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Essential oil chemical composition and antifungal effects on *Sclerotium cepivorum* of *Thymus capitatus* wild populations from Calabria, southern Italy

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Abstract: The paper reports the qualitative and quantitative composition and its antifungal activities of *Thymus capitatus* (L.) Hoffmanns. & Link, Lamiaceae, essential oils isolated by hydrodistillation from the aerial parts of plants collected in Calabria, Southern Italy. The essential oils of 22 samples were analysed by GC-Flame ionization detection and GC/MS. A total of sixty five compounds were identified. Phenols were present in highest percentage (average: 79,03%). Carvacrol was the main component (81,52%-78,40%) in all samples, confirming that *T. capitatus* is a carvacrol chemotype, according to literature data for this species. This essential oil was also characterized by high level of biogenetic precursor of the phenols: *p*-cimene (4,98%), γ-terpinene (3,13%) and by β-cariophyllene, were the most abundant sesquiterpene hydrocarbons. Antifungal activity against *Sclerotium cepivorum* Berk., a soil born fungus, was tested. At the concentration of 250 ppm there was no development of fungal mycelium. To our knowledge, studies have never been conducted on Calabria wild populations of *T. capitatus* essential oil nor were conducted studies on parasitic fungi of specific interest for crops such as *Sclerotium cepivorum*.

Article

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Introduction

In the recent years there is a renewed interest in natural products and then on plants as source of drugs, pharmaceuticals, perfumery products, cosmetics and aroma compounds used in food flavors and fragrances.

Most of the natural aromas are obtained by extraction of essential oils from officinal plants. Essential oils are volatile, natural, complex compounds characterized by a strong odour, produced by plants as secondary metabolites. The essential oils composition is strongly influenced by intrinsic factors such as species, cultivar, clone, ecotype and ecological factors as geographical origin, climatic conditions, soil, biotic and technological factors, cultivation techniques, types of collection processes, storage conditions of raw materials, processing technologies. Thus, wild plants of the same species but from different backgrounds can express different characters and chemical composition (Russo et al., 2012).

The quality of an aromatic plant and especially its biological properties are correlated to the chemical composition of its essential oils.

Furthermore, antimicrobial properties of plants have been recognized and used since ancient times for food preservation and in medicine (Conner, 1993). Because of health and economic considerations, the search for antifungal agents is extensive (Paster et al., 1995). Examination of indigenous plants material have been reported from all around the world *e.g.* Finland (Rauha et al, 2000), India (Ahmad & Beg, 2001), Argentina (Penna et al., 2001). Most of their properties are due to essential oils able to control microorganisms related to food spoilage by their antifungal (Cox et al., 1998; Cosentino et al., 1999; Elgayyar et al., 2001) and anti-bacterial (Canillac & Mourey, 2001) effects. These properties are due to many active phytochemicals, including flavonoids, terpenoids, carotenoids, coumarins and curcumins (Tepe et al., 2005).

In this work, the attention was focused on plants of the genus *Thymus*, which is one of the most important genera within the Lamiaceae family, which belong

about 215 species including several native species of the Mediterranean area, from Spain to North Africa to Turkey.

In Italy there are many wild species of the genus *Thymus*. These species are typical of garrigues, slopes and Mediterranean pine forests (Pignatti, 1982) and are characterized by a high intraspecific chemotypes variability, genetically encoded as a result of the influence of bio-pedo-climatic habitat (Russo et al., 1998). The most widespread species of thyme in Italy is *T. vulgaris*, that is present in the Mediterranean Basin with at least six different chemotypes (Panizzi et al., 1993). Other species of genus *Thymus*, pervasive in Italy, are: *T. serpyllum* L., *T. herba-barona* Loisel. (Corticchiato et al., 1998), *T. pulegioides* L. (Senatore, 1996; Russo et al., 2003), *T. longicaulis* C. Presl (Russo et al., 2003), and *T. capitatus* (L.) Hoffmanns. & Link, (Falchi Delitala et al., 1983; Ruberto et al., 1992; Biondi et al., 1993; Russo et al., 2003; Miceli et al., 2006; De Lisi et al., 2011).

In Calabria, Southern Italy, the genus *Thymus* is mainly represented by *T. capitatus* (syn. *Thymbra capitata* Cav., *Corydothymus capitatus* L. Rchb.f., *Satureja capitata* L.), a perennial, herbaceous shrub, growing at different altitude, from 0 to 1000 m above sea level, in the arid and rocky areas.

The plants of genus *Thymus* are widely used in food flavouring and culinary preparations, as well as in perfumery, folk medicine and in pharmacological sector as spasmolytic, antiseptic and expectorant (Rasooli & Mimostarfa, 2002).

Many works have focused on the chemical characterization of the essential oils of different species of *Thymus* with the aim of verifying the presence of chemotypes either for taxonomic that for biological properties: antimicrobial (Bhaskara et al., 1998, Bournatirou et al., 2007, Amarti et al., 2008), antifungal (Daferera et al., 2000; Arras & Usai, 2001; Omidbeygi et al., 2007; Marandi et al., 2011, Silva et al., 2012) antioxidant properties (Miguel et al., 2004; Sacchetti et al., 2005; Mkaddem et al., 2010). To our knowledge, studies have never been conducted on Calabria wild populations of *T. capitatus* essential oil nor were conducted studies on parasitic fungi of specific interest for crops such as *Sclerotium cepivorum* Berk.

In this work is reported the qualitative and quantitative composition of calabrian *T. capitatus* essential oil and its antifungal activity. Further investigations are underway to evaluate the antioxidant activity of this essential oil, regarded the increasing interest by consumers for its potential functional multi-purpose use (Sawamura, 2000).

Materials and Methods

Essential oil chemical composition

Plant material

Samples of wild populations of *Thymus capitatus* (L.) Hoffmanns. & Link, Lamiaceae, were collected during 2008 from four different areas of Calabria (Italy): Area A-"*Reggio Calabria*", Area B-"*Ionian Area Reggio Calabria*", Area C-"*Tyrrhenian Area Reggio Calabria*", Area D-"*Pollino Sud-West*" (Figure 1, Table 1). Six plants were harvested from each areas: A, B, and C and four plants from area D, totalling 22 samples.

Figure 1. Calabrian collection areas of wild population of thyme samples.

The samples were collected in June and July, during the "balsamic time" corresponding to the flowering stage. The plant material used for the extraction of the essential oil was air-dried at room temperature in the dark.

All samples were authenticated by Prof. Mariateresa Russo and voucher specimens were deposited at the Department of Science and Technology of Agro-Forestry and Environment, Mediterranean University of Reggio Calabria.

Essential oil extraction

Forty grams of air-dried aerial parts of each plant were hydrodistilled using a Clevenger-type apparatus for 3 h. Samples oil were dried over anhydrous sodium sulphate and stored at low temperature before analysis.

Table 1. Localization of *Thymus capitatus* wild populations, altitude and yield of essential oils.

Area	Samples	Locality	Altitude Average	Yield (% average±SD)
A *Reggio Calabria*	1 A	Gallina (RC)	140 m (a. s. l)	3,14±0,44
	2 A	Gallina (RC)		
	3 A	Lutrà Gallina (RC)		
	4 A	Arangea (RC)		
	5 A	Arangea (RC)		
	6 A	Arangea (RC)		
B *Ionian area Reggio Calabria*	7 B	Pentidattilo (RC)	250 m (a. s. l)	3,18±0,90
	8 B	Lazzaro (RC)		
	9 B	Melito Porto Salvo (RC)		
	10 B	Bova Superiore (RC)		
	11 B	Bova Inferiore (RC)		
	12 B	Pentidattilo (RC)		
C *Tyrrhenian area Reggio Calabria*	13 C	Fiumara di Muro (RC)	280 m (a. s. l)	3,44±0,56
	14 C	S. Roberto (RC)		
	15 C	Fiumara di Muro (RC)		
	16 C	S. Roberto (RC)		
	17 C	Fiumara di Muro (RC)		
	18 C	S. Roberto (RC)		
D *Pollino Sud-West*	19 D	Aquaformosa (CS)	750 m (a. s. l)	3,02±0,38
	20 D	Aquaformosa (CS)		
	21 D	Aquaformosa (CS)		
	22 D	Aquaformosa (CS)		

The essential oils were dissolved (2% v/v) in n-hexane before GC analysis.

GC and GC/MS analysis

T. capitatus essential oils were investigated by means of gas chromatography (GC) and the components were identified by GC-MS (gas chromatography-mass spectrometry) and by comparing the retention times of GC peaks with those of standards.

The quantitative GC analysis were carried out on a Shimadzu GC17A system equipped with a split/splitless injector and with a FID detector (Shimadzu, Japan) at the following conditions: fused-silica capillary column SE52 (Mega, Legnano, Italy) coated with (poly-5% diphenyl-95%-dimethyl-siloxane bonded phase column (30 m x 0.25 mm i.d.; 0.25 µm film thichness); the oven temperature increased from 60 to 80 °C at 1 °C/min, from 80 to 180 °C at 5 °C/min, holding 180 °C for 6 min, from 180 to 280 °C at 10 °C/min, holding 280 °C for 10 min; injector temperature was 250 °C in split mode; detector temperature was 270 °C; carrier gas was helium at 1 mL/min.

The quantitative composition was obtained by peak area normalization; the response factor for each component was considered equal to 1 and three replicates of each sample were made. Experimental result are expressed as the mean value.

For the identification of the compound, each sample was analysed using a Shimadzu GC-MS QP5050A quadrupole mass-selective spectrometer system.

As regards GC conditions, the column was a capillary column fused silica SE52 (30 m x 0.25 mm *i.d.*; 0.25 µm film thichness). Oven temperature and carrier flow are the same used in GC analysis. Injector temperature was 250 °C.

Following MS conditions were used: transfer line at 270 °C, ionization technique, electronic impact (EI=70 eV), acquisition mass range 40-450 amu.

Compounds were identified by comparison of mass spectra with those of standard compounds, computer matching with mass spectral libraries (NIST 21 and NIST 107) (NIST 1998) and published data (Adams, 2001); by comparison of their Linear Retention Indices (LRI) with those of authentic compounds and by comparing their LRI with literature (Mottram, 2005). LRI of the sample components were determined on the basis of the retention times of a mixture of homologue n-alkanes C6-C44 under the same GC/MS operating conditions. The value of retention indices were calculated according to

the equation proposed by Vandendool & Krantz (1963). Pure standards and homologue n-alkanes C6-C44 were purchase from Sigma Aldrich Co S.r.l Milan Italy. GC-MS Solution Ver.1.10 (Shimadzu, Japan) software allowed to measure, automatically the experimental LRI.

Antifungal activity

Microbial strain

T. capitatus essential oil was tested against the soil-borne fungus *Sclerotium cepivorum* Berk. isolated from sclerotia produced on Tropea red onion showing symptoms of white rot. Surface disinfected sclerotia were plated on potato dextrose agar (PDA, Liofilchem, Italy) amended with antibiotics (streptomycin sulphate 50 μg mL^{-1}, ampicillin sodium salt 50 μg mL^{-1}, Sigma Aldrich). The plates were incubated at 24 °C in the dark for 10 days to allow the mycelial growth. Small agar blocks containing hyphal tips were cut from the colony margins and transferred to fresh PDA and incubate at 24 °C in the dark.

Determination of antifungal effect of the essential oil on mycelial growth

The antifungal properties of essential oil was evaluated for assessing its contact phase effects towards mycelial growth of *S. cepivorum*. PDA medium was autoclaved and cooled in a water bath at 45 °C. Different concentrations of essential oil were prepared by dissolving the requisite amounts in ethanol 96% (fix amount of 25 μL), and mixed in the flasks with warm sterile molten medium to obtain a final concentration of 50, 100, 200, and 250 ppm. The PDA with essential oil was poured into sterile 90 mm Petri plates (20 mL/plate). Agar plugs (0,5 cm diameter) from the edge of 10 days-old *S. cepivorum* culture were placed at the centre of the each Petri plate and incubated at 24 °C. Two kind of control were used. In one, equal amount of ethanol was mixed in the medium; the other control consisted in pure PDA. The mean radial mycelial growth of the fungus was determined by measuring the diameter of the colony in two directions at right angles. The growth was compared to the pure PDA control plates. For each concentration and controls, four replicate plates were used. The experiments were conducted twice.

Statistical analyses

The percentage composition of the oils was computed by the normalization method from the GC peak areas. The analysis have been carried out in triplicate and the results are expressed as mean. Relative standard deviation (% RSD) was calculated for all compounds for each sample. Data for yield of essential oils and biological activity tests were presented as means±standard deviation (SD) both.

Results and Discussion

The yields of the essential oils extracted by hydrodistillation from the dried aerial part of *Thymus capitatus* (L.) Hoffmanns. & Link, Lamiaceae, collected at flowering from four distinct areas in Calabria differing for climatic conditions and altitude, were between 3,02% (w/w) to 3,44% (w/w), with a maximum of 4,65% (w/w), are showed in Table 1 (percent average on dry matter±SD).

The values were, significantly, higher than those reported in literature data for the same species of thyme (Hedhili et al., 2002; Faleiro et al., 2005; Bournatirou et al., 2007; Mkaddem et al., 2010).

The chemical composition of each sample of *T. capitatus* essential oils from the four considered areas was investigated by means of GC-FID and GC-MS in order to determine their qualitative and quantitative profiles. Table 2 shows the composition of *T. capitatus* essential oil for each sample by single compounds and then class of substances.

The compounds are listed in order of elution. A total of 65 compounds were identified in 22 samples analyzed, which constitute approximately 96% of the entire fraction. Data in Table 2 are expressed as relative percentage of the peak areas. Mean values of three replicates for each sample are given the percent relative standard deviation (%RSD).

Phenols were the components present in the highest percentage: carvacrol ranged from 76,79% to 81,52 while thymol represented 0,36-1,03%, confirming that *Thymus capitatus* is a carvacrol chemiotype, according to literature data for this species in the world (Kustrak et al., 1990; Ozek et al., 1995; Hedhili et al., 2002; Faleiro et al., 2005; Bournatirou et al., 2007; Amarti et al., 2008; Mkaddem et al., 2010) and in Southern Italy (Arras & Grella, 1992; Ruberto et al., 1992; Biondi et al., 1993; Miceli et al., 2006). Biogenetic precursor of the phenols were present in a 3,52-6,08% range *p*-cimene and 2,69-3,71% γ-terpinene. The variations between the main compounds of thyme essential oil can be explained by the biosynthetic relationship between the two phenols. The metabolic pathways for the carvacrol and thymol formation begins with the autoxidation of γ-terpinene to *p*-cymene and the subsequent hydroxylation to thymol. Instead the carvacrol originates from unsaturation of γ-terpinene to *p*-cymene followed by the hydroxylation to C-2 aromatic ring. So is evident the key role played by γ-terpinene in the process of flavoring and by *p*-cymene as precursor of oxygenates compounds. The γ-terpinene originates in the biosynthetic chain which from acetil-

Table 2. Peak identification, LRI valur and mean relative percentage peak areas of essential oil compounds of *Thymus capitatus* ([a,b]).

N°	Components	LRI esp[c,e]	LRI lit[d]	Area A	%RSD	Area B	%RSD	Area C	%RSD	Area D	%RSD
1	tricyclene	921	926	tr	-	tr	-	tr	-	tr	-
2	α-thujene	925	931	0.22	66.5	0.18	53.8	0.16	58.9	0.29	54.8
3	α-pinene	932	939	0.47	49.4	0.46	35.9	0.46	43.1	0.52	38.9
4	camphene	946	953	0.14	52.4	0.17	53.6	0.15	50.8	0.15	49.3
5	benzaldehyde	953	957	tr	-	tr	-	tr	-	tr	-
6	1-octen-3-ol	976	978	tr	-	tr	-	tr	-	0.10	26.8
7	β-pinene	975	977	0.13	33.1	0.25	19.7	0.31	27.7	0.12	26.5
8	3-octanone	984	986	tr	-	tr	-	-	-	-	-
9	β-myrcene	990	991	0.96	37.5	1.25	11.8	1.21	25.4	1.52	23.8
10	3-octanol	995	993	tr	-	tr	-	tr	-	tr	-
11	α-phellandrene	1004	1006	0.13	38.5	0.17	16.6	0.17	28.1	0.21	25.0
12	Δ^3-carene	1010	1011	tr	-	tr	-	tr	-	tr	-
13	α-terpinene	1016	1018	0.76	36.5	1.10	23.9	1.10	31.1	1.08	27.2
14	*p*-cymene	1023	1026	3.52	31.7	6.08	23.7	5.77	29.1	4.64	25.5
15	limonene	1027	1031	0.34	24.3	0.39	11.4	0.39	18.4	0.43	17.0
16	1,8-cineole	1030	1033	tr	-	tr	-	tr	-	tr	-
17	(*Z*)-β-ocimene	1036	1040	-	-	-	-	-	-	tr	-
18	(*E*)-β-ocimene	1046	1050	tr	-	tr	-	tr	-	tr	-
19	γ-terpinene	1057	1062	2.69	32.6	3.71	52.2	3.15	41.2	3.02	38.6
20	*cis*-linalool oxide	1072	1074	tr	-	tr	-	tr	-	tr	-
21	terpinolene	1087	1089	0.12	25.5	0.18	6.4	0.18	15.5	0.19	14.5
22	linalool	1099	1098	1.56	27.4	1.55	36.8	1.39	30.5	0.63	29.3
23	phenylethyl alcohol	1109	1110	tr	-	tr	-	tr	-	tr	-
24	borneol	1164	1165	0.67	23.0	0.53	36.0	0.46	29.2	0.55	29.3
25	4-terpineol	1177	1177	0.64	14.5	0.69	11.6	0.68	12.7	0.84	11.7
26	*p*-cymen-8 ol	1184	1183	tr	-	tr	-	tr	-	0.11	30.5
27	α-terpineol	1197	1199	tr	-	tr	-	0.10	27.1	tr	-
28	dihydrocarvone	1205	1207	tr	-	tr	-	tr	-	tr	-
29	thymol methyl ether	1233	1235	tr	-	tr	-	tr	-	tr	-
30	carvacrol methyl ether	1247	1244	0.14	32.5	0.13	53.7	tr	-	0.26	39.4
31	geraniol	1260	1255	0.12	11.7	tr	-	tr	-	0.13	24.6
32	geranial	1275	1270	tr	-	0.10	33.1	0.11	39.9	tr	-
33	thymol	1297	1290	0.36	10.4	0.50	20.8	0.44	15.7	1.03	21.4
34	carvacrol	1311	1298	81.52	3.7	76.79	3.7	78.78	3.6	77.65	3.7
35	α-cubebene	1354	1351	tr	-	tr	-	-	-	-	-
36	thymol acetate	1360	1355	tr	-	tr	-	tr	-	tr	-
37	α-copaene	1373	1376	tr	-	tr	-	tr	-	tr	-
38	β-bourbonene	1385	1384	tr	-	tr	-	tr	-	tr	-
39	β-cubebene	1389	1390	tr	-	tr	-	tr	-	tr	-
40	β-caryophyllene	1419	1418	2.98	16.0	3.31	9.0	2.92	13.0	4.50	13.0
41	β-gurjunene	1426	1432	tr	-	tr	-	tr	-	tr	-
42	α-bergamotene	1433	1433	tr	-	tr	-	tr	-	tr	-
43	aromadendrene	1435	1439	tr	-	tr	-	tr	-	tr	-
44	α-humulene	1454	1454	0.12	10.9	0.13	11.8	0.12	11.9	0.17	12.3
45	*allo*-aromadendrene	1462	1461	tr	-	tr	-	tr	-	tr	-
46	γ-muurolene	1480	1477	tr	-	tr	-	-	-	tr	-

47	valencene	1494	1491	tr	-	tr	-	tr	-	tr	-
48	α-muurolene	1499	1499	tr	-	-	-	-	-	-	-
49	β-bisabolene	1508	1509	0.28	42.9	0.16	76.0	-	-	tr	-
50	γ-cadinene	1513	1513	tr	-	tr	-	tr	-	tr	-
51	Δ-cadinene	1523	1524	tr	-	tr	-	tr	-	tr	-
52	spatulenol	1577	1576	tr	-	tr	-	tr	-	tr	-
53	caryophyllene oxide	1582	1581	0.84	7.6	0.83	27.3	0.69	17.5	0.82	17.4
54	viridiflorol	1591	1590	tr	-	-	-	tr	-	-	-
55	1-*epi*-cubenol	1628	1627	tr	-	tr	-	tr	-	-	-
56	T-cadinol	1636	1640	tr	-	tr	-	tr	-	tr	-
57	T-muurulol	1641	1641	tr	-	tr	-	tr	-	tr	-
58	α-cadinol	1655	1653	tr	-	tr	-	tr	-	tr	-
59	α-bisabolol oxide B	1660	1655	0.10	11.9	0.13	31.0	0.10	20.6	tr	-
60	β-bisabolol	1690	1683	tr	-	tr	-	tr	-	-	-
61	*cis*-lanceol	1760	1763	tr	-	tr	-	tr	-	tr	-

Classes of compounds of *Thymus* essential oil	Area A	%RSD	Area B	%RSD	Area C	%RSD	Area D	%RSD
Monoterpen hydrocarbons	9.56	30.4	14.06	21.4	13.15	26.7	12.31	25.8
Sesquiterpene hydrocarbons	3.68	16.5	3.87	7.1	3.23	12.0	4.96	11.7
Oxygenates compound	86.76	3.3	82.07	3.8	83.61	3.7	82.74	3.5
Phenols	82.06	3.7	77.45	3.6	79.32	3.8	78.98	3.5

[a]Identified injecting standard compounds; [b]Values are expressed as peak area percentage; [c]IUPAC 1997; [d]Mottram R, 2005. The LRI and Odour Database, Flavour Research Group, School of Food Biosciences, University of Reading, UK: http://www,odour,org,uk; [e]The average % RDS, for all experimental linear retention index (LRI) was equal or less than 0.2; tr, trace (<0.1%).

Co A leads to the synthesis of terpenoids through the formation of geranyl-pyrophosphate, just by cyclization of the latter (Russo et al., 1998). Among sesquiterpene hydrocarbons, β-cariophyllene was the most abundant and ranged from 2,92% to 3,31% (Table 2).

Figure 2 shows the typical GC profile of the volatile fraction of *T. capitatus* essential oil. In the samples from the different areas, carvacrol was the main component and the samples from area A showed the highest value (81,52%). Comparing the composition of the oils obtained from samples of different areas the major differences appears to be related to the content of phenols and their precursors, but, in general, there is a substantial homogeneity in the chemical composition of sample collected in different areas of the region for altitude, exposure and microclimate.

The antifungal activity of *Thymus* spp. essential oils have been reported from many authors (Daferera et al., 2000; Arras & Usai 2001; Pinto et al., 2006; Omidbeygi et al., 2007; Marandi et al., 2011; Silva et al., 2012; Teimouri, 2012). The use of essential oils from thyme as antifungal agent will be suitable for applications on the food industry (Viuda-Martos et al., 2007). The main reason for its suitability is its natural origin, which consumers find comforting and which is beneficial for the environment and the very low risk that pathogens will develop resistance to the mixture of components that make up the oils with its apparent diversity of antifungal mechanisms. These beneficial characteristics could increase food safety and shelf life.

In this study the *T. capitatus* essential oil have been used to test the antifungal activity against *Sclerotium cepivorum* Berk., a soil-borne pathogen responsible for the white-rot of plants of the genus Allium.

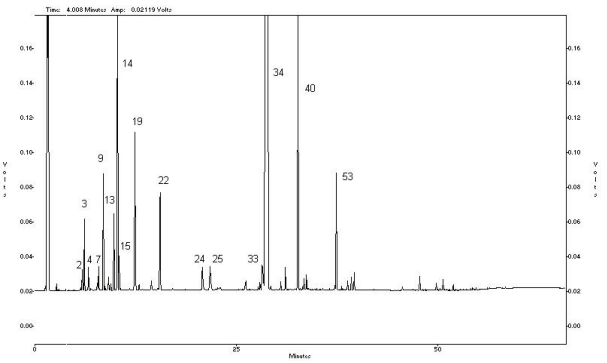

Figure 2. GC chromatogram of *Thymus capitatus* essential oil showing the major components. 2. α-thujene; 3. α-pinene; 4. camphene; 7. β-pinene; 9. β-myrcene; 13. α-terpinene; 14. p-cimene; 15. limonene; 19. γ-terpinene; 24. linalool; 27. borneol, 27. 4-terpineol; 35. Thymol, 36. carvacrol; 42. β-caryophyllene; 57. caryophyllene oxide.

This fungus causes the *Allium* White Rot (AWR), a serious and economically important disease of onions,

garlic and leeks (Coley-Smith, 1987; Entwistle, 1990a). The disease is present in most onion-growing areas of the world where environmental conditions are conducive for the pathogen (Entwistle, 1990b). In Calabria (S. Italy) *Slerotium cepivorum* is the most important disease of red onion 'Tropea' (Agosteo & Davino, 2009). Leaves of plants infected with the white rot pathogen show yellowing, leaf dieback and wilting. A semi-watery decay of the bulb scales results. Roots also rot and the plant can be easily pulled from the ground. Associated with the rot is a fluffy white mycelium, which develops around the base of the bulb with numerous small spherical sclerotia. The pathogen can attack plants from the seedling stage onwards, resulting in death before harvest or post-harvest decay in storage (Entwistle, 1990b). Sclerotia are the only means of survival and may remain dormant in the soil in the absence of host plants for more than 20 years (Coley-Smith, 1990; Entwistle, 1990b). Control of AWR has proved difficult due to the longevity of the sclerotia. Various chemical, biological and physical measures have been examined for the control of the disease, including fungicides (Ryley & Obst, 1994), soil fumigants (Entwistle, 1990a), soil solarization (Melero-Vara et al., 2000), biological control agents (Clarkson et al., 2002), vegetable wastes (Coventry et al., 2005) and aqueous extracts of plants (Montes Belmont & Prados-Ligero, 2006). In addition, a number of studies have attempted to mimic the natural phenomenon of sclerotia germination with chemicals (Coley-Smith & Parfitt, 1986; Coley-Smith, 1990).

The antifungal/fungitoxic activity of *T. capitatus* essential oil on *S. cepivorum* was evaluated *in vitro* by measuring of mycelial growth (Figure 3). Two measurements were done: after two weeks and after one month; the data are showed in the Table 3. After two weeks on growth plate, it was possible to see that the maximum reduction was obtained already at the *T. capitatus* essential oil concentration of 200 ppm where no mycelium developed. Lower the essential oil concentration greater the mycelial growth. Repeating the measurement after one month, there was growth even at 200 ppm (54,7% smaller than the control), but any mycelium develop was observed at 250 ppm (Table 3).

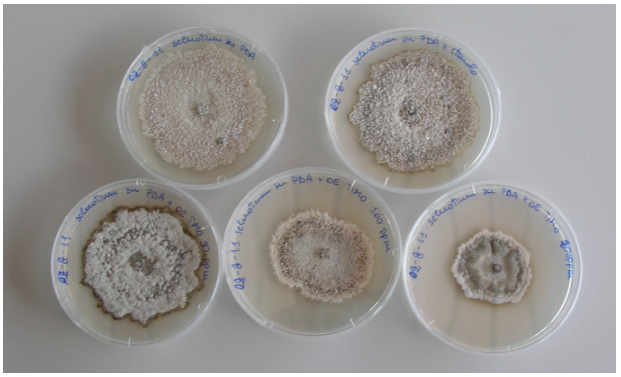

Figure 3. Mycelial growth of *Slerotium cepivorum* at different concentration of the *Thymus capitatus* essential oil after 1 month.

The complexity of the essential oils chemical composition with dozen of compounds makes very difficult the identification process for the component responsible for the antimicrobial activity. Often the antimicrobial activity result from the synergism or antagonism between several components even if Daferera et al. (2003) postulated that the antifungal activity of essential oils is mainly attributable to their main components although the possibility of other phenomena, such as synergy or antagonism with minor components.

The mechanisms of action of essential oil constituents (phenolic and terpenes) have not been completely elucidated. Prindle & Wright (1977) mentioned that the effect of phenolic compounds is concentration dependent. At low concentrations, phenols affect enzyme activity, especially of those enzymes associated with energy production; at greater concentrations, they cause protein denaturation. Phenolic compounds have also the ability to alter microbial cell permeability, permitting the loss of macromolecules from the interior. Conner & Beuchat (1984) suggested that the antimicrobial activity of the essential oils of herbs and species or their

Table 3. *Slerotium cepivorum* mycelial growth after two weeks and after 1 month on PDA woth different concentration of *Thymus capitatus* essential oil.

Effect of *Thymus capitatus* essential oil on *Slerotium cepivorum* mycelium growth				
Growth Substrate	Essential oil concentration	Avarage growth in cm after 2 weeks	Avarage growth in cm after 1 month	Growth reduction after 1 month (%)
PDA	0	6,4±3	6,4±2,5	0
PDA+EtOH	0	6,4±3	6,4±2,5	0
PDA+EtOH	50 ppm	5,3±3	6±2,5	6,3
PDA+EtOH	100 ppm	2,6±3	4,8±2,5	25
PDA+EtOH	200 ppm	0	2,9±2,5	54,7
PDA+EtOH	250 ppm	0	0	100

constituents such as thymol, carvacrol, eugenol etc., could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds.

Monoterpenes have antifungal activity on pathogenic fungi. Study on the effect of seven monoterpenes on phospholipid and sterol composition of *Sclerotium cepivorum* Berk. as well as lipid peroxidation of mycelia and sclerotia development showed that most of the monoterpenes increased the ergosterol content with a consequent diminution in the phospholipid/sterol ratio. This ratio is significantly decreased by the thymol treatment. When the fungus is grown in the presence of thymol, the saturated fatty acid content and the lipid peroxides is increased, concomitant with an increase of the sclerotial diameter. These results indicate that thymol may be promoting generation of lipid peroxides (Lucini et al., 2006). Study to determine the activity of the different components of thymus essential oils to find out the responsibility of each in the antimicrobial and antifungal activity showed that carvacrol is the most active (Pina-Vaz et al., 2004; Sokovic et al., 2009). Indeed, Arras & Usai (2001) found that *Thymus capitatus* essential oil show strong fungicidal activity against *Alternaria citri, Penicillium digitatum, P. italicum* and *Botrytis cinerea*.

From the reported result, it can be concluded that this carvacrol chemiotype *T. capitatus* essential oil exhibited, *in vitro*, antifungal activity against *S. cepivorum.* This essential oil can be used as antifungal agent, being the main reason for its natural origin that is beneficial for the environment and the very low risk that the pathogen will develop resistance to the mixture of components that make up the oil with their apparent diversity of antifungal mechanisms.

No research as been conducted yet on the antifungal activity of the essential oil *in vivo* conditions and inhibition of resting fungal structures such as sclerotia.

Further investigation are underway to evaluate different formulates for antifungal use of this *T. capitatus* essential oil as well as antioxidant activity, having regard to increasing interest by consumers and their exploitation for potential multi-purpose functional use.

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Authors contributions

RMt and PS contributed in collecting plant sample and identification, confection of herbarium. AGE and RA contributed to biological studies. RMt, PS, SF and SD contributed to chromatographic analysis running the laboratory work, analysis of the data and drafted the paper. RMt and PS designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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