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Inhibitory and stimulatory effects of essential oils and individual monoterpenoids on growth and sporulation of four soil-borne fungal isolates of *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum*, and *Verticillium dahliae*

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Abstract The effect of essential oils and individual monoterpenoids on soil-borne fungi, in pure and mixed cultures, in growth media and in the soil environment, was investigated. Essential oils were extracted from lavender (*Lavandula stoechas*), oregano (*Origanum vulgare* subsp. *hirtum*), sage (*Salvia fruticosa*) and spearmint (*Mentha spicata)*. The monoterpenoids tested were fenchone, carvacrol, 1,8-cineole, carvone, α-pinene and terpinen-4-ol. Their effect was examined on growth and sporulation of *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum* and *Verticillium dahliae* isolated from an organic cultivation of tomato. All tested essential oils and individual monoterpenoids inhibited mycelial growth in all fungi and conidial production in most fungi. The strongest inhibitory activity on mycelial growth was exhibited by oregano and spearmint oils and by carvacrol and carvone, respectively their main constituents. The inhibitory activity was clearly fungistatic in *A. terreus* and *F. oxysporum* but fungicidal in *V. dahliae.* On sporulation, clearly stimulatory effects were observed alongside inhibitory ones. Conidial production was always promoted by α-pinene in *P. expansum* and by sage oil in *F. oxysporum*. At certain dosages it was promoted by cineole and carvone in *F. oxysporum*, and by lavender oil in *A. terreus* and *V. dahliae*. Experiments with carvone and carvacrol against mixed fungal cultures in a soil environment showed that *V. dahliae* was the most sensitive and *A. terreus* the most tolerant of the four fungi. Our results demonstrate strong but divergent effects and selectivity of action of the lower terpenoids on fungal strains that can become serious pests of tomato. Of special importance is the complete inhibition of growth and conidial production of *V. dahliae*, a pathogen otherwise very resistant to chemical control.

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Abbreviations

CDA	Czapek dox agar
CFU	colony forming units
RMGR	recovery of mycelial growth
WA	water agar

Introduction

Plants produce an enormous array of secondary metabolites. It is commonly accepted that many of these compounds are part of the plant defence system, offering protection from microbial pathogens. Recently, researchers have shown a strong interest in biologically active plant products as potential alternatives to synthetic fungicides. This shift in pesticide research derives primarily from the resistance many microorganisms have developed to various synthetic compounds, and from the interference of these compounds with other organisms. In addition, use of synthetic fungicides has been increasingly restricted in many countries due to their suspected entrance into the food chain (Soković and Van Griensven 2006). Natural fungicides based on plant secondary metabolites may represent alternative crop protection agents, particularly valuable in organic farming.

Essential oils are mixtures of low molecular weight isoprenoid compounds that give fragrance to the producing aromatic plant. The antimicrobial properties of these secondary metabolites against various bacterial and fungal pathogens are well documented (Kalemba and Kunicka 2003; Karamanoli et al. 2000; Daferera et al. 2003). Due to this biological activity, essential oils and their individual constituents are promising as antimicrobial agents in various applied fields, such as pharmacology, medical and clinical microbiology, food technology, phytopathology, etc. Essential oils with antimicrobial activity are generally considered less harmful than synthetic chemicals (Soković and Van Griensven 2006), because of their natural origin, which renders them safer for the environment. In addition, their chemical properties reduce the risk of pathogens developing resistance to mixtures of compounds, since compounds involved exhibit different modes of action (Daferera et al. 2003). Due to the volatile nature of essential oils and to the different techniques employed in bioassays, determination of the active oil concentration in closed systems is expressed in various metric systems in the literature and is often not clearly defined. This situation poses a problem when comparing the activity of different compounds and classifying them according to their effectiveness.

Inhibition of mycelial growth following essential oil application has been reported for several fungi. For example, *Aspergillus* strains are reported to be inhibited by essential oils derived from thyme, cumin, clove, caraway, rosemary and sage (Farag et al. 1989). Thyme oil was the most effective, causing complete mycelial growth inhibition at a concentration of 0.4 mg ml^{-1}. Oregano, mint and basil essential oils when added in growth medium at 1,000 ppm concentration also completely inhibited *A. ochraceus* growth and ochratoxin production (Basílico and Basílico 1999). Mycelial growth for *Fusarium* spp. was retarded by 5 μl of lemongrass, cumin, fennel, cinnamon and cassia essential oils when tested by a filter paper diffusion assay (Pawar and Thaker 2007) and inhibited by oregano, thyme, dictamnus and marjoram essential oils applied in growth medium at 150, 200, 250 and 300 μg ml^{-1} respectively (Daferera et al. 2003). Regarding *Penicillium* spp., inhibition of mycelial growth was reported with the addition of 0.05% of each of eucalypt, rosemary and mugwort essential oils in broth medium (Khaddor et al. 2006), and decrease in conidial production and germination with addition of oregano, thyme, dictamnus, and marjoram essential oils at concentrations starting from 250 μg ml^{-1} (Daferera et al. 2000). The antimicrobial activity of essential oils against *Verticillium* sp. has rarely been evaluated. In a study of Soković and Van Griensven (2006), where ten different essential oils were tested against *V. fungicola*, only oregano, thyme and spearmint oils demonstrated high in vitro activity at ≥1.0, 2.0 and 5.0 μl ml^{-1} respectively. In general, the antifungal as well as the antibacterial activity of essential oils was primarily attributed by the above and other authors (e.g. Karamanoli et al. 2000; Kalemba and Kunicka 2003) to the oxygenated monoterpenoids that they contain.

From the literature survey, it is evident that in studies evaluating the effects of essential oils on fungi, the target process is primarily mycelial growth. Sporulation, which plays a major role in the fungal

life cycle, is rarely studied (Rahmani et al. 2004; Kuate et al. 2006), and the same holds true for both reversibility of the induced inhibition and conidial germination (Bång 2007). To our knowledge, no studies have been done on the effects of essential oils on mixed fungal cultures in the soil environment that would allow for interactions to be involved and expressed. Given the rich aromatic flora of the Mediterranean basin and its potential for novel uses, particularly in organic farming, the effects of essential oils from some of the most abundant aromatic plants of Greece on fungi were examined in the present study. The specific questions addressed were the following: i) investigate whether lower terpenoids, applied individually or as essential oils, affect mycelial growth and production of conidia of soil-borne fungi isolated from an organic field of tomato; ii) assess the reversibility of any inhibitory effects produced in pure fungal cultures and; iii) evaluate the performance of fungi, in mixed cultures in a soil environment, in the presence of lower terpenoids.

Materials and methods

Plant material, essential oils, and individual monoterpenoids

Plant material of lavender (*Lavandula stoechas* L.), oregano [*Origanum vulgare* L. subsp. *hirtum* (Link) Ietswaart], sage (*Salvia fruticosa* Mill.) and spearmint (*Mentha spicata* L.) was collected from populations growing wild in Greece. Dried leaves, stems and flowers were cut and a total of 50 g were placed in a 2 l flask containing 1.5 l distilled water. Essential oils were extracted by hydrostillation using a Clevenger apparatus, collected and dried over anhydrous sodium sulphate and further analyzed by gas chromatography (Varian 3700). Conditions followed were as previously described (Vokou et al. 1993). Major constituents were identified on the basis of retention times, references and previous research experience concerning the chemical profile of lavender (Vokou et al. 2002), spearmint (Kokkini and Vokou 1989), oregano (Vokou et al. 1993), and sage (Karousou et al. 1998) essential oils.

Apart from the crude essential oils, six commercial monoterpenoids representing different chemical groups were also tested: carvacrol, carvone, 1,8 cineole, fenchone, α-pinene and terpinen-4-ol. The first four are the major constituents of oregano, spearmint, sage and lavender essential oils respectively; they represent phenols (carvacrol), ketones (carvone and fenchone), and ethers (1,8 cineole). The last two, besides being common essential oil constituents, were chosen for the additional reason of representing two different groups of monoterpenoids: hydrocarbons (α-pinene) and alcohols (terpinen-4-ol).

Isolation and identification of fungi

Fungi were isolated from the soil of a tomato field where biological cultivation methods had been practiced for years. For the isolation of fungi a standard soil-dilution plate technique was used. In brief, soil suspensions were prepared by homogenizing 10 g of soil in 200 ml of 0.2% Water Agar (WA) in sterile screw-capped bottles. The contents were mixed thoroughly for 20 min. A 1-ml aliquot was then withdrawn and added to 9 ml of 0.2% WA that was further shaken for 4 min. From this, an 1-ml aliquot was removed and added to 49 ml of 0.2% WA that was further shaken for 2 min. One ml of the final suspension was transferred to each of five sterile Petri plates containing Czapek Dox Agar (CDA) or WA. The plates were incubated at 22°C for 5–6 days and the emerging fungal colonies were separated by transferring them to new plates.

Fungi were identified by the macro- and microscopic characteristics of their colonies in pure cultures using standard identification keys for *Verticillium* spp. (Goud et al. 2003) or molecular identification techniques for members of other genera. For the latter, DNA was extracted using Qia Puregene Core Kit A (QIAGEN GmbH, Hilden, Germany) according to the manufacturer protocol. Internal transcribed spacer 1 (ITS1) and ITS2 regions, including the ribosomal 5.8S RNA gene, were amplified using the universal ITS-1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS-4 (5-TCCTCCGCTTATTGATATGC-3) primers that anneal to the flanking 18S and 28S rRNA genes, according to White et al. (1990). PCR-reaction products of each isolate were purified using the Qiaquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). The purified products were sequenced in both directions using the ITS-1 and ITS-4 universal primers by Lark Technologies Inc (Essex, UK). Sequences were aligned using the computer software packages Clustal W 2. 0.9

(Thompson et al. 1997; Larkin et al. 2007) and BioEdit 7.0.9.0 (Hall 1999). The alignment of all sequences was checked visually. The sequences obtained were compared to those in the NCBI data base using BlastN 2.2.18 (Zhang et al. 2000).

Fungi selected for the antifungal assays and inoculum preparation

Following isolation and identification, four isolates of *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum* and *Verticillium dahliae* were selected to be exposed to the essential oils and their major constituents. The *V. dahliae* isolate proved to be pathogenic following artificial inoculation of tomato plants. At an inoculum concentration of 10^6 cfu ml^{-1}, 70% of the inoculated plants developed severe wilt symptoms. The same isolate caused wilt symptoms on cucumber, honey melon and watermelon after artificial inoculations. *F. oxysporum, P. expansum* and *A. terreus* were identified using molecular identification methods and all nucleotide sequences were submitted to the European Bioinformatics Institute (EMBL-EBI) under the following accession numbers: FN640476 for *F. oxysporum,* FN645431 for *P. expansum,* FN645432 for *A. terreus*. *F. oxysporum* was not pathogenic on tomato plants and its pathogenicity on other hosts or performance as a putative biocontrol agent was not further investigated. *A. terreus* and *P. expansum* caused extensive rot on artificially inoculated tomato fruits.

Apart from *A. terreus*, *F. oxysporum*, *P. expansum* and *V. dahliae*, which were selected for further experimentation, the soil of the organic cultivation of tomato hosted a number of other fungal strains that belonged to the genera of *Cladosporium*, *Gliocladium*, *Rhizopus*, *Trichoderma* etc.

In order to prepare inoculum for the antifungal assays, conidia, obtained in vitro from monoconidial cultures and maintained on CDA at 5°C, were harvested from either one-week or two-week-old cultures, depending on the strain. Ten ml of 0.05% Tween 80 in sterile distilled water were added to each plate and the conidia were dislodged by rubbing the surface of the colony with a glass rod. The washings were then filtered through several layers of sterilized cheesecloth to remove the mycelial fragments. Conidia were pelleted by centrifugation at 6,000 rpm for 10 min. The supernatant was discarded and the pellet re-suspended in 0.05% Tween 80. The concentration of conidia in the inoculum was determined using a haemacytometer and adjusted to 5×10^5 conidia ml^{-1}.

Effect on mycelial growth

The activity of essential oils and their constituents on the mycelial growth of each fungus was evaluated using a filter-paper-diffusion plate technique. Briefly, 15 ml CDA were poured into a sterile 90 mm Petri plate. The solidified medium was then overlaid with 5 ml of soft CDA (agar 50%), pre-inoculated at 40°C with a suspension of 5×10^5 conidia ml^{-1}. A sterile Whatman No. 5 filter paper disk, 5 mm in diameter, was placed in the centre of each CDA plate gently pressed down to ensure contact with the seeded medium. Then the oil or oil constituent was applied to the filter paper disk. Control sets of three plates for each fungus were concurrently run with sterile water added to filter paper disks. The plates were sealed with parafilm™ to prevent evaporation and were incubated at 22±1°C for 6–8 days. For each oil or oil constituent, three dosages were tested: 1 μl, 5 μl and 10 μl, with three plates (replicates) per dosage. Given that the volume of the Petri dishes is approximately 100 cm^3, these dosages could be considered equivalent to concentrations of 10, 50 and 100 ppm respectively. Due to the heterogeneity of the medium (air and agar), this concentration estimate is only an approximation (Vokou and Margaris 1986). Nevertheless, given the volatility of the compounds involved, this approach might provide an estimate of the essential oil concentrations in the air, thus allowing comparisons between different experiments regardless of the type of container and media used. As such, calculations in ppm are often encountered in the literature (e.g. Basílico and Basílico 1999; Olanya and Larkin 2006).

To measure mycelial growth, two lines making an angle of 90° and starting from the centre of each paper disk, were drawn at the bottom of the plates. Growth of each colony was calculated as the mean value of hyphal radial extensions, measured in mm, along the two lines marked on the Petri dish. Effects of the essential oils on mycelial growth were evaluated in two separate experiments.

Effect on conidial production

One quarter of fungal colony, separately from each plate, was transferred into a sterile 50 ml screw-

capped tube containing 10 ml of sterile water. The fungal suspension was shaken vigorously at 300 rpm for 30 min to dislodge conidia, which were then counted using a haemacytometer. Their concentration was expressed as number of conidia per cm^2 of colony grown in the plates. The experiment was conducted twice, each having three plates (replicates) per dosage, oil or oil constituent.

Estimation of mycelial recovery

To examine whether the inhibitory activity of the essential oils or individual monoterpenoids was fungistatic or fungicidal, a disk of approximately 5 mm in diameter from the edges of each colony and in the vicinity of the filter paper disk was transferred to a new plate containing pure CDA medium. In cases where complete inhibition of mycelial growth was apparent, disks were taken from the edge of the plate, where the possibility of residual fungal material was greatest. The plates were then incubated at 22±1°C for 7 days. If no mycelium re-growth occurred, the material in question was considered as fungicidal. If otherwise, it was considered to be fungistatic. Recovery of mycelial growth (RMGR) was estimated according to the formula, $\mathrm{RMGR} = [(T/C) \times 100]$, where C is the hyphal radial extension (in mm) in the control, and T is the hyphal radial extension of fungi treated with essential oils or their constituents. Three plates (replicates) per dosage, oil or oil constituent were used and the whole experiment was performed twice.

Antifungal activity of carvone and carvacrol in soil

Soil used in this assay was taken from the top layer (0–20 cm) of an organic cultivation of tomatoes. It was composed of clay (64.0%), silt (22.4%) and sand (13.6%), pH 8.2. To evaluate the antifungal activity of carvone (the main constituent of spearmint), and carvacrol (the main constituent of oregano) when added to the soil environment, 100 g of soil per treatment were autoclaved, placed in sterile 900-cm^3 glass vessels and then inoculated with a 15 ml sterile water suspension containing 5×10^5 conidia from each of the four fungi. A sterile spatula was used to mix the inocula into the soil. Carvone and carvacrol were each applied at 4.5, 22.5, 45 and 90 μl per 100 g of soil. Since the volume of the glass vessels was approximately 900 cm^3, these dosages were considered equivalent to 5, 25, 50 and 100 ppm, the two latter ones being comparable to the higher concentrations applied in the in vitro tests. This is again an approximation due to the heterogeneity of the media involved (air, soil).

Vessels with no carvone or carvacrol added to the soil were inoculated with the same conidial density and were used as positive controls. Negative controls contained only autoclaved soil mixed with sterile water. The vessels were sealed with parafilm™ and incubated in the dark at room temperature (approximately 20–25°C) for three weeks. Soil humidity was maintained by perforating the parafilm with a sterile syringe (volume 10 ml, 21 Gauge needle), adding 10 ml of sterile water once per week, and then overlaying with a new parafilm. Samples of 10 g from each vessel were taken weekly and examined for the presence of fungi by the soil-dilution plate technique, as previously described. The total population size of each fungus, expressed as colony forming units per gram of soil (CFU g^{-1} soil), was calculated after incubation at 22°C for 5–6 days. All treatments were conducted in three replicates in each of two separate experiments.

Statistical analysis

All experiments involving data for fungal growth, sporulation, and growth recovery were performed twice. A series of ANOVA F-tests (Kim and Xiao 2010) indicated that in all cases the two runs were almost identical. Thus, all further statistical analyses were applied to pooled data (average values of the corresponding two runs).

Data for fungal growth, sporulation, and growth recovery were analyzed by ANOVA, according to a full 10×3 factorial design (10 essential oils/monoterpenoids by three dosages) with three replications per treatment. Dunnett's T3 and Bonferroni's multiple comparison procedures were performed to statistically compare the treatment means (Toothaker 1993). Dunnett's T3 test was used in cases (data for fungal growth and recovery) where the homogeneity of treatment variances assumption was not met. Both post-hoc procedures keep the experiment-wise type I error rate at a predetermined significance level. For comparisons using Dunnett's method, the smallest statistically significant differences observed were reported in the tabulated results, since each comparison is based on a separate standard error and not on a

common one. Statistical analysis was run on the raw fungal growth and recovery data (expressed as mycelium length, in mm) as well as on sporulation data (expressed as number of conidia cm^{-2} of fungal colony). However, in order to be feasibly presented, tabled summary results are given as percentages (%) relative to the control values.

To analyze the effects of selected lower terpenoids on fungal populations, all CFU g^{-1} values were logarithmically (log) transformed. Given the small number of comparisons in this case, Fisher's protected Least Significant Difference-LSD criterion was adopted to separate means of different treatments (four dosages plus control of carvone and carvacrol).

The significance level for all statistical tests was predetermined at 0.05. Statistical analyses were performed using the SPSS 15.0 software (SPSS, Inc., IL. USA).

Results

In spearmint and oregano, the main constituent (carvone and carvacrol respectively) represents more than half of the oil. In lavender (fenchone) and sage (1,8-cineole) it corresponds to about 40% (Table 1).

Effects of essential oils and their constituents on mycelial growth

For all four fungal strains colony radius in control treatments was equal to 45 mm, i.e. the plate radius, as all grew out to the edge of the plate. Thus, any growth stimulating effects on the mycelia could not be assessed. Essential oils tended to exhibit a dosage-dependent effect on the fungi examined with significant interactions between fungi and compounds tested (Table 2). Compared to the other three essential oils, oregano oil caused the strongest inhibition at the low and intermediate dosages, but not always at the high one. At this dosage, spearmint oil proved more drastic against *A. terreus* and *P. expansum*, entirely inhibiting their growth. Lavender and sage oils proved drastic only against *V. dahliae*, strongly or even entirely inhibiting its growth at the high dosage.

Table 1 Main constituents of lavender, spearmint, oregano and sage essential oils (in %)

Plant species	Main constituent	%
Lavender	fenchone	37
Spearmint	carvone	55
Oregano	carvacrol	66
Sage	1,8-cineole	39

For the six monoterpenoids in general, the strongest inhibition was recorded with carvacrol, which affected all fungi at all dosages (Table 2). Carvone followed in potency, although at the high dosage its effect was stronger than that of carvacrol against *A. terreus* and *P. expansum*. Fenchone was ineffective or only slightly effective, except when applied against *V. dahliae*, the growth of which was entirely inhibited at the high dosage. The activity of terpinen-4-ol was mostly intermediate between those of carvone and fenchone. 1,8-Cineole and α-pinene were essentially inactive against all but *V. dahliae*.

Effects of essential oils and their constituents on conidial production

Strong inhibition but also strong stimulation of conidial production by the tested compounds was found (Table 3). In particular, oregano oil greatly inhibited sporulation of all fungi at all dosages. Conidial production of *A. terreus* and *P. expansum* was strongly inhibited at all dosages of spearmint essential oil while sporulation of *F. oxysporum* and *V. dahliae* was only inhibited at the intermediate and high dosages. Lavender and sage essential oils had both negative and positive effects. In particular, lavender oil strongly inhibited conidial production at all dosages in *P. expansum* and at the intermediate and high dosages in *F. oxysporum*. In contrast, it either inhibited or stimulated conidial production in *V. dahliae* and *A. terreus*, depending on the dosage applied. Sage oil, like lavender oil, inhibited conidial production almost fully in *P. expansum* at all dosages and in a dosage-dependent manner in *A. terreus* and *V. dahliae*. It promoted conidial production in *F. oxysporum*, again in a dosage-dependent manner.

In most cases, the six monoterpenoids also strongly inhibited sporulation in the tested fungi (Table 3). The strongest overall inhibition was exhibited by carvacrol, which dramatically suppressed conidial production in all fungi, even at the low dosage. Carvone also strongly inhibited production of conidia of all fungi at all dosages, with the exception of *F. oxysporum*,

Table 2 Mycelial growth of *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum* and *Verticillium dalhiae* (expressed as percentage of mycelial growth in respective control treatments), in the presence of essential oils or individual monoterpenoids in Petri plates. All controls grew to the edge of the plates, i.e. 45 mm

		Relative to the control mycelial growth[1]			
Essential oil / Monoterpenoid	Dosage (μl per disk)	*Aspergillus terreus*	*Fusarium oxysporum*	*Penicillium expansum*	*Verticillium dalhiae*
Lavender	1	100.0 a	100.0 a	100.0 a	91.0 abcd
	5	97.3 a	100.0 a	89.0 bc	63.0 gh
	10	88.0 b	98.0 ab	85.0 cd	0.0 k
Spearmint	1	100.0 a	100.0 a	91.0 abc	91.0 abcd
	5	51.0 d	91.5 abc	64.0 fg	49.0 i
	10	0.0 i	90.0 bcd	0.0 k	11.0 k
Oregano	1	87.0 b	80.0 e	71.0 ef	56.0 hi
	5	45.0 f	17.0 g	41.0 hi	0.0 k
	10	27.0 h	6.0 h	32.0 i	0.0 k
Sage	1	100.0 a	100.0 a	100.0 a	96.0 ab
	5	100.0 a	100.0 a	96.0 ab	71.0 fg
	10	99.0 a	100.0 a	91.0 abc	33.0 j
Carvacrol	1	58.5 c	82.6 de	76.1 de	46.7 i
	5	33.9 g	54.3 f	39.6 hi	0.0 k
	10	28.2 h	48.6 f	22.2 j	0.0 k
Carvone	1	100.0 a	100.0 a	90.2 bc	74.1 efg
	5	48.1 e	91.5 abc	59.3 g	0.0 k
	10	0.0 i	54.4 f	0.0 k	0.0 k
1,8-Cineole	1	100.0 a	100.0 a	100.0 a	100.0 a
	5	100.0 a	100.0 a	100.0 a	91.6 abcd
	10	100.0 a	100.0 a	100.0 a	81.7 def
Fenchone	1	100.0 a	100.0 a	100.0 a	100.0 a
	5	100.0 a	100.0 a	100.0 a	91.4 abcd
	10	100.0 a	97.8 ab	100.0 a	0.0 k
α-Pinene	1	100.0 a	100.0 a	100.0 a	93.0 abc
	5	100.0 a	100.0 a	100.0 a	84.4 cde
	10	100.0 a	100.0 a	100.0 a	82.2 cde
Terpinen-4-ol	1	100.0 a	100.0 a	100.0 a	95.6 ab
	5	100.0 a	94.8 ab	72.2 ef	86.7 bcd
	10	85.9 b	85.6 cde	45.2 h	0.0 k
Smallest observed statistically significant difference ($P<0.05$) according to Dunnett's T3 multiple comparison procedure		>2.7	>8.5	>9.0	>11.1

[1] Relative mycelial growth values within the same column followed by the same letter(s) do not differ significantly according to Dunnett's T3 multiple comparison test at significance level 0.05

where a stimulatory effect was recorded at the high dosage. Terpinen-4-ol had an effect comparable to those of carvacrol and carvone. Fenchone strongly inhibited conidial production in all fungi mainly at the intermediate and high dosages. Like all other oxygenated compounds, effects of 1,8-cineole were very drastic against *P. expansum*, less effective against *A. terreus* while it stongly enhanced conidial production

Table 3 Sporulation of *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum* and *Verticillium dalhiae* (expressed as percentage of conidia produced in respective control treatments), in the presence of essential oils or individual monoterpenoids

Essential oil / Monoterpenoid	Dosage (μl per disk)	Conidial production relative to control[1,2]			
		Aspergillus terreus	*Fusarium oxysporum*	*Penicillium expansum*	*Verticillium dalhiae*
Lavender	1	111.5 b	71.4 gh	3.7 efg	121.5 a
	5	135.2 a	9.7 mno	2.0 g	7.3 l
	10	52.4 d	6.9 mno	0.7 g	
Spearmint	1	10.6 hij	94.7 e	4.9 defg	98.5 b
	5	0.4 j	32.7 k	2.3 fg	16.1 kl
	10		28.9 k		13.1 kl
Oregano	1	32.0 ef	43.9 j	26.1 b	26.2 ij
	5	18.6 gh	1.3 o	4.0 defg	
	10	15.2 ghi	0.0 o	4.2 defg	
Sage	1	85.9 c	146.2 c	5.8 defg	69.2 d
	5	60.3 d	178.5 b	2.7 fg	32.4 hi
	10	24.9 fg	196.1 a	2.7 fg	8.1 l
Carvacrol	1	6.1 ij	14.4 lmn	29.6 b	20.0 jk
	5	5.0 ij	0.0 o	15.2 c	
	10	2.2 j	0.0 o	0.6 g	
Carvone	1	7.3 hij	115.8 d	11.0 cd	29.2 i
	5	6.5 ij	22.5 kl	1.3 g	
	10		10.3 mno		
1,8-Cineole	1	84.6 c	181.0 b	10.7 cde	80.1 c
	5	79.5 c	49.7 j	3.1 fg	40.1 fgh
	10	58.8 d	15.4 lmn	1.3 g	26.4 ij
Fenchone	1	107.6 b	45.6 j	9.2 cdef	87.7 c
	5	56.2 d	54.5 ij	4.4 defg	42.0 fg
	10	15.0 ghi	5.0 no	0.7 g	
α-Pinene	1	39.5 e	86.0 ef	136.4 a	47.7 ef
	5	26.6 fg	75.5 fg	139.6 a	33.9 ghi
	10	6.9 hij	52.5 ij	142.7 a	33.7 ghi
Terpinen-4-ol	1	10.9 hij	61.8 hi	5.1 defg	52.3 e
	5	0.7 j	44.4 j	1.2 g	10.7 l
	10	6.5 ij	17.1 lm	1.2 g	
Least Significant Difference ($P<0.05$) according to Bonferroni's multiple comparison procedure		>11.7	>11.0	>7.1	>9.0

[1] Actual number of conidia in control treatments was $3.0\pm0.1\times10^6$ for *A. terreus*, $1.6\pm0.03\times10^6$ for *F. oxysporum*, $32.7\pm1.02\times10^6$ for *P. expansum* and $5.9\pm0.18\times10^6$ for *V. dalhiae* per cm^2 of fungal colonies

[2] Relative sporulation values within the same column followed by the same letter(s) do not differ significantly according to Bonferroni's multiple comparison test at significance level 0.05

Blank cells indicate that no data could be taken due to complete mycelial growth inhibition

in *F. oxysporum* at the low dosage. Inhibition of conidial production of *F. oxysporum* and especially of *A. terreus*, by α-pinene was dosage-dependent. In contrast, it was almost equally effective at all dosages against *V. dahliae*. However, it promoted production of conidia in *P. expansum*, which other-

wise was the most sensitive fungus to the other monoterpenoids tested.

Mycelial recovery after exposure to essential oils and their constituents

All essential oils caused irreversible inhibition of fungal growth in *V. dahliae* at the intermediate and high dosages (Table 4). Inhibition by lavender and sage oils at any dosage was fully reversible in *A. terreus*, *P. expansum* and *F. oxysporum*. In contrast, inhibition was only partially reversible in *A. terreus* and *P. expansum*, when caused by the oregano and spearmint oils, mainly after exposure at the intermediate and high dosages, and in *F. oxysporum* when induced by the oregano oil.

All compounds, except for 1,8-cineole, produced irreversible inhibition in *V. dahliae,* at least at the high dosage, with carvone and carvacrol inducing such inhibition at all dosages. *A. terreus, P. expansum* and *F. oxysporum* fully recovered after exposure to 1,8-cineole or α-pinene (at any dosage). The same holds true for the treatments of *F. oxysporum* with carvone, fenchone or terpinen-4-ol, and those of *A. terreus* with fenchone or terpinen-4-ol. None of the fungi treated with carvacrol, at the intermediate or high dosages, fully recovered after its removal.

Antifungal activity of carvone and carvacrol

The antifungal activity of carvone and carvacrol, when applied in mixed cultures of the four fungi in the soil contained in sealed vessels, is shown in Fig. 1a and b respectively. Populations of all fungi remained almost stable in the control samples. *A. terreus* and *P. expansum* populations were more sizeable over the three-week experimental period than those of the other two fungi. No growth was observed in *V. dahliae* until the third week. In general, both carvone and carvacrol at the dosage of 22.5 μl 100 g^{-1} of soil reduced populations of all fungi by at least 10^2 CFU g^{-1} of soil. Doubling the dosage of the two oxygenated monoterpenoids resulted in reducing the fungal population by at least 10^4 CFU g^{-1} of soil. Stimulatory effects were also observed at least for *A. terreus* when the two compounds were applied at the 4.5 μl dosage, whereas fungal growth was entirely inhibited when they were applied at the 90 μl dosage.

Discussion

In the present study, essential oils derived from aromatic plants growing wild in Greece as well as their monoterpenoid constituents variously affected soil-borne fungi isolated from an organic tomato field. Oregano essential oil proved the most inhibitory against the fungi examined with respect to both mycelial growth and sporulation. Spearmint oil was also very drastic, except against *F. oxysporum*. Results are in accordance with those reported by Soković and Van Griensven (2006), who concluded that oregano and spearmint essential oils are very drastic against fungi like *V. fungicola* and *Trichoderma harzianum*. Lavender oil presently caused negligible to low inhibition of *A. terreus*, *F. oxysporum* and *P. expansum*, yet was very drastic against *V. dahliae.* Strong activity has been recorded for lavender oil when tested against *F. oxysporum,* though it was only moderately effective against *A. flavus* (Angioni et al. 2006) at a dosage of 60 μl oil (paper diffusion assay), much higher than the ones tested in our bioassays. For sage oil, which in the present study demonstrated negligible activity on all fungi tested but *V. dahliae*, there are reports of weak antifungal activity against *Fusarium* sp. and *Botrytis cinerea* (Daferera et al. 2003), and of high effectiveness against *F. solani* f. sp. *cucurbitae*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Pitarokili et al. 2003). Such contrasting results can be attributed either to the different fungal strains tested and dosages applied and/or to the different types of oils used, as the oil qualitative and quantitative composition may vary depending on genetic, developmental and environmental factors to which the producing plant is exposed (Vokou 1999).

Strong antifungal activity has been reported for carvone and carvacrol, as well as for carvacrol's isomer, thymol, against food storage pathogens (Thompson 1989) and the phytopathogenic fungi *F. moniliforme*, *R. solani*, *S. sclerotiorum*, and *Phytophthora capsici* (Müller-Riebau et al. 1995; Kalemba and Kunicka 2003). Of the monoterpenoids tested in this study, carvacrol and carvone, respectively the major constituents of oregano and spearmint oils, also exhibited the strongest antifungal activity. In general, there was a match between the magnitude of effect induced by essential oils and by their main constituents for both growth and sporulation, as in the above-mentioned case of oregano oil/carvacrol and spearmint/carvone. Yet both far stronger and far milder effects were

Table 4 Recovery of mycelial growth after re-culture of *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum* and *Verticillium dalhiae* in a terpenoid-free environment

		Recovery of mycelial growth (RMGR)[1]			
Essential oil / Monoterpenoid	Dosage (μl per disk)	*Aspergillus terreus*	*Fusarium oxysporum*	*Penicillium expansum*	*Verticillium dalhiae*
Lavender	1	100.0 a	100.0 a	100.0 a	68.2 bc
	5	100.0 a	100.0 a	100.0 a	0.0 f
	10	100.0 a	100.0 a	100.0 a	0.0 f
Spearmint	1	100.0 a	100.0 a	100.0 a	45.5 de
	5	81.8 b	100.0 a	44.5 c	0.0 f
	10	50.0 e	100.0 a	5.6 e	0.0 f
Oregano	1	100.0 a	100.0 a	77.8 ab	36.4 de
	5	77.3 bc	94.0 a	38.9 cd	0.0 f
	10	68.2 cd	74.0 c	38.9 cd	0.0 f
Sage	1	100.0 a	100.0 a	100.0 a	72.7 b
	5	100.0 a	100.0 a	100.0 a	0.0 f
	10	100.0 a	100.0 a	100.0 a	0.0 f
Carvacrol	1	100.0 a	100.0 a	77.8 ab	0.0 f
	5	77.3 bc	84.0 b	72.2 b	0.0 f
	10	59.1 de	60.0 d	0.0 e	0.0 f
Carvone	1	100.0 a	100.0 a	100.0 a	0.0 f
	5	77.3 bc	100.0 a	77.8 ab	0.0 f
	10	59.1 de	100.0 a	0.0 e	0.0 f
1,8-Cineole	1	100.0 a	100.0 a	100.0 a	72.7 b
	5	100.0 a	100.0 a	100.0 a	50.0 cd
	10	100.0 a	100.0 a	100.0 a	45.5 de
Fenchone	1	100.0 a	100.0 a	100.0 a	97.7 a
	5	100.0 a	100.0 a	38.9 cd	77.3 ab
	10	100.0 a	100.0 a	38.9 cd	0.0 f
α-Pinene	1	100.0 a	100.0 a	100.0 a	77.3 ab
	5	100.0 a	100.0 a	100.0 a	27.3 e
	10	100.0 a	100.0 a	100.0 a	0.0 f
Terpinen-4-ol	1	100.0 a	100.0 a	100.0 a	68.2 bc
	5	100.0 a	100.0 a	55.6 bc	45.5 de
	10	100.0 a	100.0 a	16.7 de	0.0 f
Smallest observed statistically significant difference ($P<0.05$) according to Dunnett's T3 multiple comparison procedure		>9.1	>6.0	>27.7	>22.7

[1] Values within the same column followed by the same letter(s) do not differ significantly according to Dunnett's T3 multiple comparison test at significance level 0.05

recorded. For instance, although *P. expansum* treated with lavender oil fully recovered, it did not when treated with fenchone, the main constituent of lavender oil. This suggests interference from other, less abundant compounds in the oil, which might act synergistically or competitively to the effect of the main constituent.

The inhibitory effect of essential oils and monoterpenoids on sporulation was in general stronger than that on fungal growth, even at the low dosage applied.

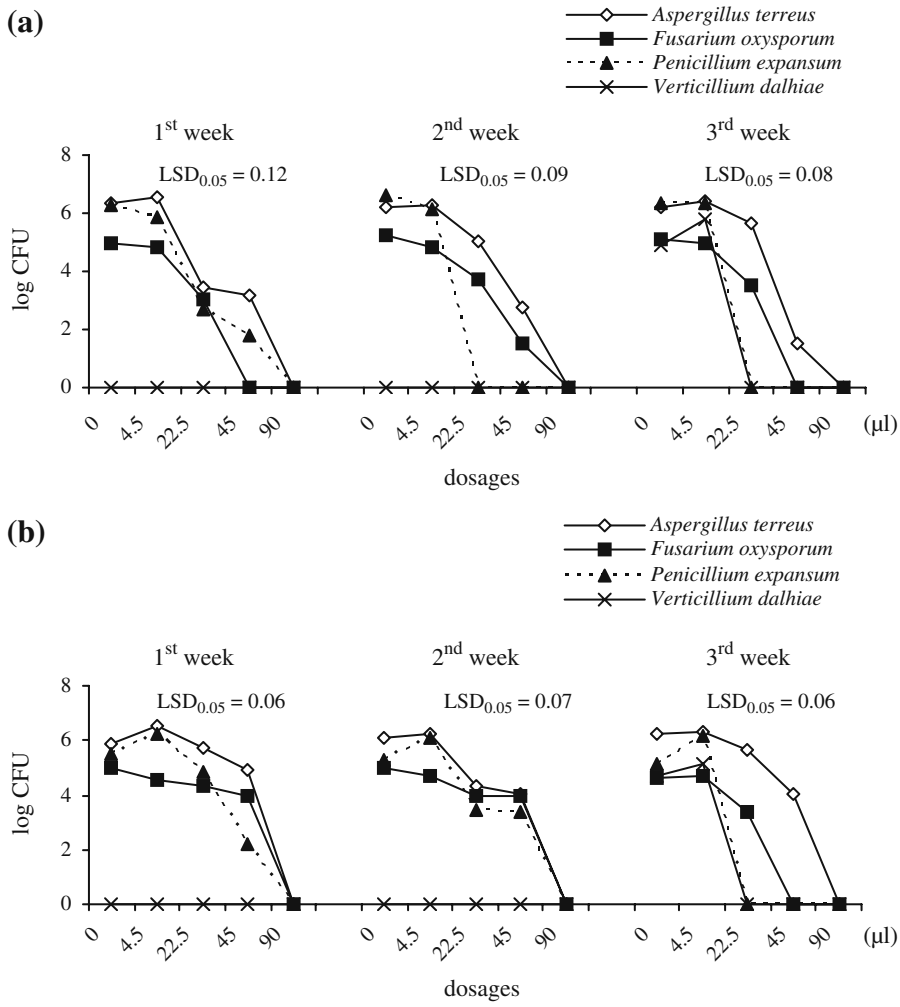

Fig. 1 Growth of *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum* and *Verticillium dalhiae* during a three-week period. The fungi were inoculated into 100 g of soil (placed in 900 cm^3 glass vessels) where 0 µl, 4.5 µl, 22.5 µl, 45 µl or 90 µl of carvone (**a**) and carvacrol (**b**) were added. Each point represents pooled data (mean values) from two experiments, three replications per experiment

However, clearly stimulatory effects of certain essential oils and/or their constituents were repeatedly observed on sporulation. Kuate et al. (2006) also reported that essential oils from citrus enhanced sporulation of the fungus *Phaeoramularia angolensis* by 1.36% to 701%. Calvo et al. (2002) speculated that seed fatty acids, like linoleic acid, can increase conidial development in *Aspergillus* by mimicking and/or interfering with signals that regulate fungal sporogenesis. There is evidence that abiotic stresses, such as excess of light, chemicals or mechanical damage, also increase fungal sporulation and/or conidial germination (Hountondji et al. 2006). In our results, whenever a stimulatory effect on sporulation was apparent, the corresponding inhibitory effect on growth was absent, negligible or very low (0 to 9%). This might suggest that sporulation per se is part of a defence mechanism enabling fungi to overcome the chemical stress caused by the monoterpenoids in their growth medium. The above suggestion is weakened by the fact that this connection was not always observed, implying that different mechanisms of resistance were involved.

Reversibility of fungal growth inhibition was observed in some cases. For *A. terreus, F. oxysporum* and *P. expansum,* reversibility results indicated that the inhibitory-to-growth activity was fungistatic rather than fungicidal. This was not the case for *V. dahliae*, against which the activity of essential oils and individual monoterpenoids was primarily fungicidal.

The four fungi differed regarding their performance in mixed culture in a soil environment, with *V. dahliae* being the weakest competitor of all. Addition of carvacrol and carvove did not change the profile of

competitive interactions and resulted in total inhibition of *V. dahliae* growth. In these soil experiments, unlike in the ones in culture media, stimulation of growth (e.g. *A. terreus*) was observed at the lowest dosage of monoterpenoids tested. The observation supports previous reports of conflicting results obtained when different test systems were applied to evaluate the effects of potential antifungal substances (Bång 2007).

The results of this study pointed to *A. terreus* and *F. oxysporum* as the most tolerant fungi and *V. dahliae* as the most sensitive to the tested compounds: *V. dahliae* was the only fungus in which both growth and sporulation were negatively affected by almost all oils and their constituents to a greater degree than were the other three fungi. It was the only fungus for which fungicidal effects were exerted by almost all individual monoterpenoids, and it was the least competitive regarding growth in a soil environment in the presence of either carvone or carvacrol. These are important findings, given that *V. dahliae* is a serious plant pathogen of global importance and is very resistant to the common agronomic practices. Consequently, these data justify subsequent in vivo experimentation using effective essential oils for controlling *Verticillium* wilt, at least in tomato crops. The volatile nature of these compounds is an additional advantage for their potential use as antifungal agents, particularly in crops where organic cultivation methods are applied, since following their application little residue is left on the produce or in the environment. Yet this property does not bar them from persisting in the soil environment long enough to exert their effects. For example, in previous works by our team (Karamanoli et al. 2008; Chalkos et al. 2010), it was reported that spearmint essential oil persists long after addition to the soil, exhibiting a modified yet active composition.

In practice, application of essential oils or their main constituents in the field presents limits since dosages required are quite high. For instance, treating 1 ha of a field at a 20 cm depth with an active carvacrol dosage of 22.5 μl 100 g^{-1} soil, would require not less than 5 t of carvacrol, i.e. approximately the whole biomass produced by 1 ha of oregano cultivation. Alternative practices were proposed, such as regular fumigations of tomatoes when oils are used as biofumigants or through drip irrigation under polyethylene covers, providing adequate aeration to avoid phytotoxicity in tomato plants per se (Pradhanag et al. 2003). Nevertheless, essential oils under the active concentrations tested in this work could be efficiently used at a much smaller scale in tomato seedbeds to protect seedlings during the highly vulnerable stage that precedes transplanting.

Results of the present study revealed more divergent effects caused by the lower terpenoids on fungal sporulation than on fungal growth. Besides inhibitory effects, strong stimulatory effects were recorded in the former case. This is a parameter of concern for the future in vivo trials, since sporulation of various pathogenic fungi might be promoted. In addition, these terpenoids should be tested for their ability to stimulate or inhibit sporulation of beneficial fungal strains that may control harmful ones via their competitive interactions or production of antibiotics. Further experimentation on the potential use of the lower terpenoids as natural pesticides must address these issues, if the active compounds are to be applied to the soil environment.

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