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CHARACTERIZATION AND USE OF ESSENTIAL OIL FROM *THYMUS VULGARIS* AGAINST *BOTRYTIS CINEREA* AND *RHIZOPUS STOLONIFER* IN STRAWBERRY FRUITS

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Key Word Index—*Thymus vulgaris*; Labiatae; thyme; *Fragaria ananassa*; Rosaceae; strawberries; volatile components; antifungal; *Botrytis cinerea*; *Rhizopus stolonifer*.

Abstract—The essential oils from two clonal types of *Thymus vulgaris* (Laval-1 and Laval-2) were characterized and tested for antifungal activity. 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. The essential oil volatiles from two clonal types exhibited antifungal activity against *Botrytis cinerea* and *Rhizopus stolonifer*, two common storage pathogens of strawberries (*Fragaria ananassa*). The inhibition of *B. cinerea* and *R. stolonifer* ranged from 26.5 to 63.5% and 5.5 to 50.5% respectively by oil from Laval-1, when exposed to concentrations of 50 to 200 ppm, while values of 36.9 to 90.5% and 11.5 to 65.8% were observed from oil from Laval-2. The decay of strawberry fruits caused by *B. cinerea* and *R. stolonifer* was controlled up to 73.6 and 73.0% respectively by volatiles from maximin concentration of Laval-1, and up to 75.8 and 74.8% from Laval-2. No visual phytotoxic symptoms were noticed for the observed period. Essential oil from Laval-2 exhibited higher antifungal activity which was related to its relatively higher content of antimicrobial compounds.

INTRODUCTION

Thyme (*Thymus vulgaris* L.), a member of the Labiateae family, is an aromatic and medicinal plant of increasing importance in horticulture. In recent years, studies on the antifungal activity of essential oil components have been reported by numerous investigators [1–5]. Further essential oils derived from many aromatic plants are known to possess insecticidal [6] and antimicrobial activities [7]. Among them, thyme essential phenolic oil has been reported to have antibacterial, antimycotic, antioxidative and food preservative properties [8, 9]. Of the 50 plant essential oils examined by Deans and Ritchie [8], thyme oil was the most inhibitory against 25 genera of bacteria. A concentration of 500 μg ml^{-1} of an ethanolic extract of thyme inhibited the growth of *S. aureus* [10], and the growth of *V. parahaemolyticus* was inhibited at 1000 μg ml^{-1}. It has also been reported that thyme serves as a flavouring agent for a variety of food products, sauces, meats, canned foods, and used as an antiseptic agent for its antimicrobial properties [7, 11]. Analysis of the essential oils of *T. vulgaris* L. growing in the wild in northern Italy showed 44 components, with the main constituents being *p*-cymene, γ-terpinene and thymol [12]. Among these compounds, thymol was shown to be the most effective against a wide spectrum of microbes. Thymol has also been shown to inhibit the growth and toxin production by mycotoxigenic molds [13–15]. Thymol concentrations of over 500 μg ml^{-1} were shown to fully inhibit the growth of *A. parasiticus* [16], while concentrations lower than 0.4 μg/ml completely inhibited the growth of both *Aspergillus flavus* and *Aspergillus versicolor* [12]. Substantial antifungal activities of volatile oils from thyme, organum and savory against 18 pathogenic and non pathogenic fungi were reported, when tested *in vitro* using a standard-zone inhibition test [17].

Post-harvest decay of strawberries caused by *Botrytis cinerea* Pers.: Fr. (gray mold rot) and *R. stolonifer* (Ehrenb.: fr.) Vuill. (soft rot) represent major loss during storage and shipment [18]. Control of these fungi during storage can be achieved by physical and chemical methods. Exposure to high CO_2 and low temperature storage are effective in reducing the fungal development [19, 20]. However exposure to high

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CO_2 for prolonged periods could cause off-flavours of strawberries even though they can tolerate relatively high CO_2 levels [21], while low temperature storage alone is not adequate for prolonged storage life required to service distant markets. Application of fungicides proves to be the most effective method in reducing post-harvest diseases in strawberries [22], but the consumers are increasingly concerned with the chemical residues in the fresh produce [23]. Furthermore, the development of resistance of post-harvest pathogens to chemical fungicides is also drawing attention [24]. Thus in the present study, we have analyzed the essential oil components from two clonal types such as Laval-1 and Laval-2 of *Thymus vulgaris*, and explored the possibilities of using them for the control of two common post-harvest pathogens of strawberries.

RESULTS AND DISCUSSION

Oil composition

Analysis of essential oils from two clonal types, Laval-1 and Laval-2, revealed 85 different compounds (Table 1). It was also observed that the oils are rich in monoterpene hydrocarbon and phenolic monoterpenes. In both types, the most abundant monoterpenes were γ-terpinene and *p*-cymene, and the biogenetic precursors of the phenolic terpenes thymol and carvacrol. Though the GC-MS analysis showed a similar nature of the monoterpene hydrocarbons and oxygenated monoterpenes that make up most of the two oils, there are significant differences in the relative quantities of their main constituents. The four principal constituents are carvacrol, *p*-cymene, terpinen-4-ol and thymol, which amount to 50.3% in Laval-1 and 52.9% in Laval-2 (Table 1). A higher proportion of thymol and carvacrol in Laval-2 (27.0%) compared to Laval-1 (12.2%) may suggest a stronger effect of oil from Laval-2 in fungal inhibition. Furthermore, there is a significant difference in linalool content (3.2% in Laval-1 and 13.3% in Laval-2) between two clonal types. This compound has an antimicrobial action [17], but may also synergize the antimicrobial action of thymol and carvacrol. Differences in antimicrobial properties of essential oils from *Mentha* species were observed, and such differences were related to variation in the chemical composition [7]. The chemical polymorphism of *Thymus* essential oil is well known [25], and 8 chemotypes (linalool, thymol, carvacrol, geraniol/geranyl acetate, 1,8-cineole/linalool, 1,8-cineole/linalool/thymol) have been demonstrated in Portuguese *T. zygis* sp. *sylvestris* [26]. Based on essential oil analysis, the 2 clones Laval-1 and Laval-2 used in this study can be classified into chemotypes thymol/carvacrol and thymol/carvacrol/linalool of *T. vulgaris* respectively.

Effect on radial growth

Incorporation of different concentrations of two types of thyme oil into potato dextrose agar (PDA)

Table 1. Relative composition of thyme essential oils from Laval-1 and Laval-2 clonal types

		Composition (%)†	
R_I	Compound*	Laval-1	Laval-2
Monoterpene hydrocarbons		27.8	29.2
1015	α-pinene*	1.4	2.6
1018	α-thujene	0.5	0.9
1049	Camphene*	0.2	0.4
1088	β-pinene	1.0	0.2
1105	Sabinene	0.3	0.1
1116	3-carene*	—	1.1
1160	β-myrcene*	0.8	0.9
1167	α-terpinene*	2.2	0.4
1187	*d*-limonene*	0.8	1.2
1194	β-phellandrene*	1.3	—
1238	γ-terpinene*	2.1	0.2
1262	*p*-cymene*	16.3	20.8
1269	Terpinolene	0.8	0.2
1429	*p*-cymenene	0.1	0.2
Oxygenated monoterpenes		50.4	52.8
1192	Cineole*	1.9	1.1
1495	Camphor*	—	0.3
1499	*cis*-pinocamphone	2.3	—
1534	*trans*-pinocamphone	4.2	—
1541	*cis-p*-2-menthen-1-ol	0.1	0.2
1549	Linalool*	3.2	13.3
1555	Linalyl acetate*	0.3	1.2
1566	Bornyl acetate*	—	0.5
1592	Terpinen-4-ol*	21.8	5.1
1612	*trans-p*-2-menthen-1-ol	0.2	0.2
1655	Isoborneol	0.3	0.4
1670	Verbenol	0.3	0.5
1691	α-terpineol*	3.2	2.5
1788	Myrtenol	0.1	—
1847	Tolyldimethylcarbinol	0.3	0.4
2110	Cuminyl alcohol	—	0.1
2185	Thymol*	9.5	18.1
2211	Carvacrol	2.7	8.9
Sesquiterpene hydrocarbons		8.3	3.7
1479	α-copaene	0.1	0.3
1512	α-gurjunene	0.5	—
1577	Caryophyllene	1.1	1.4
1597	*FW* = 204	0.3	—
1618	*FW* = 204	—	0.2
1623	Aromadendrene	1.0	0.1
1639	*FW* = 204	0.2	—
1647	α-caryophyllene	0.2	0.2
1679	*FW* = 204	1.3	0.2
1710	β-selinene	0.2	—
1716	α-selinene	0.1	—
1731	α-muurolene	0.2	0.2
1718	β-bisabolene	—	0.4
1752	δ-cadinene	1.8	0.7
1819	Calamenene	0.5	—
1859	*FW* = 206	0.2	—
2066	*FW* = 204	0.3	—
2169	*FW* = 204	0.3	—
Oxygenated sesquiterpenes		4.3	3.6
1964	*FW* = 220	0.1	0.1
1973	Caryophyllene oxide	1.1	1.1
1992	Mayurone	0.1	—
2010	*FW* = 222	0.2	—

Table 1—*continued.*

R_I	Compound*	Composition (%)† Laval-1	Laval-2
2014	Carotol	—	0.2
2026	Ledol	0.2	—
2077	Globulol	0.6	0.1
2084	Guaiol	0.7	0.1
2091	*cis*-α-copaene-8-ol	—	0.1
2125	Spathulenol	1.3	1.1
2220	β-eudesmol	—	0.8
Miscellaneous		5.9	6.5
1364	bis(2-chloroisopropyl)ether	0.4	—
1447	1-octen-3-ol	—	0.4
1458	Unknown	—	0.2
1459	*FW* = 154	0.2	0.3
1684	Unknown	—	0.2
1703	α-trimethyldodecane	2.1	0.2
1822	Anethol*	—	0.1
1842	Unknown	—	0.2
1911	Unknown	0.2	—
1954	(*Z*)-3-hexenyl butyrate	0.1	0.1
2038	Unknown	0.2	0.2
2056	Unknown	0.5	0.4
2062	Oxacyclotetradecan-2-one	—	0.9
2086	Unknown	0.5	—
2101	Unknown	0.1	—
2107	Unknown	0.2	—
2116	Unknown	0.3	—
2158	Unknown	0.3	—
2172	3-methyl-3-cyclohexen-1-ol	0.3	—
2198	Unknown	0.3	1.2
2257	Unknown	—	1.5
2367	Unknown	—	0.3
2444	Unown	—	0.3
2658	2-ethyl-4,5-dimethylphenol	0.2	—
Total		96.7	95.8

* Authentic reference compounds used in identification.
† Compounds less than 0.1% are not reported.

showed significant reduction ($P < 0.05$) on the growth of two common pathogens of strawberries, *B. cinerea* and *R. stolonifer* (Table 2). Complete inhibition, however, was not achieved at the concentrations tested in this study, indicating that the essential oils are fungistatic rather than fungicidal at these concentrations. In general, the rate of inhibition was less in *R. stolonifer* exposed to different concentrations of thyme oil. Also the growth of *R. stolonifer* was faster than that of *B. cinerea*, and the mycelium reached the edge of the untreated PDA plates within 4 days of inoculation compared to 7 days for *B. cinerea*. There was a significant difference ($P < 0.05$) between the oils from Laval-1 and Laval-2 in their inhibitory action, with oil from Laval-2 exhibiting a comparatively higher antifungal activity which can be related to its high thymol, carvacrol and linalool contents. Thymol has been shown to inhibit the growth and toxin production by mycotoxigenic molds [13–15], and earlier investigation showed that carvacrol completely inhibited mycelial growth of all *Rhizopus* spp. with a minimum of 5 μl [27].

Effect on inoculated strawberries

The effect of thyme oil volatiles on the control of fungal growth in inoculated strawberries was evaluated. Thyme oil volatiles were highly effective in reducing gray mold and soft rot incidence in strawberry fruits caused by *B. cinerea* and *R. stolonifer*, respectively (Table 3). Fungal infection was noticed after 6 days with thyme treatments, while in the control, the infections were evident after 2 days of storage at 13°. In addition, significant increases in decay were noticed from day 7 to day 14 in the control experiments. Exposure to oil volatiles at 200 ppm from Laval-1 reduced gray mold and soft rot incidences by more than 70% after 14 days of storage. The control of decay in fruits subjected to volatiles from Laval-2 was significantly ($P < 0.05$) higher compared to the oil from Laval-1, which was again related to its higher composition of antimicrobial compounds. The gray mold and soft rot incidences were reduced by 75.8 and 74.8% respectively at 14 days of storage by exposure to maximum concentration of volatiles from Laval-2. No visible phytotoxic symptoms were noticed on treated strawberry fruits. As observed in the inhibition test on PDA, the effect of thyme oil on the control of gray mold and soft rot was dependent on the oil concentration. Significant decreases in the decay with increases in the oil concentrations was observed. It has been reported that most of the antifungal volatiles and vapors reduce conidial germination, subsequently killing the fungi [28]. In this study, the inoculated spores on strawberry surface were directly exposed right from the inoculation without allowing them to germinate. At this point, it can be said that the oil volatiles were not totally cidal to spores, but reduced spore germination and mycelial growth, resulting in decreased decay with increases in oil volatile concentration.

In conclusion, this study demonstrates the potential of thyme oil volatiles as an antifungal preservative for strawberry fruits that are quire susceptible to decay caused by *B. cinerea* and *R. stolonifer*. However further studies are required to determine the optimal concentration of thyme oil and exposure time for decay control, and the sensory quality of the treated fruits.

EXPERIMENTAL

Thyme oil

Thyme oil from two clonal-stock thyme lines from the breeding program of the Horticultural Research Center, Laval University, named Laval-1 and Laval-2 were used. The harvested plants were dried in a forced-air drier at 39° for 72 h. The air dried plant

Table 2. Effect of thyme oil on the radial growth of *Botrytis cinerea* and *Rhizopus stolonifer* on potato-dextrose agar

	Inhibition (%)*			
	Laval-1		Laval-2	
Concentration (ppm)	*B. cinerea*	*R. stolonifer*	*B. cinerea*	*R. stolonifer*
0	0.0	0.0	0.0	0.0
50	26.5(±0.7)	5.5(±0.9)	36.9(±0.6)	11.5(±0.5)
100	32.3(±0.6)	9.5(±0.3)	49.0(±0.9)	20.1(±0.8)
200	63.5(±0.9)	50.5(±0.6)	90.5(±1.5)	65.8(±1.3)

*Measurement of radial growth was performed 7 and 3 days after PDA plates were inoculated with *B. cinerea* and *R. stolonifer*, respectively.

Values in the parentheses represent standard errors of the mean.

Table 3. Effect of thyme oil volatile exposure on the incidence of gray mold and soft rot in strawberry fruits

		Decay (%)*			
		Laval-1		Laval-2	
Concentration (ppm)	Storage (days)	*B. cinerea*	*R. stolonifer*	*B. cinerea*	*R. stolonifer*
0	7	43.5(±1.5)	55.3(±2.1)	43.5(±1.3)	55.3(±2.1)
	14	85.3(±0.9)	90.5(±2.5)	85.3(±3.1)	90.5(±3.3)
50	7	15.5(±1.3)	25.3(±1.9)	12.5(±1.1)	20.5(±1.5)
	14	18.3(±0.9)	33.7(±2.1)	17.5(±1.5)	31.7(±1.7)
100	7	12.3(±1.1)	19.7(±0.9)	10.5(±0.7)	18.7(±0.9)
	14	16.5(±1.5)	25.5(±1.3)	14.7(±1.1)	23.7(±1.1)
200	7	6.1(±0.9)	10.5(±0.7)	5.1(±0.3)	7.9(±0.6)
	14	11.7(±1.2)	17.5(±0.9)	9.5(±0.6)	15.7(±1.1)

* Percentage of infected strawberries was based on five replicates of 10 fruits each.

Values in the parentheses are standard errors of the mean.

material (aerial parts from 10 plants) was pulverized and the essential oil content was extracted by steam distillation using a clevenger-type apparatus.

Essential oil analyses

Essential oils were analyzed by GC using a fused silica capillary nitroterephthalic acid FFAP column (25 m × 0.20 mm, film thickness 0.33 μm) with an oven temperature programmed as follows: from 40° (isothermal for 5 min) to 170° at 4° min^{-1}, and from 170° to 240° at 8° min^{-1} and isothermal period of 12 min at 240°. Injector and detector temperatures were 250° for analysis with both FID and MS detectors. The latter was of selective quadrupole type and an ionization voltage of 70 eV was used in MS analysis. Samples of 1.0 μl were injected with a split ratio of 1 : 50. The linear velocity of the carrier gas (hydrogen) was 35 cm s^{-1} at 100° isothermal. Qualitative analysis was based on comparison with mass library (NBS75K) and Kovats indices (R_I) [29–31]. As indicated in Table 1, authentic reference compounds were also used in identification.

Fungal culture

Fungi *B. cinerea* and *R. stolonifer* were isolated from diseased berries on PDA by the single spore procedure. Inoculated plates were held at 23° for 4 days. The cultures were transferred to PDA slants and maintained at 4° until use.

Antifungal assay

The antifungal assay was determined on PDA plates amended with three concentrations of thyme oil (50, 100 and 200 ppm). The oil was added to sterile molten PDA to obtain the desired concentrations. Aliquots of 20 ml of the solution were immediately dispensed into petri plates which were seeded with 6 mm diameter mycelial plugs from the edge of 4-day-old *B. cinerea*, and 2-day-old *R. stolonifer*. Plates in four

replicates were used for each treatment, and the inoculated plates were incubated in the dark at 24°. Growth measurements were determined when the growth in the control plate reached the edge of the plate. The experimental design was a completely randomized block and the assay was repeated twice. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control.

Strawberries and inoculation

Strawberry fruits were obtained from commercial market. The berries of uniform size, free of physical damage and fungal infection were selected. A quantity of 50 fruits were randomly distributed into five replicates of 10 fruits each for each treatment. Fruits were surface sterilized with 2.5% sodium hypochlorite for 3 min, followed by washing with distilled water $\times 3$. Fruits were arranged by groups of 10 in plastic containers having three layers of moistened blotters at the bottom. Conidia of *B. cinerea* or *R. stolonifer* were recovered from 2- and 1-week-old cultures respectively by adding 10 ml of sterile water to each plate. The mycelial suspension was filtered through three layers of sterile cheese cloth. The concentration of the conidial suspension was adjusted to 2×10^5 conidia per ml, and a drop of Tween 80 was added to the suspension. Each fruit was inoculated with 20 μl of conidial suspension near the stem scar region and stored at 13°.

Thyme volatiles

The inoculated fruits were arranged on moistened blotter in plastic containers (500 ml) at the beginning of the exposure. Aqueous solutions of thyme oils were prepared at 50, 100 and 200 ppm levels and stored in glass vials. Sponge dics (4 mm diameter, 2 mm thick) were saturated with different concentrations of thyme oil by immersion of the discs for 5 min. One disc was placed at the center of each plastic box containing 10 fruits, while discs saturated with sterile water were placed in the control containers. The boxes were sealed to obtained an atmosphere saturated with volatiles. After 8 h of exposure, the lids of the containers were replaced with ones having openings of 1–2 mm. These conditions prevented CO_2 accumulation and O_2 depletion in the atmosphere. After 4 days of exposure, the sponges were removed from the containers to avoid over exposure of the fruits to thyme oil volatiles. Strawberries were evaluated daily for symptoms, and decayed fruits were removed from the lot to avoid secondary infection. The data are presented as percentage of decayed fruits at 7 and 14 day intervals. The equipment was repeated three times, pooled data were analyzed by analysis of variance procedure and the means were separated by Duncan's multiple range test [32].

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