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Antimicrobial activity of essential oils and their components against the three major pathogens of the cultivated button mushroom, *Agaricus bisporus*

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Abstract

Essential oils of *Matricaria chamommilla, Mentha piperita, M. spicata, Lavandula angusti folia, Ocimum basilicum, Thymus vulgaris, Origanum vulgare, Salvia officinalis, Citrus limon* and *C. aurantium* and their components; linalyl acetate, linalool, limonene, α-pinene, β-pinene, 1,8-cineole, camphor, carvacrol, thymol and menthol were assayed for inhibitory activity against the three major pathogens of the button mushroom, *Agaricus bisporus*, i.e. the fungi *Verticillium fungicola* and *Trichoderma harzianum* and the bacterium *Pseudomonas tolaasii*. The highest and broadest activity was shown by the *Origanum vulgare* oil. Carvacrol possessed the highest antifungal activity among the components tested.

Abbreviations: Iz – Inhibition zones; MBC – Minimal bactericidal concentration; MFC – Minimal fungicidal concentration; MIC – Minimal inhibitory concentration; MMC – Minimal microbial concentration

Introduction

Bacterial and fungal diseases are a major problem in mushroom cultivation; a high percentage of product is lost due to lower productivity, decrease of quality and shortened shelf life.

The white button mushroom *Agaricus bisporus* (Lange) Imbach is highly sensitive to bacterial, fungal and viral diseases. Major pathogens are the bacterium *Pseudomonas tolaasii*, the fungi *Verticillium fungicola* and *Trichoderma harzianum* and La France virus; they are all highly infectious and all cause serious damage (Geels et al., 1988). In Western countries the average annual damage due to those four diseases accounts for approximately 25% of the total production value. Although careful farm management and extreme hygiene may prevent major attacks, some diseases are very difficult to control on the farm. Also shelf life quality is severely affected by diseases that are still asymptomatic at the time of harvest. The use of disinfectants such as chlorine (household bleach) and formalin and the application of selected fungicides is general practice in the cultivation of mushrooms. This not only requires significant costs but the use of chemicals in cultivation leaves unwanted residues and several have become forbidden to be used. Most chemicals that are still permitted have failed to adequately control the major diseases of mushrooms because resistance is easily induced (Gea et al., 2005). Therefore good alternatives have to be found.

Pseudomonas tolaasii is a bacterium which causes bacterial blotch in the button mushroom

Agaricus spp., in Oyster mushroom *Pleurotus* spp., and in Shi-take *Lentinus edodes* (Munsch et al., 2000). The disease is characterized by the formation of yellow to brown lesions on mushroom caps and by bacterial growth in and discolouration of the stipes. Typically, spotting occurs at or near the edge of mushroom caps. Blotch occurs when mushrooms remain wet for a period of 4–6 h after watering. Controls include lowering the relative humidity of the air, and watering with a low percentage chlorine solution (calcium hypochlorite products are used since sodium hypochlorite products may burn caps). When the mushrooms stay wet, however, chlorine has little effect since the bacterial population reproduces at a rate that neutralizes the effect of the oxidizing agent (Geels et al., 1988).

Verticillium fungicola (Preuss) Hassebr. var. *fungicola* is the cause of dry bubble disease (Van Zaayen and Gams, 1982) of button mushrooms. The pathogenic fungus causes superficial, cinnamon-brown lesions of the mushrooms caps. Infection of the stem results in a bent and/or split stipe. The second major symptom is a dry bubble, i.e. a small, puffball-like fungal mass where the mushrooms should be. The disease is commonly prevented by hygiene and disinfection of the casing soil. Chemical controls include the application of chlorinated aromatic compounds like prochloraz i.e. Sporgon, (Geels et al., 1988). Many *Verticillium* isolates have become resistant to this fungicide (Gea et al., 2005). Also prochloraz is banned from use in many countries.

Green mold caused by *T. harzianum* is characterized by an aggressive, white mycelium that rapidly grows over the casing soil and onto the mushroom fruitbody, causing a soft decay. Masses of spores that eventually form are emerald green. The disease is rapidly spread due to the vast sporulation of this pathogen (Geels et al., 1988). Green mold can be prevented by hygiene and disinfection. Common disinfectants are ethanol, chlorine, formaldehyde, iodine, phenol, and quaternary ammonium compounds. Some of those agents are harmful to both mushrooms and humans. No cure exists for the disease. When severely infected, mushroom houses are usually heated by steam for 12 h (i.e. cooked out) to prevent further spread of the disease.

It was therefore necessary to search for new and effective antimicrobial substances that lack the drawbacks mentioned above. A solution for the problems may be found in some plants and the essential oils they contain. Essential oils have been used in traditional medicine because of their therapeutic effects against infectious diseases. Essential oils are active against several microbial pathogens of man and animals and also against those of plants. This has been confirmed by a multitude of papers (Knobloch et al., 1986; Thompson, 1990; Sivropoulou et al., 1996; Soković et al., 2002).

In this paper we describe the *in vitro* effects of essential oils of different plants on the pathogens of the three major diseases of the white button mushroom *A. bisporus*. For this study we used disc-diffusion and microdilution methods to determine the *in vitro* antibacterial activity of a wide variety of essential oils and their components. For antifungal activity we used microatmospheric, macro- and microdilution methods.

Materials and methods

Plant material

Plant material of *Salvia officinalis* was collected in Risan, Montenegro; *Origanum vulgare* was collected from the field near Paraćin, Serbia and Montenegro. All the plant samples were collected in the flowering period, July 2001. Voucher specimens for each plant have been deposited in the Herbarium of the Institute of Botany and Botanical Garden, Faculty of Biology, University of Belgrade.

Bacterial and fungal pathogens

P. tolaasii var. *tolaasii, V. fungicola* var. *fungicola* and *T. harzianum* were gifts of Dr. F. P. Geels, (Mushroom Experimental Station, Horst, The Netherlands) and were derived from the large culture collection of this research Institute.

Oil isolation and analysis

Essential oils from *Matricaria chamommilla*, *Mentha piperita*, *M. spicata*, *Lavandula angustifolia*, *Ocimum basilicum*, *Thymus vulgaris*, *Citrus limon* and *C. aurantium* were prepared by water-distillation and donated by the Institute of

Medicinal Plant Research "dr Josif Pančić", Belgrade. All the components tested (linalyl acetate, linalool, limonene, α-pinene, β-pinene, 1,8-cineole, camphor, carvacrol, thymol, menthol) were from the same Institute. Basically, dried leaves and flowering tops were ground to a powder; 50 g dry material was distilled for 2 h using a Clevenger-type apparatus. Analyses of the oil were performed by GC(-FID) and GC/MS on fused silica capillary column PONA (crosslinked methyl silicone gum, $50\ m \times 0.2$ mm, 0.5 μm film thickness). For this purpose a Hewlett–Packard, model 5890, series II gas chromatograph equipped with split-splitless injector was used (Soković et al., 2005; Glamočlija et al., 2006). Sample solution in ethanol (0.2%) was injected in split mode (1:100) at 250 °C. Detector temperature was 300 °C (FID), while column temperature was linearly programmed from 40–280 °C, at a rate of 2 °C min^{-1}. In the case of GC/MS analysis, a Hewlett–Packard, model 5971A MSD was used. The transfer line was kept at 280 °C. Identification of each individual compound was made by comparison of their retention times with those of pure components, matching mass spectral data with those from the Wiley library of 138,000 MS spectra (Adams, 1995). For library search a PBM based software package was used.

Tests for antibacterial activity

The antibacterial assays were carried out by the disc-diffusion (Verpoorte et al., 1983) and microdilution method (Hanel and Raether, 1988; Daouk et al., 1995; Espinel-Ingroff, 2001) in order to determine the antibacterial activity of oils and their components against the mushroom pathogenic bacterium *P. tolaasii*.

The bacterial suspension of *P. tolaasii* was adjusted with sterile saline to a concentration of 1.0×10^5 CFU ml^{-1}. The inocula were daily prepared and stored at +4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum.

Disc-diffusion test

Compounds were investigated by disc diffusion using a 4 mm filter disc. *Pseudomonas tolaasii* was cultured overnight at 28°C in LB medium and then adjusted with sterile saline to a concentration of 1.0×10^5 CFU ml^{-1}. The suspension was added to top agar (6 ml) and dissolved in Petri dishes (2 ml per agar plate) with solid Agar F (King's B medium). Filter discs with different concentrations of essential oils and main components (1.0–20.0 μl ml^{-1}) were placed on agar plates (five discs per agar plate). After 24 h of incubation at 28 °C the diameter of the growth inhibition zones was measured. A mixture of 10 mg ml^{-1} streptomycin (Sigma P 7794) and 10e4 I.U ml^{-1} penicillin (Sigma S0890) was used as a positive control. Five microlitre of the mixture was applied to each disc. All tests were carried out in duplicate; replicates were done for each oil and each component. Each experiment was repeated twice.

Microdilution test

MICs and MBCs were determined using 96-well microtitre plates. The bacterial suspension of *P. tolaasii* was adjusted with sterile saline to a concentration of 1.0×10^5 CFU ml^{-1}. Compounds to be investigated were dissolved in broth LB medium (100 μl) with inoculum of *P. tolaasii* (1.0×10^4 CFU per well) to achieve the required concentrations (0.5–15.0 μl ml^{-1}). The microplates were incubated for 24 h at 28 °C. The lowest concentrations without any visible growth under the binocular microscope were defined as MICs. MBCs were determined by serial sub-culture of 2 μl suspension into microtitre plates containing 100 μl of broth per well and further incubation for 72 h. MBC defined 99.5% mortality of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank and the positive control. A mixture of streptomycin + penicillin was used as a positive control using the same concentrations as in the disc diffusion test. Two replicates were done for each oil and each component. The experiment was repeated twice.

Test for antifungal activity

The essential oils and components were tested for antifungal activity by the microatmosphere test (Zollo et al., 1998), macrodilution test (Ishii, 1995) and microdilution test method (Hanel and

Raether, 1988; Daouk et al., 1995; Espinel-Ingroff, 2001) against three strains of mushroom pathogenic fungi *T. harzianum* and *V. fungicola*.

Microatmosphere test

The essential oils and their components were investigated by the microatmosphere method which allows the determination of the antifungal activity of the vapour phase of the essential oils which diffuse towards the agar in an inverted Petri dish method (Zollo et al., 1998). The assay was performed using mushroom pathogenic fungi, with three isolates of *T. harzianum* and *V. fungicola*. Petri dishes were filled with malt agar (MA), and then seeded with a 7 day-old mycelial culture of the tested fungi. The Petri dishes were then inverted and the determined amount of essential oils impregnated on sterile filter paper discs (4 mm diam) attached to the inverted lid (1 disc per lid). The Petri dishes were wrapped with parafilm along the rim, inverted and incubated for 21 days at 25 °C in an incubator. MIC of essential oils that inhibit the total growth of fungi was noted every 7 days. Bifonazole was tested at concentration of 50 μl per disc. Two replicates were done for each oil and each component. The experiment was repeated twice.

Macrodilution test

The essential oils (0.5–35.0 $\mu l\ ml^{-1}$) and components (0.05–13.0 $\mu l\ ml^{-1}$) investigated were mixed with 0.01% Tween 20 surfactant and dissolved in molten MA medium. The fungi were inoculated in the centre of Petri dishes and incubated for 21 days at 25°C. Mycelial growth was observed every 7 days and compared with the control. The commercial fungicide prochloraz (Sporgon) was used as a positive control (Ishii, 1995). Two replicates were done for each oil and components and the experiment was repeated two times.

Microdilution test

The microdilution test for antifungal activity was carried out as described under tests for antibacterial activity, with the following exceptions. Compounds investigated were dissolved in broth Malt medium (100 μl) with fungal inoculum (1.0×10^5 CFU per well) to achieve the required concentrations (0.125–20.0 $\mu l\ ml^{-1}$ for essential oils and 0.02–11.0 $\mu l\ ml^{-1}$ for components). The microplates were incubated for 24 h at 25 °C. MFC defined 99.5% mortality of the original inoculum (Hanel and Raether, 1988; Daouk et al., 1995; Espinel-Ingroff, 2001). Prochloraz (Sporgon) was used as a positive control. Two replicates were done for each oil and each component. The experiment was repeated twice.

Statistical analyses

All the MIC, MBC and MFC values were logarithmically transformed. The effects of antimicrobial activity of essential oils and components were analysed by two factorial analysis of variance (ANOVA). Also the Post-hoc LSD test (least significant difference) was done to detect the differences of antifungal activity between the oils and the components, and between respective oil components and the control. The Package programme Statistica (release 4.5, Copyright StatSoft, Inc. 1993) was used for statistical evaluation.

Results

The results of the chemical analyses of essential oils investigated are presented in Table 1. The main components in *M. spicata* oil are menthon (21.92%) and carvon (49.52%). In *M. piperita* the main components are menthon (12.70%), menthol (37.40%) and methyl acetate (17.37%). Limonene is the most abundant component in *C. limon* (59.68%) and *C. aurantium* (90.01%) oils. In *M. chamommilla* oil *trans*-β-pharnesene is the major component (43.47%). Linalool (27.21%) and linalyl acetate (27.54%) are the most abundant components in *L. angustifolia* oil. Linalool is also the main component in *O. basilicum* oil with 59.25%. Camphor (16.67%) and α-thujone (31.65%) are the main components in *S. officinalis* oil. In *O. vulgare* oil carvacrol (64.50%) is the dominant component. The major components of *T. vulgaris* oil are p-cymene (18.99%) and thymol (64.50%).

The results of antifungal activity of essential oils investigated obtained by the macrodilution method are presented in Figure 1. The best antifungal activity can be seen for *O. vulgare* and *T. vulgaris* oils. The MIC for *O. vulgare* oil is 0.5–1.0 $\mu l\ ml^{-1}$

Table 1. Chemical composition of essential oils investigated

Components	*M. s.* %	*M. p.* %	*C.l.* %	*C.a.* %	*M.c.* %	*L. a.* %	*O.b.* %	*S.o.* %	*O.v.* %	*T.v.* %	RI
Tricyclene	0.31	–	0.39	–	0.15	0.04	–	–	–		926
α-Thujene	0.07	–			–	0.58	–	0.14	1.9	1.17	931
α-Pinene	0.99	–	2.85	–	–	0.19	0.10	4.77	–	1.21	939
Camphene	–	–	–	–	0.08	–	0.06	6.90	–	0.83	948
Sabinene	0.71	2.52	–	–	0.35	–	–	0.12	2.20	0.58	973
β-Pinene	0.40	–	17.25	–	–	–	–	1.74	–	0.41	980
β-Myrcene	2.28	0.50	1.72	–	–	–	0.31	1.09	–	1.06	991
3-Octanol	–	0.13			–	–	–	–	–		993
δ-3-Carene	–	–	–	6.20	–	–	–	–	2.20		1011
α-Terpinene	–	0.12	–	–	0.10	0.25	0.05	–	–	0.65	1018
p-Cymene	0.49	0.10	–	–	0.16	–	–	0.99	10.90	18.99	1026
Limonene	5.77	6.85	59.68	90.01	0.29	8.50	0.91	2.56	–	0.46	1030
1,8-Cineole	3.06	5.59	–	–	0.38	3.34	0.82	8.70	–	0.76	1031
cis-Ocimene	–	0.13	0.13	–	1.65	–	0.12	–	–		1040
trans-Ocimene	–	0.19	–	–	1.92	–	0.46	–	–	1.30	1050
γ-Terpinene	1.36	0.28	11.21	–	0.12	–	–	0.35	10.80	4.08	1068
cis-Linalool oxide	–	–	–	–	–	2.44	–	–	–		1072
Henchone	–	–	–	–	–	0.59	–	–	–		1087
α-Terpinolene	0.30	0.10	0.29	–	–	–	0.43	0.29	–		1088
trans-Sabinene hydrate	–	–	–	–	0.32	–	–	–	–		1097
Linalool	–	0.17	–	–	–	27.21	69.25	–	–	0.74	1098
α-Thujone	–	–	–	–	–	–	–	31.65	–	–	1102
Endo-fenchol	–	–	–	–	–	0.09	–	–	–		1112
β-Thujone	–	–	–	–	–	–	–	4.61	–		1114
Iso-3-thujanol	–	–	0.21	–	–	–	–	–	–		1133
trans-Limonene oxide	–	–	0.17	–	–	–	–	–	–		1137
Camphor	–	–	–	–	–	1.07	0.30	16.67	–	0.17	1143
Menthon	21.92	12.70			–	–	–	–	–		1154
Menthofuran	–	6.82			–	–	–	–	–		1164
Borneol	–	–	–	–	–	2.51	0.27	2.64	–	1.72	1165
Menthol	0.52	37.40			–	–	–	–	–		1173
Terpin-4-ol	0.68	–	–	–	–	2.09	–	0.37	–	1.78	1177
α-Terpineol			0.27			4.20	0.63	0.11	–		1189
cis-Dihydrocarvon	0.33	–	–	–	–	–	–	–	–		1193
Methyl chavicol	–	–	–	–	–	–	2.38	–	–		1195
trans-Dihydrocarvon	0.45	–	–	–	–	–	–	–	–		1200
trans-Carveol	0.22	–	–	–	–	–	–	–	–		1217
Nerol	–	–	–	–	–	–	0.39	–	–		1228
Thymol-Methyl-eter	–	–	–	–	–	–	–	–	–	0.16	1235
Neral	–	–	0.84	–	–	–	–	–	–		1240
Carvone	49.52	–	–	–	–	–	0.06	–	–		1242
Pulegone	–	1.23	–	–	–	–	–	–	–		1243
Carvacrol-methyl-ether	–	–	–	–	–	–	–	–	–	1.73	1244
Piperitone	0.57	0.81	–	–	–	–	–	–	–		1252
Geraniol	–	–	–	–	–	–	1.87	–	–		1253
trans-Anethole	0.48	–	–	–	–	–	–	–	–		1283
Linalyl acetate	–	–	–	–	–	27.54	–	–	–		1257
Bornyl acetate	–	–	–	–	–	0.06	0.30	–	–		1285
Lavandulyl acetate	–	–	–	–	–	6.54	–	–	–		1289
Thymol	–	–	–	–	–	–	–	–	3.50	48.92	1290
Menthyl acetate	–	17.37	–	–	–	–	–	–	–		1294
trans-Pinocarvyl acetate	–	–	–	–	–	0.16	–	–	–		1297
Carvacrol	–	–	–	–	–	–	–	–	64.50	3.45	1298
Eugenol	–	–	–	–	–	–	1.42	–	–		1356
Neryl acetate	–	–	0.64	–	–	2.02	–	–	–		1365
α-Copaene	–	–	–	–	–	–	0.38	–	–		1376

Table 1. (continued)

Components	*M. s.* %	*M. p.* %	*C.l.* %	*C.a.* %	*M.c.* %	*L. a.* %	*O.b.* %	*S.o.* %	*O.v.* %	*T.v.* %	RI
Geranyl acetate	–	–	0.55	–	–	2.95	–	–	–		1383
β-Bourbonene	1.27	0.41	–	–	–	–	–	–	–		1384
β-Elemene	–	–	–	–	–	–	0.82	–	–		1391
β-Caryophyllene	0.71	0.29	0.44	–	0.35	–	0.56	2.20	2.50	3.45	1418
α-*trans*-Bergamotene	–	–	0.87	–	–	–	1.02	–	–		1436
α-Guaiene	–	–	–	–	–	–	1.11	–	–		1439
(Z)-β-Farnesene	–	0.69	–	–	–	–	–	–	–		1443
α-Humulene	–	–	–	–	–	–	0.51	3.41	–	0.30	1454
trans-β-Pharnesene	–	–	–	–	43.47	–	–	–	–		1458
Germacrene D	0.26	0.48	–	–	0.39	–	–	–	–	0.33	1480
β-Selinene	–	–	–	–	–	–	1.05	–	–		1485
α-Selinene	–	–	–	–	–	–	1.66	–	–		1494
Bicyclogermacrene	–	1.29	–	–	5.21	–	–	–	–		1495
α-Zingiberene	–	–	–	–	–	–	0.58	–	–		1496
α-Muurolene	–	–	–	–	–	–	0.09	–	–		1499
trans-β-Guaiene	–	–	–	–	–	–	2.10	–	–		1500
Germacrene A	0.49	0.47	–	–	–	–	–	–	–		1503
β-Bisabolene	–	–	1.29	–	–	–	–	–	–		1509
γ-Cadinene	–	–	–	–	–	–	2.48	0.03	–		1513
δ-Cadinene	–	0.79	–	–	–	–	1.13	0.07	–		1524
trans-γ-Bisabolene	–	–	–	–	8.48	–	–	–	–		1533
cis-Nerolidol	–	–	–	–	–	–	0.11	–	–		1534
α-Cadinene										2.23	1538
Caryophyllene oxide	–	–	–	–	–	–	–	0.30	–		1581
Viridiflorol	–	0.17	–	–	–	–	–	3.03	–		1590
Epi-α-muurolol	–	–	–	–	–	–	0.43	–	–		1641
α-Cadinol	–	–	–	–	–	–	2.56	–	–		1653
Bisabolol oxide B	–	–	–	–	9.09	–	–	–	–		1655
Bisabolon oxide	–	–	–	–	6.06	–	–	–	–		1682
Chamazulene	–	–	–	–	5.62	–	–	–	–		1725
cis-Farnesol	–	–	–	–	–	–	–	–	–		1713
Bisabolol oxide A	–	–	–	–	8.50	–	0.19	–	–		1744
Total	97.08	97.60	98.8	96.21	92.78	97.47	96.96	93.11	98.50	96.08	

M.s. – Matricaria spicata, *M.p.* – Mentha piperita, *C.l.* – Citrus limon, *C.a.* – Citrus aurantium, *M.c.* – Matricaria chamommilla, *L.a.* – Lavandula angustifolia, *O.b.* – Ocimum basilicum, *S.o.* – Salvia officinalis, *O.v.* – Origanum vulgare, *T.v.* –Thymus vulgaris.

while *T. vulgaris* oil showed MIC at 1.5–2.0 μl ml^{-1}. Oils isolated from *M. spicata* and *M. piperita* exhibited antifungal activity at 3.5–5.0 μl ml^{-1}, and 3.0–4.0 μl ml^{-1}, respectively. *Ocimum basilicum* oil possessed antifungal activity at 15.0–20.0 μl ml^{-1}, while MIC for sage and lavender oils were 20.0–25.0 μl ml^{-1}. *Citrus* oils showed MIC at 25.0–35.0 μl ml^{-1}, and oil from *M. chamommilla* showed the lowest antifungal potential with MIC at 30.0–35.0 μl ml^{-1}. Bifonazole showed antifungal effect at 20.0–30.0 μl ml^{-1}. *Citrus* oils and *M. chamommilla* oil showed lower potential than bifonazole. The LSD test showed a significant difference between the antifungal activity of all the oils tested except for *Citrus* oils and *M. chamommilla* oil; these possessed almost the same activity (Figure 1).

The active concentration of the essential oils obtained by the microdilution method was lower than in the macrodilution method (Figure 2). However, Oregano oil showed the best antifungal potential in this method, with a MIC of 0.125–0.5 μl ml^{-1} and an MFC 0.25–1.0 μl ml^{-1}. *Thymus vulgaris* oil inhibited the fungal growth at 0.5–1.5 μl ml^{-1} and was fungicidal at 1.0–1.5 μl ml^{-1}. MIC for *M. spicata* oil was 0.5–2.5 μl ml^{-1} and MFC was 1.5–2.5 μl ml^{-1}, while *M. piperita* oil showed inhibitory effect at 2.5–3.5 μl ml^{-1} and fungicidal effect at 3.0–4.0 μl ml^{-1}. Basil oil exhibited inhibitory activity at 1.0–4.0 μl ml^{-1} and MFC was 2.0–4.0 μl ml^{-1}. MIC and MFC for Lavender oil were 2.0–2.5 μl ml^{-1} and 4.0–5.0 μl ml^{-1}, respectively. Sage oil showed better activity in this method with MIC at 5.0–

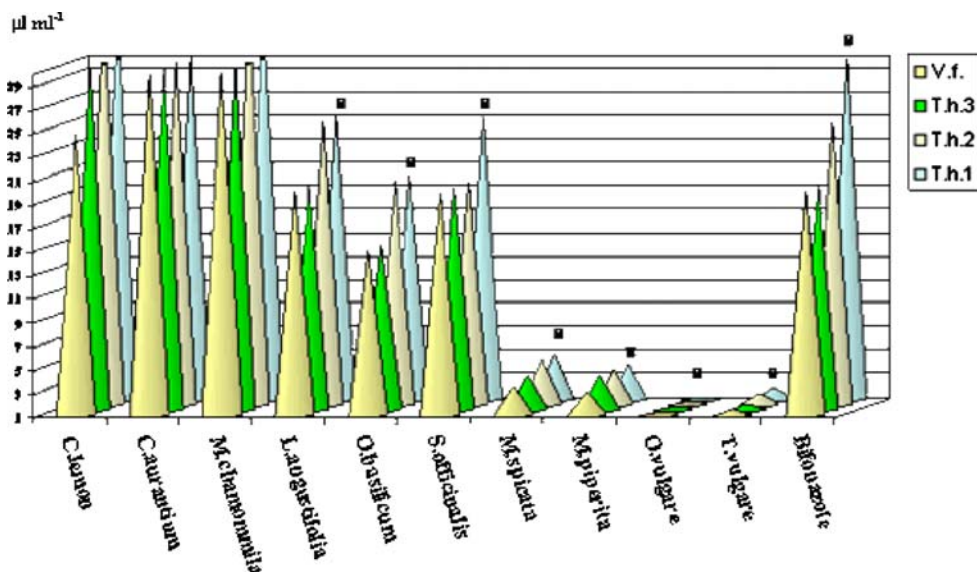

Figure 1. Minimal inhibitory concentration (MIC – μl ml^{-1}) of essential oils and bifonazole in macrodilution method (a – significant differences between the effect of essential oils, $P < 0.001$, ANOVA test).

10.0 μl ml^{-1} and MFC at 10.0 μl ml^{-1}. Both *Citrus* oils possessed the same activity, with inhibitory and fungicidal activity at the equal concentration of 10.0–15.0 μl ml^{-1}. As in the macrodilution method the *M. chamommilla* oil showed the lowest antifungal potential, with a MIC of 10.0–20.0 μl ml^{-1} and MFC of 15.0–20.0 μl ml^{-1}. The commercial fungicide, bifonazole, used as a positive control, showed MIC at 7.0–15.0 μl ml^{-1} and MFC at 8.0–15.0 μl ml^{-1}. It can be seen that all the oils tested showed better antifungal potential than bifonazole, with the exception of *Citrus* and *M. chamommilla* oils.

All the oils investigated possessed lower antibacterial than antifungal potential. MIC of Oregano oil against *P. tolaasii* was 0.5 μl ml^{-1}, and MBC was 1.0 μl ml^{-1}. Thyme oil showed bacteriostatic activity at 1.0 μl ml^{-1}, and was bactericidal at 1.5 μl ml^{-1}. *Mentha* oils showed bacteriostatic activity at 0.5–1.5 μl ml^{-1}, and was

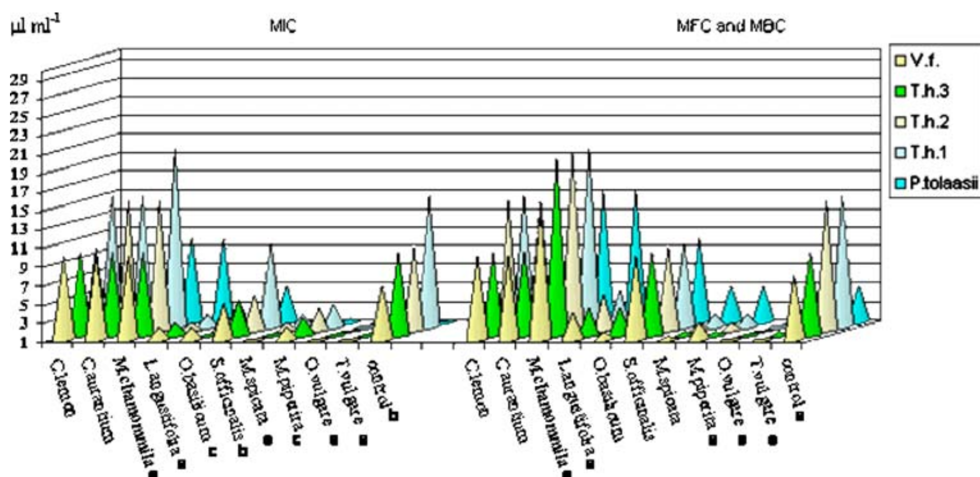

Figure 2. Antifungal and antibacterial activity (MIC, MBC and MFC – μl ml^{-1}) of essential oils in microdilution method (significant differences between the effect of essential oils a – $P < 0.001$, b – $P < 0.005$, c – $P < 0.05$, ANOVA test).

bactericidal at 5.0 μl ml^{-1}. Basil oil exhibited MIC at 2.5 μl ml^{-1}, and MBC at 5.0 μl ml^{-1}. Sage oil showed inhibition of bacterial growth at 5.0 μl ml^{-1}, and MBC at 10.0 μl ml^{-1}. *Citrus* oils showed bacteriostatic activity at 7.0 μl ml^{-1}, and were bactericidal at 10.0 μl ml^{-1}. *Lavandula angustifolia* and *M. chamommilla* oils were active at the same concentration, i.e. MIC at 10.0 μl ml^{-1} and MBC at 15.0 μl ml^{-1}. Streptomycin + penicillin showed antibacterial activity at 1.0 μl ml^{-1} (MIC) and 5.0 μl ml^{-1} (MBC). From the observed results it can be seen that only *O. vulgare, T. vulgaris* and *M. spicata* oils possessed better antibacterial activity than the commercial preparation that we used as a positive control. *Mentha piperita* and *O. basilicum* oils showed almost the same activity as streptomycin + penicillin. The LSD test showed that the MICs of essential oils in the microdilution method are statistically similar for the *Citrus* oils, *O. basilicum* and *M. piperita*, while Sage oil showed almost the same activity as the control. All the other oils showed statistically significant differences in activity. According to the LSD test oils of *O. basilicum* and *M. spicata* showed statistically similar activity, while *S. officinalis* and *C. aurantium* exhibited almost the same MMCs (Figure 2).

Among the components tested in the macrodilution method, carvacrol, thymol and menthol showed the best antifungal potential, with very low MIC of 0.05–0.5 μl ml^{-1}, 0.125–0.5 μl ml^{-1} and 0.25–1.5 μl ml^{-1}, respectively. Linalool also possessed very strong activity with MIC at 2.0–8.0 μl ml^{-1}. α-pinene, 1,8-cineole and camphor showed antifungal potential at 4.0–9.0 μl ml^{-1}, while linalyl acetate and β-pinene were active against fungi at 6.0–11.0 μl ml^{-1}. Limonene exhibited the lowest activity with MIC at 8.0–13.0 μl ml^{-1}. All the components investigated showed better antifungal potential than bifonazole (20.0–30.0 μl ml^{-1}). The LSD test showed that there was no statistically significant difference in MIC for camphor and α-pinene, 1,8-cineole and α-pinene, while thymol and carvacrol possessed almost the same MIC, which means that these components reacted very similarly (Figure 3).

The results of antimicrobial activity of essential oil components obtained by the microdilution method are presented in Figure 4. As in the macrodilution method carvacrol, thymol and menthol showed the best activity with MIC at 0.02–0.25 μl ml^{-1}, 0.05–0.25 μl ml^{-1} and 0.05–1.5 μl ml^{-1}, respectively, and MFC at 0.05–0.25 μl ml^{-1}, 0.125–0.5 μl ml^{-1} and 0.25–1.5 μl ml^{-1}. Linalool and 1,8-cineole showed also very good antifungal activity with MIC 2.0–5.5 μl ml^{-1} and 2.0–7.0 μl ml^{-1} and MFC at 2.0–6.0 μl ml^{-1} and 3.0–7.0 μl ml^{-1}. α-pinene, β-pinene and camphor possessed almost the same activity with MIC at 3.0–8.0 μl ml^{-1}, 4.0–7.0 μl ml^{-1} and 3.0–8.0 μl ml^{-1}, while MFCs were at 3.0–

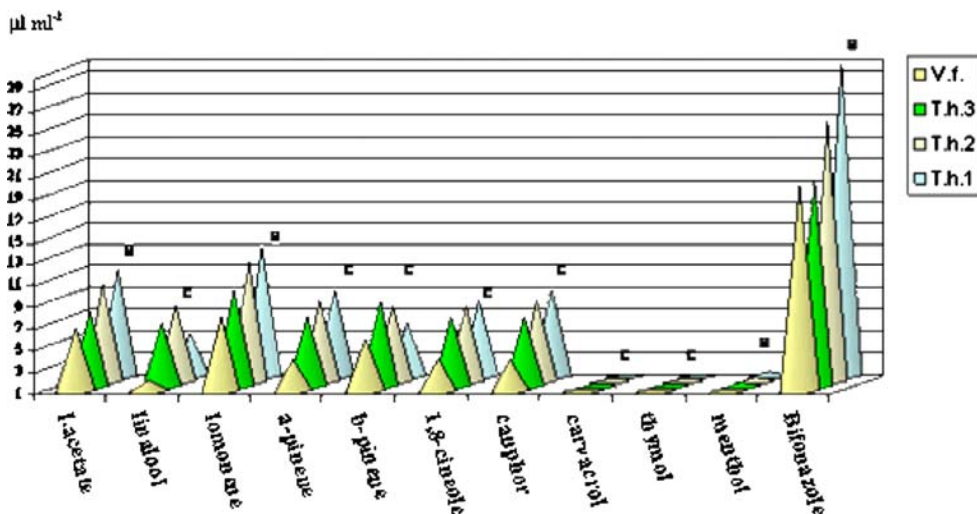

Figure 3. Minimal inhibitory concentration (MIC – μl ml^{-1}) of essential oils components in macrodilution method (significant differences between the effect of essential oils a – $P < 0.001$, c – $P < 0.05$, ANOVA test).

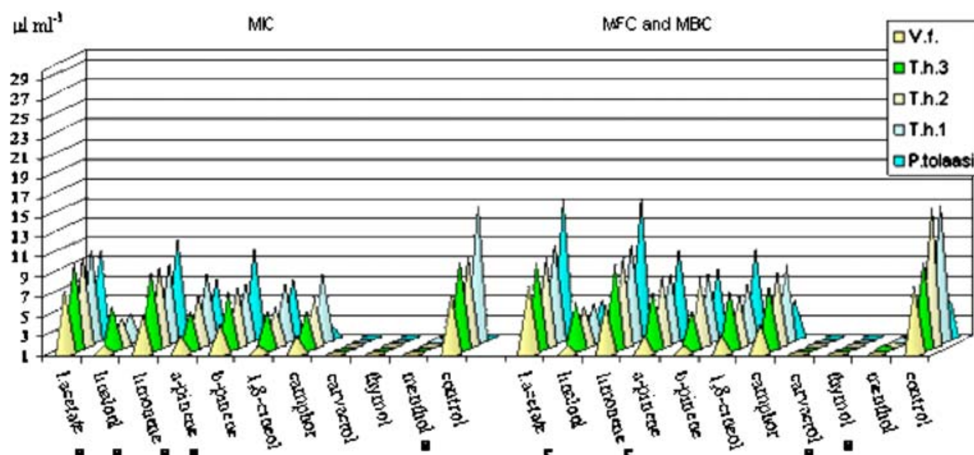

Figure 4. Antifungal and antibacterial activity (MIC, MBC and MFC – μl ml^{-1}) of essential oils components in microdilution method (significant differences between the effect of essential oils a – $P < 0.001$, c – $P < 0.05$, ANOVA test).

8.0 μl ml^{-1}, 5.0–8.0 μl ml^{-1} and 4.0–9.0 μl ml^{-1}, respectively. Linalyl acetate and limonene were the components with the lowest antifungal potential in this method with MICs at 7.5–10.5 μl ml^{-1} and 5.0–9.0 μl ml^{-1}, and MFCs at 8.0–11.0 μl ml^{-1} and 6.0–11.0 0 μl ml^{-1}. MIC for bifonazole was 7.0–15.0 μl ml^{-1}, and MFC at 8.0–15.0 μl ml^{-1}. All the components showed better antifungal potential than bifonazole.

Carvacrol, thymol and menthol showed the best antibacterial activity in the microdilution method, with very low MICs at 0.5 μl ml^{-1}, 0.5 μl ml^{-1} and 1.0 μl ml^{-1}, while MBCs were 0.5 μl ml^{-1}, 0.5 μl ml^{-1} and 1.5 μl ml^{-1}, respectively. MICs for linalool and camphor were 1.5 μl ml^{-1} and 2.5 μl ml^{-1}, respectively, while MBCs were the same, 5.0 μl ml^{-1}, α-pinene and 1,8-cineole possessed bacteriostatic activity at 7.0 μl ml^{-1} and were bactericidal at 10.0 μl ml^{-1}. Linalyl acetate, limonene and β-pinene showed bacteriostatic activity at 10.0 μl ml^{-1}, and were bactericidal at 15.0 μl ml^{-1}. The penicillin + streptomycin solution used as a positive control showed a MIC at 1.0 μl ml^{-1} and MBC at 5.0 μl ml^{-1}. Carvacrol, thymol and menthol showed better antibacterial activity than the positive control, linalool and camphor almost the same activity as the antibiotic, and all the residual components tested showed lower activity than streptomycin + penicillin. Camphor and 1,8-cineol showed statistically almost the same MIC, while carvacrol and thymol possessed the same activity. MMCs for linalyl acetate and limonene were similar to the MMC for the control, indicating that they have the same activity as the control. Thymol and carvacrol showed the same activity with very similar MMCs (Figure 4).

We investigated the antifungal activity of essential oils and their components also by the microatmospheric method (Figures 5, 6). All the oils and components tested showed almost the same values as in the macrodilution method. Against *Verticillium fungicola* the values for MIC were the same as in macrodilution method. Sporgon did not react by evaporation at all. According to the LSD test *Citrus* oils showed statistically almost the same activity as *M*. *chamomilla* oil; all the other oils tested showed statistically significant differences between their activities. Among the components tested 1,8-cineol and linalool showed similar activity. All the other components showed statistically significant differences in activity (Figures 5, 6).

The results of antibacterial activity of oils and components tested in four concentrations, i.e. 1.0, 5.0, 10.0 and 20.0 μl per disc, obtained by disc diffusion are presented in Figures 7 and 8. Oregano and Thyme oils showed the best antibacterial activity. Even at the lowest concentration, 1.0 μl per disc, the are large (20.0 and 16.0 mm). At the higher volume tested, the iz are 30.0 and 24.0 mm, respectively. *Mentha* oil also possessed strong antibacterial activity with iz of 10.0–18.0 mm. The iz for basil oil were 8.0–12.0 mm. Lavender oil showed activity in all tested concentrations with iz of 6.0–10.0 mm. *Matricaria chamommilla* and

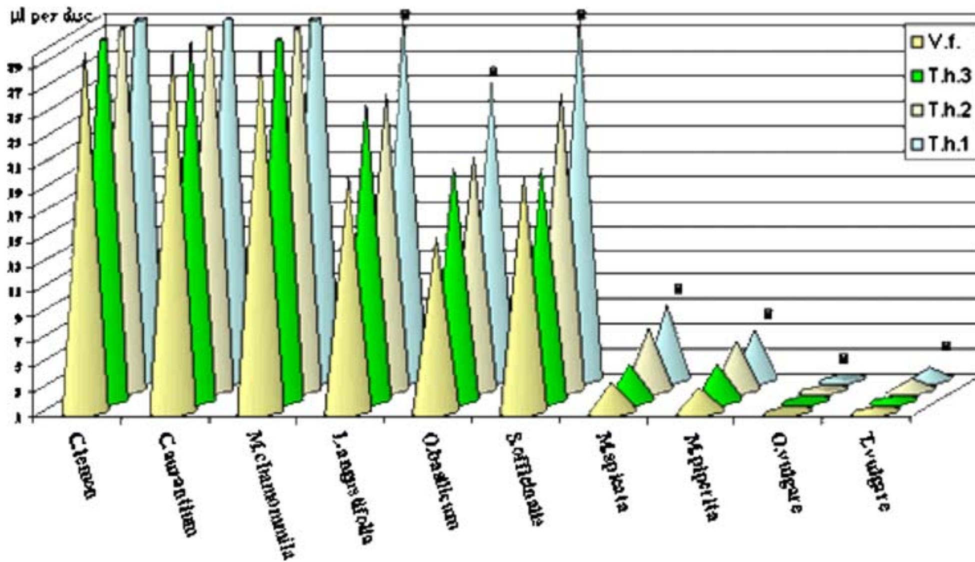

Figure 5. Antifungal activity of essential oils by microatmospheric method (MIC – μl per disc) (significant differences between the effect of essential oils a – $P < 0.001$, ANOVA test).

S. *officinalis* oils exhibited activity only at 10.0 and 20.0 μl per disc with iz of 6.0 mm, while *Citrus* oils were active only at the highest concentration (20.0 μl per disc) with iz of 6.0 mm. Streptomycin + penicillin showed antibacterial activity in all tested concentrations but with much smaller iz, i.e. 6.0–10.0 mm. It was seen that *Citrus, M*. *chamommilla* and *S*. *officinalis* oils showed lower antibacterial activity against *P*. *tolaasii* than the commercial antibiotics. All the other oils tested possessed better activity than the control. LSD test showed that *M*. *piperita* possessed the same activity as *M*. *spicata* oil, while *Citrus* oils reacted similarly; also Sage oil showed a similar effect as Basil oil. All the other oils tested showed statistically significant differences in activity (Figure 7).

Carvacrol showed very good antibacterial activity at all concentrations tested; the iz were

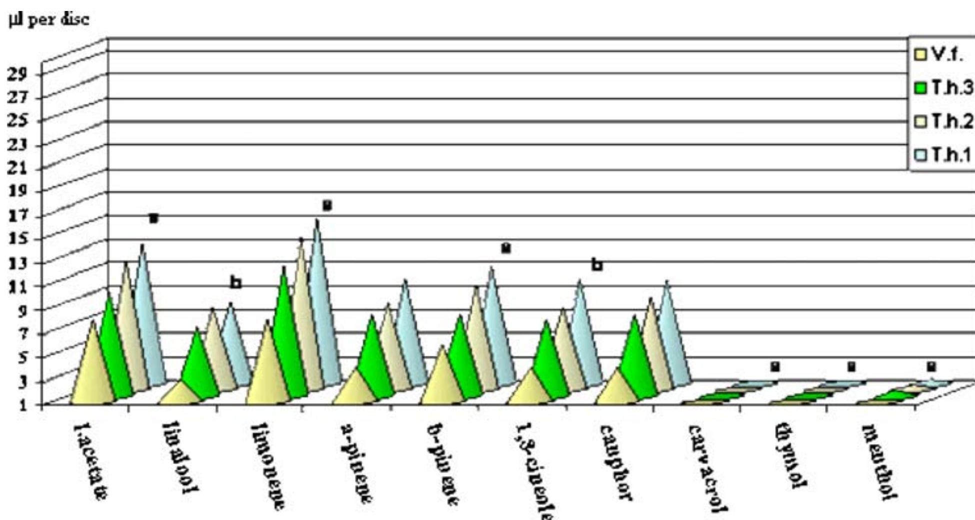

Figure 6. Antifungal activity of essential oils components by microatmospheric method (MIC – μl per disc) (significant differences between the effect of essential oils a – $P < 0.001$, b – $P < 0.005$, ANOVA test).

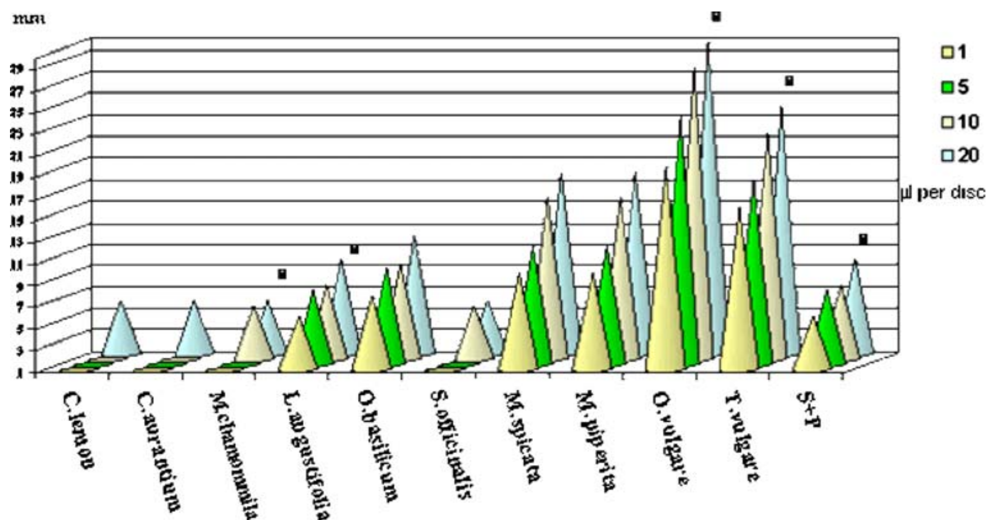

Figure 7. Antibacterial activity of essential oils in disc-diffusion method, inhibition zones in mm (significant differences between the effect of essential oils a – $P < 0.001$, ANOVA test).

20.0–32.0 mm. Thymol and menthol also possessed strong activity with iz of 12.0–22.0 mm, and 10.0–20.0 mm, respectively. Linalool, 1,8-cineole and camphor exhibited similar activity with iz of 6.0–12.0 mm, 6.0–12.0 mm and 8.0–14.0 mm. α-pinene and β-pinene were active at 10.0 and 20.0 μl per disc, with iz of 8.0–10.0 mm. Limonene was active also at the same concentration but with lower iz of 8.0 mm. Linalyl acetate possessed the lowest antibacterial potential in this method; the activity was seen at the highest concentration (20.0 μl per disc) with iz of 8.0 mm. Only linalyl acetate and limonene showed lower antibacterial activity than the positive control. According to the LSD test linalool and 1,8-cineol possessed the same activity as the control while α-pinene, β-pinene and limonene exhibited the same activity. All the other components showed statistically significant differences in their activity (Figure 8).

When comparing the antimicrobial activity of the compounds in the different methods used here, it was seen that the lowest MIC and MMC (MBC

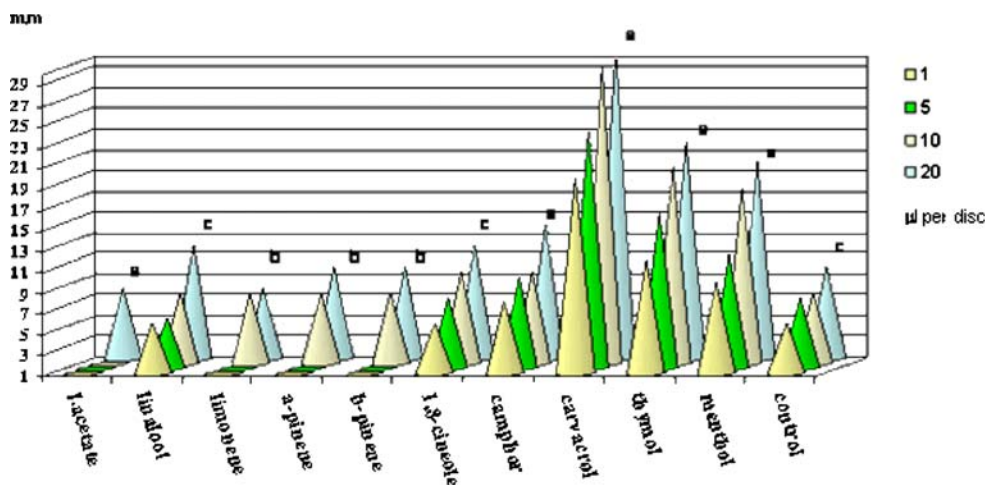

Figure 8. Antibacterial activity of essential oils components in disc-diffusion method, inhibition zones in mm (significant differences between the effect of essential oils a – $P < 0.001$, b – $P < 0.005$, c – $P < 0.05$, ANOVA test).

and MFC) were obtained in the microdilution method. This was particularly the case for essential oils isolated from *Citrus*, *M. chamommilla*, *L. angustifolia*, *O. basilicum* and *S. officinalis*.

Trichoderma harzianum 1 was the most resistant strain among the fungi tested, while *V. fungicola* was the most sensitive strain in this experiment. It was noticed that there were large differences in the sensitivity of the different *Trichoderma* strains. The resistance of *P. tolaasii* was the same as that of the *T. harzianum* 1 strain.

The results obtained by twofactorial analysis of variance for antimicrobial activities of 10 essential oils against four pathogens showed statistically significant effects of oils tested, and statistically significant effects of interaction of oils and pathogens. Also the ANOVA test showed that there were statistically significant differences in activity of 10 essential oil components tested against four pathogens and statistically significant differences in the effects of the interaction of oil components and pathogens.

Discussion

The results of our experiments make clear that the essential oils from *O. vulgare, T. vulgaris* and *M. spicata* have the best *in vitro* effect of a large series of oils we tested against the three major mushroom diseases. Oregano oil showed the highest activity against the microorganisms tested and in all methods used. This activity of Oregano and Thyme oils is not new: important fungal plant pathogens such as *Botrytis cinerea*, and *Fusarium solani* var. *caeruleum* were found to be strongly inhibited by the oils of *Origanum* spp. and of *T. capitatus* (Dafarera et al. 2003). Also *Oregano* and *Thyme* oils were reported as highly effective against *Phytophthora infestans* causing late blight in tomato (Soylu et al. 2006). A series of older publications reported analogous findings (Knobloch et al., 1986; Thompson, 1990; Sivropoulou et al., 1996, Soković et al., 2002).

The concentration at which Oregano oil was active in our study was very low, ranging from 0.125–1.0 μl ml^{-1}. Carvacrol which is the main component in Oregano oil had very strong activity, the best among the components tested, ranging from 0.02–0.5 μl ml^{-1}. The essential oil of *T. vulgaris* also showed strong activity, the active concentrations were 0.5–2.0 μl ml^{-1}. The main component in this oil is thymol, which was active against all the microorganisms we tested, ranging from 0.05–0.5 μl ml^{-1}. The lowest activity was observed for the essential oils from *Citrus* and *M. chamommilla* oil; the active concentrations were 7.0–40.0 μl ml^{-1}. The main components in *Citrus* oils are limonene with moderate activity, 5.0–15.0 μl ml^{-1} and β-pinene, 4.0–15.0 μl ml^{-1}. Oils that are rich in phenolic components, like Oregano and Thyme oils, were very active against microorganisms, while oils that possess a high content of monoterpenic hydrocarbons, (Lemon, Orange, and Chamomile) showed only low activity.

The results indicate that different essential oils have different efficacy. Also, the modes of action of essential oils are different for bacterial and fungal species. Different oil components may be active against different microorganisms. There seems to exist a correlation of the chemical structure of the essential oil constituents with antimicrobial activity (Villar et al., 1986). The site of action of terpenoids is the cell membrane. Several monoterpenes were found to affect the structural and functional properties of the lipid fraction of the plasma membrane of bacteria and yeasts, causing intracellular materials to leak (Trombetta et al., 2005). Also respiratory enzymes were inhibited (Cox et al., 2000). Most of the terpenoids tested were found to inhibit microbial oxygen uptake and oxidative phosphorylation. In particular, the phenolic and non-phenolic alcohol had the strongest inhibitory effects, followed by aldehydes and ketones. The monoterpene hydrocarbons had lower activity. It was suggested that the free OH group of the phenol and alcohol might be a key to their activity (Griffin et al., 2000). Our results confirmed that oils containing phenolic compounds (*Oregano* and *Thymus* species) were the most active. MIC and MMC values were lower in the microdilution method. The low water solubility of the oils and their components limited their diffusion through the agar medium. Only the more water-soluble components, such as 1,8-cineole diffused into the agar. The hydrocarbon components either remained on the surface or evaporated. This could be a reason why some essential oils rich in monoterpenic components (*Citrus, M. chamommilla, L. angustifolia, O. basilicum* and

S. officinalis) showed lower activity in the macrodilution method.

Broth methods used in microtitre trays, had the advantage of lower workloads for a larger number of replicates and used small volumes of the test substance and growth medium. In this method dilution of the oil was better, there was no agar in the medium, and there was better diffusion through the liquid medium. We observed that some compounds that we tested acted not only as fungicidal agents; they also inhibited sporulation of *T. harzianum*, which is a major factor determining the virulence of this fungus. Treatment was not only effective in solution or by contact, but even a vapour treatment was very effective allowing the microbial growth to be inhibited by a smaller amount of essential oil while also acting as a potent inhibitor of sporulation. Vapour concentration and the duration of exposure are important. The gaseous contact activity was demonstrated mainly by the maximum vapour concentration at an early stage of incubation: maintaining a high vapour concentration for long periods of time appeared not to be necessary.

Essential oil vapours might serve to control proliferation of mould and bacteria in mushroom houses that are now treated with other sanitizing agents. The oils and their components have high vapour pressures and are relatively volatile. Solutions and emulsions used in the form of sprays with or without a carrier therefore represent the preferred form in which those compounds should be applied to large areas of casing soil surface with minimal effort. Also evaporation by heating could be considered. Further, Oregano oil seems suited to prevent bacterial growth during cultivation and after harvesting.

The synthetic fungicides Benomyl and Sporgon (prochloraz) that are commonly used in mushroom cultivation at present are very effective and inexpensive. However, it is suggested that they may cause side effects, including carcinogenicity, teratogenicity and residual toxicity, although this seems very doubtful scientifically (Kato et al., 1995; McCarroll et al., 2002). The use of most of the synthetic fungicides has been restricted in many countries since the early sixties of the last century. Microbicidal essential oils are generally considered less harmful than synthetic chemicals. Indeed, the oral LD_{50} values in rats for carvacrol (810 mg kg^{-1} body weight) and thymol (980 mg kg^{-1} body weight) (Jenner et al., 1964) show this. Moreover, for thymol a no-effect level has been calculated in a 19-week oral rat study (Hagan et al., 1967). The mutagenicity study of Azizan and Blevins (1995) gave negative results for thymol with the Ames tester strain TA97, irrespective of metabolic activation. An additional advantage of essential oils is their volatile nature which implies that no or only little residue will be left on the produce after treatment.

Results of the present study indicate that Oregano and Thyme oils and their derivatives may be used as an alternative for the synthetic chemicals that are applied at present in mushroom cultivation to prevent and cure the most important diseases. Further studies are required to develop strategies for practical application.

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