol	–	–	0.7	1416.0	(E)-β-Caryophyllene	0.02	–	–	1415.0
α-Terpineol	–	–	0.5	1176.0					

RI: retention index

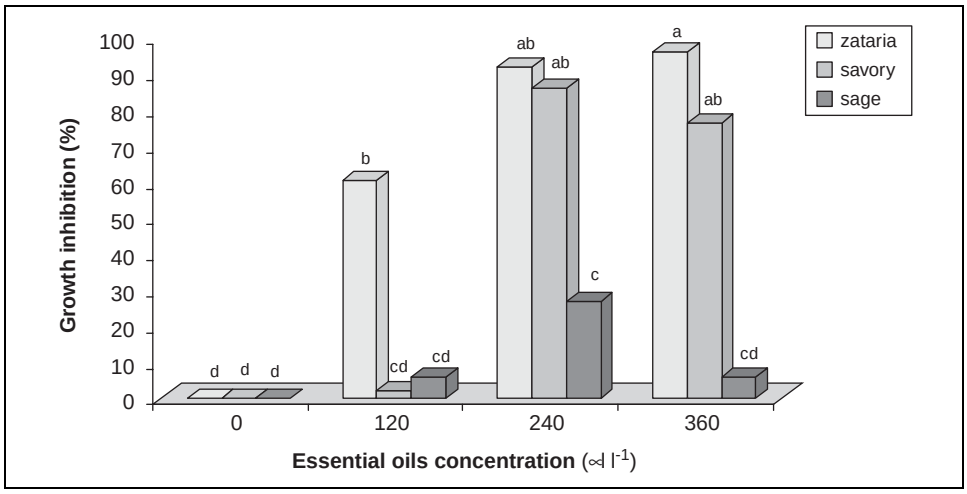


Fig. 1. Effect of essential oils on mycelial growth of *Rhizopus stolonifer* (agar dilution method). Colony radius was measured after 3 d at 25 °C. Different letters above columns indicate significant differences according to Duncan's Multiple range tests at P≤0.01.

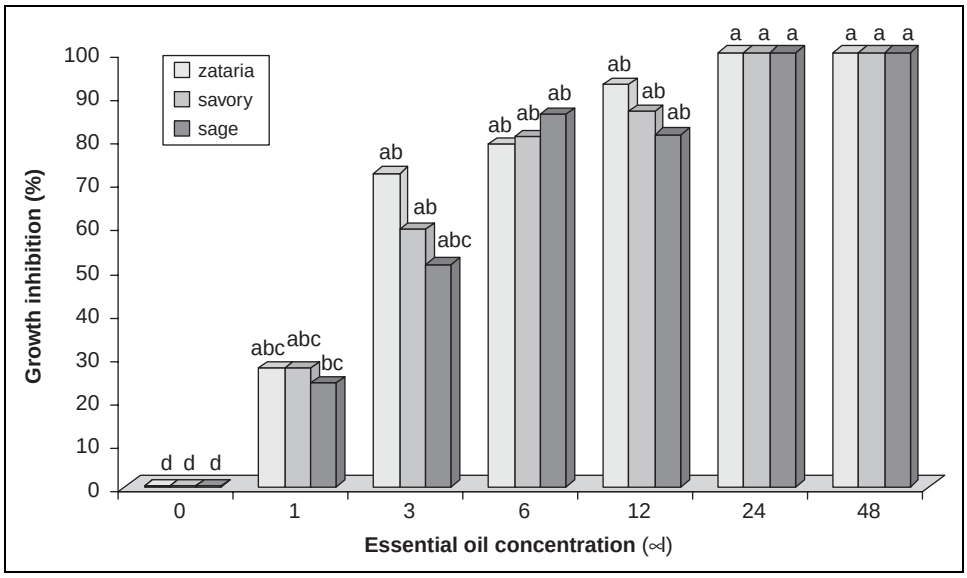


Fig. 2. Effect of volatiles of essential oils on mycelial growth of *Rhizopus stolonifer*. Colony radius was measured after 7 d at 25 °C. Different letters above columns indicate significant differences according to Duncan's Multiple range tests at P≤0.01.

Nielsen (2003), who reported that the major components of thyme essential oil are thymol, carvacrol, linalool and α-pinene.

According to Bhaskara-Reddy et al. (1998) the essential oils from two clone types of *Thymus vulgaris* (Laval-1 and Laval-2) that their contents were high in *p*-cymene, linalool, terpinen-4-ol and thymol which constituted 53.5 % and 66.2 % of Laval-1 and Laval-2 essential oils respectively, had antifungal activity against *Botrytis cinerea* and *Rhizopus stolonifer*. Lopez et al. (2007) screened eighty eight extracts of different polarity obtained from 18 *Lamiaceae* medicinal and aromatic plants for their antioxidant and antifungal properties. A thin layer chromatography (TLC) analysis combined with two bioassays showed that in most cases the highest radical scavenger capacity was detected in methanol and aqueous extracts and polyphenols may be responsible.

Terpenoids related structurally to phenol like carvacrol, thymol, eugenol and isoeugenol exhibit powerful antiseptic properties probably because of their lipophilic properties and specific functional groups such as free OH groups. (Scora and Scora 1998). Several studies have shown that the phenols show stronger antifungal activity than other major oil components such as alcohols, aldehydes, ketones and ethers (Faid et al. 1996). Apparently, the oils of zataria and savory inhibited mycelial growth were due to their high content of the phenolic compounds thymol and carvacrol. It is important to note that the zataria and savory oils showed the strongest antifungal activity against mycelial growth of *R. stolonifer* in the vapour phase (Fig. 2) perhaps due to the high volatility of phenols.

In vitro studies on essential oils of *Salvia officinalis*, *Satureja hortensis* and *Zataria multiflora* indicated their potentiality as ideal antifungal compounds against postharvest fruits spoilage fungi. We suggest further research on the efficiency of studied oils in the prevention of fruit rotting such as Rhizopus rot.

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