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In vivo antifungal activity of two essential oils from Mediterranean plants against postharvest brown rot disease of peach fruit

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ABSTRACT

Fresh fruits of several plants are susceptible to infection by several pathogenic fungi after harvest. Some synthetic fungicides are known to be highly effective in their control on various vegetables and fruits. In the present study the potential fungicidal activity of the essential oils obtained by thyme (*Thymus vulgaris*) and vervain (*Verbena officinalis*), respectively, against *Monilinia laxa*, *Monilinia fructigena*, and *Monilinia fructicola* was tested at various concentrations *in vivo*. The oil of thyme was mainly composed by *o*-cymene (56.2%), while the main components of the oil of vervain were citral (44.5%) and isobornyl formate (45.4%). The higher concentrations of both studied EOs from vervain (1000 ppm) and thyme (500 ppm) significantly reduced the brown rot lesion diameter. The lower concentrations of vervain (500 ppm) and thyme (250 ppm) EOs resulted in low effectiveness. This research revealed the potential fungicidal role *in vivo* of the essential oils on peach fruits postharvest. Moreover, the application of essential oils could be combined with other innovative postharvest treatments such as biocontrol agents.

1. Introduction

Fresh fruits of several plants are susceptible to infection by several pathogenic fungi after harvest. Some synthetic fungicides are known to be highly effective in their control on various vegetables and fruits. However, the use of synthetic fungicides is limited by the emergence of resistant fungus strains and, in some cases, their use is prohibited by law in postharvest phase. The growing of public concern over the health and environmental hazards associated with the increased levels of pesticide use in fruit orchards and the lack of continued renewed approval of some of the most effective active molecules have lead to develop alternative, safe and natural methods postharvest control (Lopez-Reyes et al., 2013). Recently, there has been a great interest in using essential oils (EOs) and plant extracts as possible natural substitutes for conventional synthetic pesticides. This has been mainly due to the concern over ecosystem pollution and pesticide resistance in pests and fungal pathogens (Holmes and Eckert, 1999; Camele et al., 2010).

EOs are volatile compounds produced in many plant species. These oils are thought to play a role in plant defense mechanisms against phytopathogenic microorganisms (Liu and Chu, 2002). EOs have been reported to control *Monilinia laxa* (Aderh. & Ruhland) Honey in stone fruit (Neiri et al., 2007) postharvest diseases in tomato, citrus, molds, food-borne, various bacteria (Banihashemi and Abivardi, 2011) and weeds (Macias et al., 2007). EO from *Thymus capitatus* (L.) Hoffm. & Link displayed antifungal activity on stored foods and inhibited the growth of both *Botrytis cinerea* (de Bary) Whetzel and *Monilinia fructicola* (G. Winter) Honey (Tsao and Zhou, 2000). The potential use of EOs by spraying or dipping techniques to control postharvest decay has been studied in vegetables, fruits and cut flowers (Dixit et al., 1995).

In vivo antifungal activity of some EOs derived from some Mediterranean plants such as *Thymus vulgaris* L., *Verbena officinalis* L. and *Origanum vulgare* (Link) Ietswaart, has been proved against some postharvest fungal pathogens such as *B. cinerea*, *Penicillium italicum* Wehmer, *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian and *Rhizopus stolonifer* (Ehrenb. Fr.) Vuill. In particular, the most effective EO was extracted from *T. vulgaris*, which controlled at 2000 ppm dose, the fruit rot caused by *B. cinerea*, *P. citrophthora* and *R. stolonifer* but was ineffective against *P. italicum*. The EOs extracted from *V. officinalis* inhibited infection caused by *B. cinerea*, *P. citrophthora* and O. *vulgare* oil was effective only against *P. citrophthora* (Camele et al., 2010). *Monilinia* spp. are responsible for brown rots (De Cal and Melgarejo, 1999) serious postharvest

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stone fruits diseases which cause significant losses in most temperate regions of the world. In particular, *M. fructicola* is included in the EPPO (European and Mediterranean Plant Protection Organization) A2 list of quarantine pest. *M. fructicola*, regularly present in Asia, North America and Australia, has been recently detected in Italy (Pellegrino et al., 2009) and other European countries, where it is rapidly replacing *M. laxa* as the main agent of brown rot on stone fruits (Lopez-Reyes et al., 2013). In this study, the potential fungicidal activity of two EOs, previously selected in tests *in vitro* by Camele et al. (2010), was investigated *in vivo* against three

Table 1
Percent compositions of thyme and vervain essential oils.

N.	Compound	Ki[a]	Ki[b]	*T. vulgaris*(%)	*V. officinalis*(%)	Identification[c]
1	α-Thujene	930	1035	t[d]	-[e]	1, 2
2	α-Pinene	938	1032	2.5 ± 0.1	0.2 ± 0.0	1, 2, 3
3	(-)-Camphene	953	1076	1.0 ± 0.1	-	1, 2, 3
4	Sabinene	973	1132	t	0.5 ± 0.0	1, 2, 3
5	Hepten-3-one	975		-	0.2 ± 0.1	1, 2
6	β-Pinene	978	1118	-	t	1, 2, 3
7	Verbenene	982	1131	t	-	1, 2
8	Myrcene	993	1174	0.1 ± 0.0	-	1, 2, 3
9	α-Phellandrene	995	1176	t	-	1, 2, 3
10	α-Terpinene	1012	1188	0.1 ± 0.0	t	1, 2, 3
11	*o*-Cymene	1020	1187	56.2 ± 0.2	0.1 ± 0.0	1, 2, 3
12	*p*-Cymene	1024	1280	0.1 ± 0.0	-	1, 2, 3
13	β-Phellandrene	1029	1218	0.2 ± 0.1	0.7 ± 0.2	1, 2, 3
14	Limonene	1030	1203	0.6 ± 0.0	2.3 ± 0.9	1, 2, 3
15	1,8-Cineole	1034	1213	t	0.4 ± 0.1	1, 2
16	(Z)-β-Ocimene	1038	1246	t	t	1, 2, 3
17	(E)-β-Ocimene	1049	1280	t	0.3 ± 0.1	1, 2, 3
18	γ-Terpinene	1057	1255	0.4 ± 0.0	0.1 ± 0.0	1, 2, 3
19	Terpinolene	1086	1265	0.7 ± 0.1	t	1, 2
20	Linalol	1097	1553	0.4 ± 0.1	0.1 ± 0.0	1, 2, 3
21	*cis*-Thujone	1105	1430	t	-	1, 2, 3
22	*trans*-Pinocarveol	1138	1654	t	t	1, 2
23	(-)-Citronellal	1143	1491	0.5 ± 0.1	-	1, 2, 3
24	*iso*-Borneol	1144	1633	0.1 ± 0.0	-	1, 2, 3
25	Camphor	1145	1532	t	-	1, 2, 3
26	*iso*-Pinocamphone	1153	1566	t	0.2 ± 0.0	1, 2
27	*trans*-Pinocamphone	1159	1160	t	t	1, 2
28	*iso*-Menthone	1163	1503	0.1 ± 0.0	-	1, 2, 3
29	Pinocarvone	1165	1587	t	t	1, 2
30	Borneol	1167	1719	0.2 ± 0.0	0.1 ± 0.0	1, 2, 3
31	Terpinen-4-ol	1176	1611	t	0.2 ± 0.0	1, 2, 3
32	dihydro-Carveol	1177	1755	0.2 ± 0.0	-	1, 2
33	*p*-Cymen-8-ol	1185	1864	t	t	1, 2
34	α-Terpineol	1189	1706	0.3 ± 0.0	0.3 ± 0.1	1, 2, 3
35	Myrtenal	1193	1648	0.3 ± n0.0	-	1, 2
36	Myrtenol	1196	1804	0.3 ± 0.0	-	1, 2
37	Isobornyl formate	1228	1596	-	45.4 ± 0.9	1, 2
38	*cis*-Anethole	1262	1780	-	0.2 ± 0.0	1, 2
39	(E)-Citral	1270	1727	-	44.5 ± 0.9	1, 2, 3
40	Isobornyl acetate	1277		t	t	1, 2
41	Bornyl acetate	1284	1591	t	t	1, 2
42	Thymol	1290	2198	8.7 ± 0.9	-	1, 2, 3
43	Carvacrol	1297	2239	24.4 ± 0.9	-	1, 2, 3
44	Methyl eugenol	1369	2023	-	t	1, 2
45	α-Copaene	1377	1497	t	0.2 ± 0.1	1, 2
46	Isoledene	1382	1367	t	0.1 ± 0.0	1, 2
47	β-Elemene	1387	1600	t	0.2 ± 0.1	1, 2
48	Longifolene	1411	1576	t	t	1, 2
49	β-Caryophyllene	1418	1612	0.1 ± 0.0	0.1 ± 0.1	1, 2
50	β-Cedrene	1424	1638	-	0.4 ± 0.1	1, 2
51	Aromadendrene	1437	1628	t	-	1, 2
52	α-Humulene	1455	1689	t	0.2 ± 0.0	1, 2
53	allo-Aromadendrene	1463	1661	t	0.1 ± 0.0	1, 2
54	γ-Gurjunene	1473	1687	t	t	1, 2
55	Bicyclogermacrene	1491	1756	-	0.1 ± 0.0	1, 2
56	*cis*-Muurola-4(14),5-diene	1510	1675	t	0.2 ± 0.1	1, 2
57	α-7-*epi*-Selinene	1518	1740	t	0.2 ± 0.1	1, 2
	Total			97.5	97.6	
	Monoterpenes hydrocarbons			61.9	4.2	
	Oxygenated monoterpenes			35.5	91.2	
	Sesquiterpenes			0.1	1.8	
	Oxygenated sesquiterpenes			-	-	
	Other compunds			-	0.4	

[a] Kovats retention index on HP-5 MS column.
[b] Kovats retention index on HP Innowax column.
[c] 1 = Kovats retention index, 2 = mass spectrum, 3 = co-injection with authentic compound.
[d] t = trace, less than 0.05%.
[e] - = not detected.

postharvest fungal pathogens of peach fruits with the aim to find an alternative mode of postharvest fruit disease control.

2. Material and methods

2.1. Plant material

T. vulgaris and *V. officinalis* were grown at the garden of Medicinal Plants in Salerno, State University Campus. Samples from the above plant species were collected at full flowering stage, in July 2012. Voucher specimens of the plants were deposited in the Herbarium of the Medical Botany Chair, Department of Pharmacy, Salerno University.

2.2. Extraction of essential oils

Five hundred grams of freshly picked aerial parts of *T. vulgaris* and *V. officinalis* were cut into small pieces and then subjected to hydrodistillation for 3 h, following the standard procedure described in the European Pharmacopoeia (2004). The oils were solubilized in *n*-hexane, dried over anhydrous sodium sulphate and stored under N_2 at 4 °C in the dark until tested and analyzed. All extractions were done in triplicate.

2.3. GC–FID and GC–MS analysis

Essential oils were analyzed by gas chromatography–flame ionization detector (GC–FID) and gas chromatography–mass spectrometry (GC–MS). GC–FID analyses were performed using a PerkinElmer Sigma-115 gas chromatograph with a data handling system and a FID. Analyses were carried out using a DB-1 fused silica column (30 m × 0.25 mm i.d.; 0.25 μm film thickness). The operating conditions were as follows: injector and detector temperatures, 250 °C and 280 °C, respectively; oven temperature program, 5 min isothermal at 40 °C, then at 2 °C/min up to 250 °C and finally held isothermally for 20 min. Aliquots of 1 μL were injected manually at 250 °C and in the splitless mode. Analysis was also run by using a fused silica HP Innowax polyethylene glycol capillary column (50 m × 0.20 mm i.d.; 0.20 μm film thickness). In both cases, helium was used as the carrier gas (1 mL/min). Diluted samples (1/100 v/v, in *n*-hexane) of 1 μL were injected manually at 250 °C and in the splitless mode. GC–MS analyses were carried out using a Hewlett-Packard 5890 A gas chromatograph connected on line to a HP mass selective detector (MSD 5970HP), equipped with a HP-1 fused-silica column (25 m × 0.25 mm i.d.; 0.33 μm film thickness); GC and GC–MS conditions: ionization voltage 70; electron multiplier energy 2000 V. Gas chromatographic conditions were as reported above; transfer line was kept at 295 °C.

Most constituents were identified by GC by comparison of their Kovats retention indices (R_i) [determined relative to the t_R of *n*-alkanes (C_{10}–C_{35})], with either those of the literature (Jennings and Shibamoto, 1980; Davies, 1990; Adams, 2007; Goodner, 2008) and mass spectra on both columns with those of authentic compounds available in our laboratories by means NIST 02 and Wiley 275 libraries (Wiley, 1998). The components' relative concentrations were obtained by peak area normalization. No response factors were calculated.

2.4. Fungal isolates

The plant pathogenic fungi tested derived from monoconidic cultures stored at 4 °C as pure culture-maintained in the mycotheca of the School of Food, Forestry and Environmental Science, Basilicata University, Potenza, Italy. The fungal species were cultured on potato dextrose agar (PDA) at 24 °C ± 2 °C. The micromycetes used were the following: *M. laxa* (isolate number 1517 from pear), *M. fructicola* (isolate number 1561 from plum) and *M. fructigena* (Aderh. & Ruhl.) Honey (isolate number 1521 from apple). The morphological identification was attempted using light microscope. In addition, DNA was extracted from the anamorphs of each tested species of *Monilinia* and amplified with primer ITS4/ITS5 (White et al., 2007). The amplicons obtained were directly sequenced and the resulting sequences were compared with those available in GenBank using BLAST software (Altschul et al., 1997). Potato dextrose agar (PDA), was used for the fungal culture media.

2.5. In vivo antifungal activity

Different series of peach fruits of cv. "Springcrest", each composed of 12 mature individual fruits (144 fruits total) not subjected to any pre- and postharvest chemical treatment, were superficially sterilized by a 10 min immersion in a 2% sodium hypochlorite solution, repeatedly washed with sterile distilled water, and then dried on sterile filter paper before being inoculated with one of the above mentioned three phytopathogenic fungi. Artificial fungal inoculation was performed at room temperature by puncturing with a sterile needle each fruit in three points apart 5 cm from each other and putting on each wound 10 μL of a suspension containing 10^6 CFU (Colony Form Unit)/mL spores of each tested fungus. Spore suspensions were obtained from 7–10 day old cultures grown in 9 cm diameter Petri dishes containing 14 mL PDA by adding two loopful of fresh fungal mycelium to 10 mL of sterile distilled water. The suspensions were filtered through a sterile muslin cloth and the desired spore concentration was adjusted by a further serial dilution in sterile distilled water. One day after inoculation, the single fruit series (36 fruits for each variant) were sprayed with an emulsion containing sterile distilled water, 0.2% Tween-20, and 250, 500 of thyme EO or 500, 1000 ppm of vervain EO. Each experiment was replicated twice. Twelve fruits after being wounded with a sterile needle, were sprayed only with sterile distilled water and used as negative control. Twelve fruits not inoculated fruits for each oil concentration were used as a control to determine the possible EOs phytotoxicity. All the fruit series were kept in moist chamber under light at high relative humidity (about 95%) for 4 days at room temperature before being observed for eventual symptoms appearance. The presence of inoculated *Monilinia* species was verified by reisolating them from the edge of lesions and morphologically identified using light microscope. The fungitoxicity effectiveness was expressed as diameter of brown rot lesion in mm on fruit respect to control.

2.6. Statistical analysis

Results were statistically processed and subjected to analysis of variance. Means significantly different were separated by the Tukey test using SPSS a software program, version 13.0 (2004).

3. Results and discussion

Table 1 shows the chemical composition of the two studied EOs; compounds are listed according to their elution order on a HP-5MS column. In *T. vulgaris* oil, forty-nine components were identified, accounting for 97.5% of the total oil. *o*-Cymene and carvacrol are the main constituents, accounting for 56.2% and 24.4% in thyme oil. Our data on thyme oil composition agree with the available literature. In fact, thyme oil was characterized by the high level of phenolic precursors (*p*-cymene and *o*-cymene) and phenolic compounds (Li et al., 2011; Nezhadali et al., 2012; Cheurfa et al., 2013) In the vervain EO, forty components were identified, accounting for 97.6% of the total oil. The oil is mainly constituted by monoterpenes (95.4%), of which oxygenated compounds represent 91.2%; citral and isobornyl formate are the main constituents,

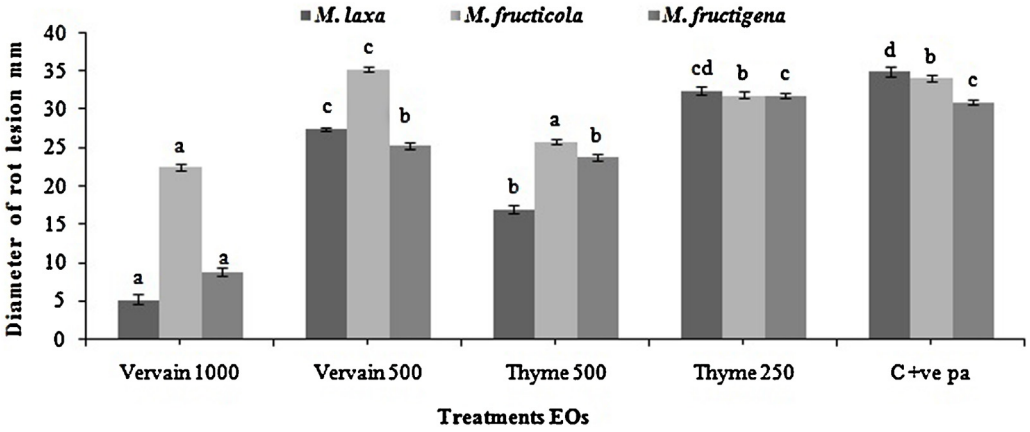

Fig. 1. Diameter of brown rot lesions on peaches with *Monilinia laxa, M. fructicola* and *M. fructigena*. Bars with different letters indicate means values significantly different at $P < 0.05$ according to Tukey test. Data are expressed as mean of three replicates ± SD. *: C + ve pa: is positive control where the fruits were challenged only with pathogens.

accounting for 44.5% and 45.4%, respectively. The composition of this EO differs from others reported in literature. In fact, Ardakani et al. (2003) reported 1-octen-3-ol and verbenone as the main constituents of the EO from *V. officinalis* collected in Iran. On the other hand, spathulenol, limonene and 1,8-cineole have been reported as the principal constituents of an EO of *V. officinalis* from Morocco (Chalchat and Garry, 1996).

The DNA sequences obtained from the anamorphs of each tested species of *Monilinia*, compared with those present in GenBank, confirmed the morphological identification by microscopic technique. One sequence of each species of *Monilinia* tested was submitted to GenBank with accession code HF678387 (*M. laxa*), HF678388 (*M. fructicola*) and HF678389 (*M. fructigena*). The higher concentrations of both studied of EOs from vervain (1000 ppm) and thyme (500 ppm) significantly reduced the brown rot lesion diameter. The lower concentrations of vervain (500 ppm) and thyme (250 ppm) EOs resulted scarcely effective (Fig. 1). In addition, the highest significant activity ($P < 0.05$) was observed against *M. laxa* with vervain EO at 1000 ppm, whereas the lowest significant ($P < 0.05$) effective EOs concentration was that of thyme EO at 250 ppm against the same micromycete.

The EOs did not show any phytotoxic effect on the fruits tissues at all studied concentrations. According to the above mentioned results of previous experiment (Camele et al., 2010), the present study is a complementary study to verify the *in vivo* antifungal activity of these EOs against serious postharvest pathogens like *Monilinia* spp., in particular for stone fruits peaches. The results showed that the two studied EOs inhibited fungal growth but their effectiveness varied.

The two EOs exhibited an inhibitory activity against the three target pathogenic fungi in different way. Probably their activity is related to the high percent amounts of monoterpenes and phenolic compounds. In fact, the major constituents of the EOs were *o*-cymene, isobornyl formate, citral, carvacrol and thymol. In recent years, interest has been generated in the development of safe antifungal agents such as plant-based EOs and extracts to control phytopathogens in agriculture (Adebayo et al., 2013).

Plant EOs and extracts have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies (Prabuseenivasan et al., 2006). Lopez-Reyes et al. (2013) reported the efficacy of different EOs (among which thyme essential oil) and their major components (α-pinene, *p*-cymene, carvacrol and thymol). Their results confirmed the bioactivity of those substances for *in vivo* postharvest treatment on stone fruit against brown rot and grey mold rot caused by *M. laxa* and *B. Cinerea*. Moreover, they reported the fungicidal effects of their single active components (citral, carvacrol, thymol and *o*-cymene) and/or by their synergic effect (Lopez-Reyes et al., 2010). Camele et al. (2012) studied *in vitro* the efficacy of β-phellandrene, β-pinene, camphene, carvacrol, citral, *o*-cymene, γ-terpinene and thymol, extracted from some Mediterranean aromatic plants, for controlling fruit rot disease caused by five postharvest pathogens and suggested the possibility that phenol components (carvacrol and thymol) can interfere with cell wall enzymes, like chitin synthase/chitinase, as well as with the α- and β-glucanases of the target species. Results obtained in this study agree with Adebayo et al. (2013) who showed the antifungal activity of EOs from oregano and monarda species. On the other hand, some commercial formulations mainly contain as main active ingredients thymol and carvacrol and are used to inhibit the mycelium growth and spore germination of *B. cinerea*.

4. Conclusion

The obtained results showed that the two tested EOs can be effectively utilized for controlling infections caused by *M. laxa*, *M. fructicola* and *M. fructigena*. In particular, the studied EOs proved to be effective in controlling all tested pathogenic fungi on peach fruit, but their efficacy is referred to the utilized concentrations and application time. On the other hand, the application of EOs could be combined with other innovative postharvest treatments such as biocontrol agents. In fact, the implementation of EOs treatment especially for controlling the postharvest diseases of stone fruit, is very promising because EOs are compatible with other conventional and new technologies and may be used, probably, also for some other fruit and vegetables. In the next future, trials will be carried out either to study the antifungal efficacy of the single components of each studied EO or to isolate chemically the most bioactive component(s) and to evaluate their possible use on the large scale.

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