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**Antifungal activity and potential use of essential oils against
Fusarium culmorum and Fusarium verticillioides**

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3 **Antifungal activity and potential use of essential oils against *Fusarium culmorum***
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5 **and *Fusarium verticillioides***
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20
21 **Abstract**
22

23 Essential oils of bay leaf, cinnamon, clove and oregano were tested *in vitro* and *in vivo* against two
24 foodborne fungi belonging to the dominant mycobiota of stored rice, *Fusarium culmorum* and *Fusarium*
25 *verticillioides*, collected from the Albufera rice-producing Mediterranean area near Valencia
26 (Spain). Chemical composition was identified by gas chromatography-mass spectrometry. Essential oils
27 presented a high percentage of oxygenated components: 78.8% in bay leaf (eucalyptol 51%); 90.3% in
28 clove (eugenol 89.8%); 92% in cinnamon (eugenol 60% and eugenyl acetate 18.3%); 71.8% in oregano
29 (carvacrol 49.6% and thymol 21.2%). Monoterpenes and sesquiterpenes were: 18% in bay leaf, 9% in
30 clove, 5% in cinnamon, 25% in oregano. This research showed that essential oils have a great potential to
31 control both fungal pathogens. In the *in vitro* test, the essential oils of cinnamon, clove and oregano
32 reduced fungal growth by 90% and almost 100%, being oregano the most effective essential oil to inhibit
33 fungal growth. The effect of the oregano essential oil on fungal development in inoculated rice grains
34 demonstrated its effectiveness.
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41 **Keywords**
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43 Antifungal activity, *Fusarium culmorum*, *Fusarium verticillioides*, essential oils, rice.
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46 **Introduction**
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48 Rice is the second most cultivated crop worldwide in cultivated areas after wheat, and the first most
49 important in the human diet. This crop is a primary food source for more than one third of the world
50 population. Rice is an important crop in the Valencian autonomous region (east Spain), with an origin
51 denomination Rice of Valencia, and is grown according to environmental conditions and characteristics in
52 the la Albufera Natural Park. The incidence of fungi *Fusarium culmorum* and *Fusarium verticillioides* on
53 stored rice samples is high. These fungi cause considerable grain and seed loss, and are producers of
54 mycotoxins (including fusarin C, trichothecenes, fusaric acid, fumonisins, moniliformin, culmorin and
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3 zearalenone), which are extremely dangerous for human and animal health (Logrieco, et al., 2003;
4 Sempere, 2009). Furthermore, *F. culmorum* causes foot and root rot and head blight in cereals.
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7 Fungal contamination is a chronic problem in food products and has a negative effect on their quality and
8 quantity. Pathogenic fungi can reduce yields of major foods and cash crops by nearly 20% (Agrios, 2000).
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12 New methods to control spoilage fungi in food are being investigated because the application of synthetic
13 fungicides has led to a number of environmental and health problems. In line with public demand, it is
14 necessary to study natural compounds. In recent years, numerous studies have documented the antifungal
15 effects of essential oils of plants to control food spoilage fungi *in vitro* and *in vivo* (Amiri, et al., 2008;
16 Dikbas, et al., 2008; Dubey, et al., 2008; El Bouzidi, et al., 2012; Feng and Zheng, 2007; Marei et al.,
17 2012; Tolouee, et al., 2010; Tzortzakis, 2007, 2009).
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22 Our study objectives were to: (i) determine the chemical composition of the essential oils of bay leaf,
23 cinnamon, clove and oregano; (ii) evaluate the *in vitro* effect of these oils against *Fusarium culmorum*
24 and *Fusarium verticillioides*, isolated from rice, (iii) and assess *in vivo* the effect of oregano essential oil
25 on rice grain conservation to develop natural antifungal products.
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28 29 **Materials and methods**

30 31 32 Essential oils and chemical characterization

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35 The essential oils used were those of bay leaf (*Laurus nobilis* L.) extracted from leaves, oregano
36 (*Origanum compactum* Benth.) extracted from the plant in flower, clove (*Syzygium aromaticum* (L.)
37 Merr. & Perry), extracted from leaves, and cinnamon (*Cinnamomum zeylanicum* Blume) extracted from
38 branches, naturally extracted by first cold pressing. Harvesting tool place at the appropriate time and
39 extraction was carried out by steam distillation in order to obtain 100% natural oil. At work were used
40 four commercial essential oils in order that the results of the antifungal activity were reproducible, and
41 avoid variations.
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45 46 Gas chromatography-mass spectrometry (GC-MS) analysis conditions

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49 Essential oils were analyzed by GC-MS. The GC-MS analysis was performed in an Agilent 5973 N
50 system and an HP-5MS UI (Agilent) column (30 m x 0.25 mm x 0.25 μ m film thickness) with helium.
51 The GC oven temperature was 60°C for 5 min, which was increased to 180°C at 3°C/min, and further
52 increased to 280°C at 20°C/min for 10 min. The split flow was adjusted at 1mL/min, and the injector
53 temperature was 250°C. Mass spectra were recorded at 70eV. The mass range went from 30 to 500 m/z.
54 n-Alkanes (C₈-C₃₀) were used as reference points to calculate the retention indices (RIs).
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Essential oil components were identified by comparing their relative retention times with those of authentic samples or by comparing their relative retention index (RI) with a series of n-alkanes. Computer matching against commercial (Nist, Nist_msms, mainlib, replib, Wiley7n) libraries, built by genuine compounds and components of known oils, as well as GC-MS literature data, were also used for identification purposes. Quantification was performed by percentage peak area calculations via GC-FID.

Fungi

Strains of the fungi *Fusarium culmorum* (G. W. Smith) Sacc. LBEA 2160, LBEA 2161 and *Fusarium verticillioides* (Sacc.) Nirenberg LBEA 2163, LBEA 2164 were isolated at the Botany Laboratory of the Department of Agroforest from samples of bomba rice grains collected in the Albufera rice-producing Mediterranean area near Valencia (Spain). Their classification was confirmed by the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Institute of the Royal Netherlands Academy of Arts and Sciences.

Bioassays on essential oils activity

The essential oil was dissolved, mixed and homogenized by agitation in laboratory flasks with a previously sterilized PDA growth medium at 45-50°C when still in a liquid form. It was added at the 300 µg/mL (Tween 20, 0.1%) concentration and distributed into Petri capsules (90x15mm). Petri dish control contained equal amounts of sterilized water/Tween 20 (0.1%) on PDA was employed. The antifungal activity of the essential oils was tested following the poisoned food technique of Singh et al. (2008), which was modified.

Explants (8 mm in diameter), taken by a punch from a 7-day old colony, were placed in the centre of the Petri plates containing the essential oil. Plates were incubated at 25°C for 7 and 21 days. Fungal growth was evaluated by measuring two perpendicular diameters of the colony daily, and speed of growth was calculated. Six repetitions were undertaken per treatment. The Petri plate control contained only PDA.

Effect of oregano essential oil (OEO) on rice grain conservation

Healthy bomba rice grains, were collected from the Albufera rice-producing Mediterranean area. Kernels were washed with sodium hypochlorite (0.1%) for 10 min, rinsed twice with distilled water and air-dried at room temperature (25±2°C). Rice grains were placed into 15x10cm polystyrene containers (100 grains per container). The containers with rice grains were sprayed with 5 mL of a spore suspension of 5x10⁵ conidia mL⁻¹ of each fungi to be tested, and were air-dried to completely dry.

Two concentrations (100 and 200 µg/mL) of OEO solutions were prepared in Tween 20 (0.1%). Then 2 mL of each solution was vaporized (sprayed) into the containers spontaneously at 20°C. Controls were prepared similarly except for the volatile treatment. Filter paper, moistened with 0.5 mL sterilized water, was placed into each container and high relative humidity (90-95%) was maintained during the storage

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3 period. All the containers were then transferred to storage at 28°C for 30 days. The percentage of infected
4 rice grains was recorded after 30 days of incubation with a magnifying glass mod. Olympus SZX10.
5 There were five replicates per treatment.
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8 9 Statistical analysis

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11 The fungal growth results were submitted to variance analysis (ANOVA) with significant values at
12 $P \leq 0.05$. STATGRAPHICS Plus 5.0 software (Stat Point, Inc., Herndon, Virginia, USA) was used in the
13 study.
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16 17 18 Results

19 20 Chemical composition of the essential oils of bay leaf, cinnamon, clove and oregano

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22 The identified components are shown in Table 1, with their relative percentages. The composition of the
23 studied essential oils is typically high in their percentages of oxygenated components, which include
24 epoxides, esters, phenols and ketones.
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28 In bay leaf, the oxygenated components amounted to 78.8% of their entire composition, the majority
29 being epoxide 1,8-cineole (eucalyptol), at a percentage of almost 51% and ester α -terpinenyl acetate,
30 found at a percentage of 12.9%. Hydrocarbons (monoterpenes and sesquiterpenes) accounted for 18% of
31 their composition. Clove essential oil presented 90.3% of oxygenated compounds, and eugenol had the
32 greatest proportion (89.8%). Hydrocarbons (monoterpenes and sesquiterpenes) accounted for only 9%;
33 of these, 6.7% were β -caryophyllene and 1.9% were α -caryophyllene. In cinnamon essential oil, the
34 percentage of oxygenated compounds was 92%. The highest percentage was for eugenol, at
35 approximately 60%, followed by esters eugenyl acetate (18.3%) and cinnamyl acetate (5.6%), while
36 hydrocarbons (monoterpenes and sesquiterpenes) represented only 5%, of which the greatest proportion
37 was for β -caryophyllene (1.9%), followed by limonene (1.0%), α -pinene (0.8%), p-cymene (0.7%) and α -
38 caryophyllene (0.4%). Eugenol was the main volatile component of both extracts and, in our case, was
39 more abundant in clove than in cinnamon.
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43 Oregano essential oil contained 71.8% of oxygenated compounds and was characterized by its high
44 content of phenols carvacrol (49.6%) and thymol (21.2%). Monoterpenes and sesquiterpenes accounted
45 for 25.1%, of which the most outstanding were p-cymene (11%), γ -terpinene (9.2%), β -caryophyllene
46 (1.5%), α -terpinene (1.4%) and α -pinene (0.5%). The high content of carvacrol, thymol and their
47 precursors, γ -terpinene and p-cymene, has been previously reported as a common characteristic of all the
48 oregano species (Poulose and Croteau, 1978; Kokkini, 1996).
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51 52 Antifungal activity of the essential oils of bay leaf, cinnamon, clove and oregano

Study of oils on *Fusarium culmorum*

Fusarium culmorum presented growth rates of 11.75 mmd^{-1} when it grew in PDA growth medium. A slight increase in growth was observed when the fungus grew on PDA-bay leaf oil, and it reached a value of 11.88 mmd^{-1} . No significant differences were found. The essential oils of cinnamon, clove and oregano significantly lowered the speed of growth of *F. culmorum* ($P \leq 0.05$), which was 1.54 mmd^{-1} for cinnamon oil and 1.49 mmd^{-1} for clove oil, which meant a reduction of 87% in speed in both cases if compared to the control. Oregano essential oil totally inhibited *Fusarium culmorum* growth until day 17 (Figure 1).

Study of oils on *Fusarium verticillioides*

The speed of growth of *Fusarium verticillioides* in the PDA and PDA-bay leaf oil growth media was 6.6 and 4.82 mmd^{-1} , less than those presented for *Fusarium culmorum*. The behaviour of the fungus was practically the same when the essential oils of clove or cinnamon were added to the growth media, with growth values of 2.2 and 2.1 mmd^{-1} . Oregano essential oil also inhibited the growth of this fungus until day 18, and was practically null up to reading day 24, a reduction of 97.4% if compared to the control, (Figure 2). The essential oils of cinnamon, clove and oregano significantly reduced the growth of *Fusarium sambucinum* ($P \leq 0.05$).

The addition of oregano essential oil (OEO) not only reduced this fungi's speed of growth, but also modified the form, colour, sporulation and texture of the fungal colonies. Treatment with OEO totally inhibited the formation of the conidial genesis of *F. verticillioides* and *F. culmorum* (Figure 3).

Effect of oregano essential oil on rice grain conservation

Oregano essential oil was selected for the good results it obtained. The results obtained using OEO on rice grain conservation are shown in Figure 4. The results indicate that the percentage of infected rice grains significantly ($P \leq 0.05$) lowered by this essential oil at 28°C for 30 days. OEO at a concentration of 200 $\mu\text{g/mL}$ showed the greatest inhibition of fungal infection with a value of 90% (*F. culmorum*) and 95% (*F. verticillioides*), as compared with the control.

Discussion

The responses of the tested fungal species differed, but the same behaviour pattern was observed for the different essential oils studied.

The bibliography is contradictory as far as studies on the antifungal activity of bay leaf oil are concerned. In some studies, bay leaf oil activates fungal growth, whereas it acts as a fungicide and fungistatic in others (Atanda, et al., 2007; De Corato, et al., 2010).

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4 The chemical composition of bay leaf oil, which in our study had no inhibitory effect on the fungi tested,
5 was 50.7% of 1.8 cineole (eucalyptol), whereas there was a small proportion in cinnamon and oregano,
6 0.068 and 0.037 respectively, which was not detected at all in clove. The 1.8-cineole compound has been
7 described by various authors for its antifungal power against plant pathogenic fungi (García, et al., 2008;
8 Kordali, et al., 2007; Zhao, et al., 2011). However, we found no activity against the fungi tested.
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12 The essential oils that displayed the strongest antifungal activity in our study were those of oregano, clove
13 and cinnamon, which contained a high percentage of different phenols (eugenol, carvacrol and thymol).
14 Eugenol is abundant in clove (90%) and in cinnamon (60%), and is the main volatile component in these
15 oils, whereas in oregano, we found a high percentage of phenols carvacrol (50%) and thymol (21%). The
16 anti-fungal action of the extracts can be proven, but their action has yet to be elucidated (Holley and
17 Patel, 2005; Knaak, et al., 2013).
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24 Various authors have studied the effect of cinnamon, clove and oregano oils on the growth of the
25 *Fusarium* species isolated from plants, and their application in disinfecting seeds, without reducing their
26 germinating capacity, and have shown the potential of these oils with a dose ranging from 100 to 400
27 $\mu\text{g/mL}$ (Barrera and Garcia, 2008; Cueto et al., 2010; Dambolena et al., 2012; García-Camarillo et al.,
28 2006). However, it is not easy to correlate the fungicidal activity that cinnamon and clove oils present
29 with any of its single compounds, although it is with the complex mixture of compounds that essential
30 oils form, which evidences a synergetic effect (Burt, 2004; Ranasinghe, et al., 2002; Wink, 2003). The
31 effect of clove leaf extracts on the spore germination of fungi has been studied and a sharp drop was
32 found in the spore germination of *Alternaria alternata*, *Aspergillus niger*, *Fusarium solani* and *Rhizopus*
33 sp. (Shafique, et al., 2007).
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39 The OEO studied, with a high percentage of carvacrol and thymol, exhibited highly inhibited fungal
40 growth. This result coincides with the work of other authors who indicate that compounds carvacrol and
41 thymol present major antifungal activity against food spoilage fungi and phytopathogenic fungi (Ávila-
42 Sosa, et al., 2012; Cueto, et al., 2010; Dambolena, et al., 2012; Filipowicz, et al., 2003; Kordali, et al.,
43 2007; Muller-Ribeau, et al., 1995; Tabanca, et al., 2006; Terzi, et al., 2007; Tzortzakis and Economakis,
44 2007). Moreover other compounds such as γ -terpinene and p-cymene possess fungicidal activity
45 (Filipowicz, et al., 2003; Terzi, et al., 2007). Nevertheless, this activity is always poorer than the activity
46 that the essential oil presents, which can be attributed to the synergetic effect of the compounds found in a
47 lower proportion to the majority compounds.
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53 The results obtained in this work have been highly satisfactory against *Fusarium culmorum* and *Fusarium*
54 *verticillioides*, in addition to the complete inhibition of growth and the sporulation of the fungus at days
55 20 and 25, when oregano oil was used.
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These results led us to believe that the inhibited growth of the studied fungi is related to the content of phenols, eugenol, carvacrol and thymol and the ester eugenyl acetate in essential oils, and also to the synergetic effect of the compounds found in a lower proportion to the majority compounds. The higher percentage of non-oxygenated monoterpenes and sesquiterpenes (25.1%), among them p-cymene (11%) and γ -terpinene (9.2%), found in OEO is also noteworthy, as opposed to (9%) clove and (5%) cinnamon. The results suggest that essential oils are an alternative for practical applications in stored products. The addition of oregano, clove and cinnamon oils can provide an alternative for controlling *Fusarium culmorum* and *Fusarium verticillioides* in stored products, thus extending their shelf life. They can be used as preservatives and additives in foodstuffs, and can be applied to store grain and seeds.

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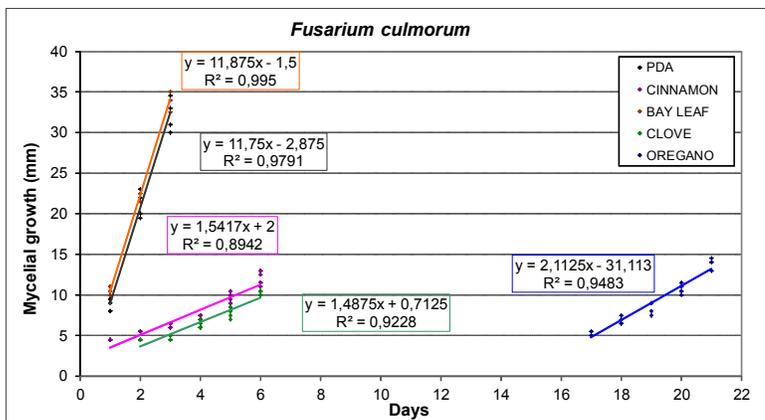


Figure 1 Mycelial growth of *Fusarium culmorum* on PDA (◇), PDA-Bay leaf (◆), PDA-Cinnamon (◇), PDA-Clove (◇) and PDA-Oregano (◆).

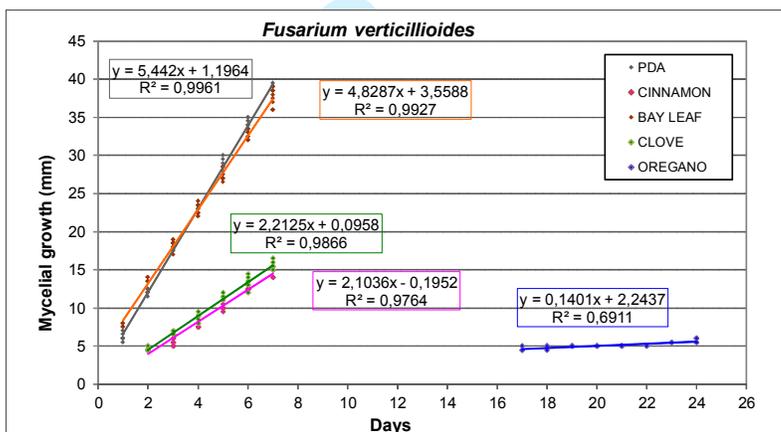


Figure 2 Mycelial growth of *Fusarium verticillioides* on PDA (◇), PDA-Bay leaf (◆), PDA-Cinnamon (◇), PDA-Clove (◇) and PDA-Oregano (◆).

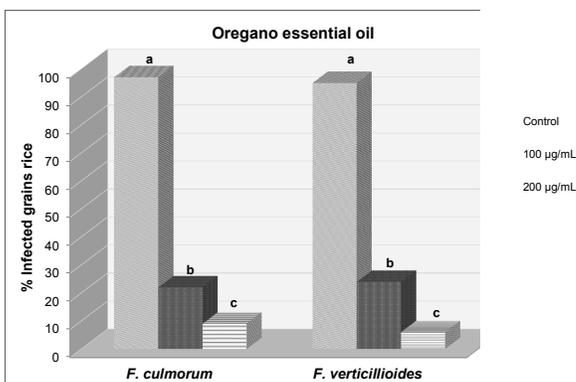


Figure 4 Efficacy of oregano essential oil on fungal development in inoculated rice grains. Significant difference $P < 0.01$, ANOVA test.

Table 1 Majors components of comercial essential oils of bay leaf (*Laurus nobilis*), cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*) and oregano (*Origanum compactum*).

KI ^a	COMPOUND ^b	CINNAMON (%) ^c	CLOVE (%) ^c	BAY LEAF (%) ^c	OREGANO (%) ^c
1273	2-propenal, 3-phenyl- ^b	1.999			
1362	eugenol ^b	60.403	89.757		
1420	β -caryophyllene ^{a,b}	1.913	6.715		1.513
1447	cinnamyl acetate ^b	5.567			
1538	eugenyl acetate ^{a,b}	18.33			
1453	α -caryophyllene ^{a,b}		1.917		
979	sabinene ^{a,b}			7.474	
980	β -pinene ^{a,b}			3.255	
1035	1,8-cineole (eucaliptol) ^{a,b}			50.654	
1063	γ -terpinene ^{a,b}			1.203	9.216
1001	linalool ^{a,b}			3.655	
1180	terpinen-4-ol ^{a,b}			2.165	
1193	α -terpineol ^{a,b}			2.301	
1357	α -terpinenyl acetate ^b			12.917	
1409	methyl eugenol ^b			3.803	
939	α -pinene ^{a,b}			3.95	
1020	α -terpinene ^{a,b}				1.405
1029	p-cymene ^{a,b}				11.03
1290	thymol ^{a,b}				21.174
1298	carvacrol ^{a,b}				49.548

^a Kovats index relative to *n*-alkanes (C₈-C₃₀). ^b a, identification based on retention times of genuine compounds on the HP-5MS agilent column; b, tentatively identified on the basis of computer matching of the mass spectra of peaks with the (Nist, Nist_msms, mainlib, replib, wiley7n) libraries. ^c Calculated from flame ionization detector data; tr, trace (< 0.05%).

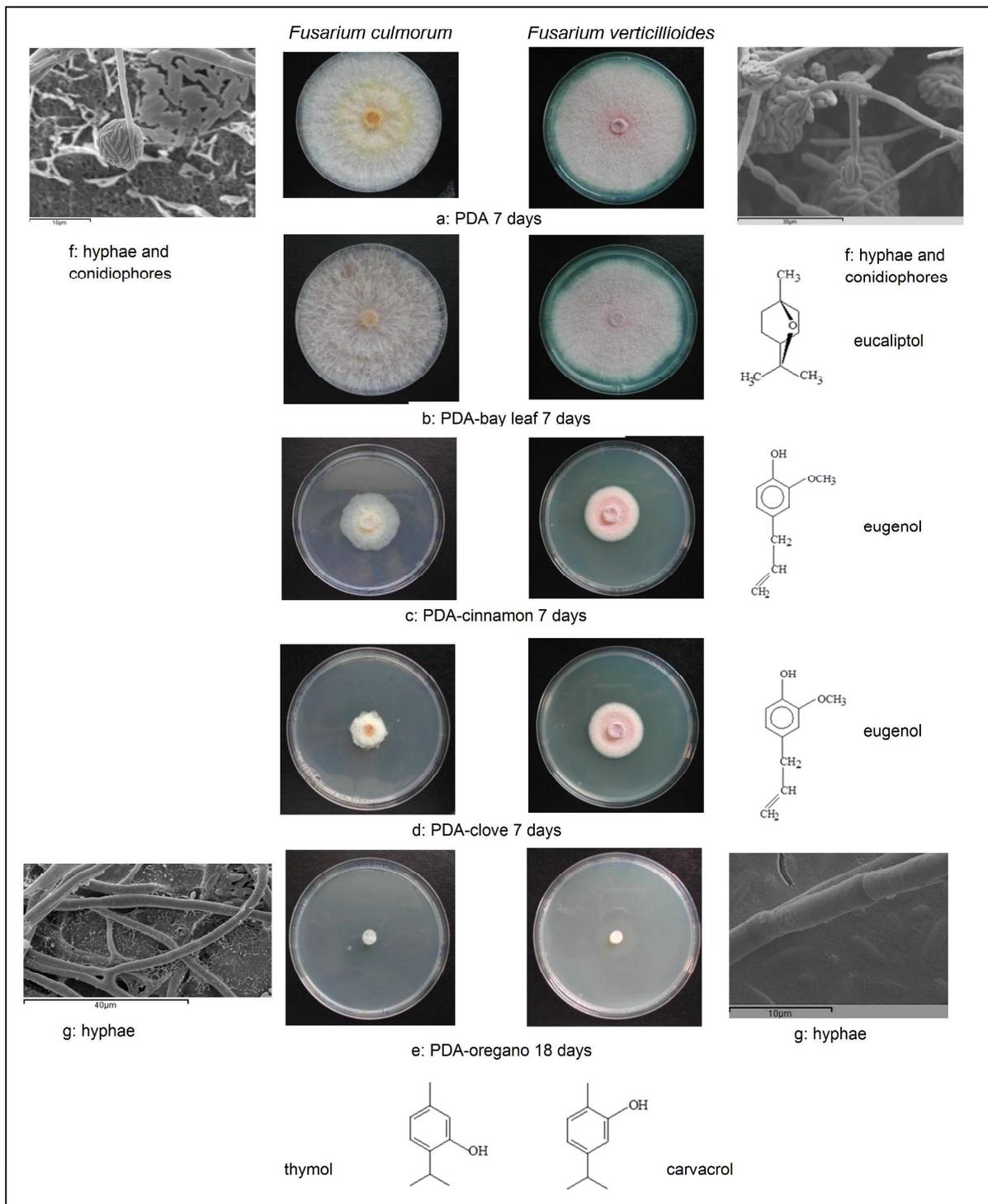


Figure 3 Mycelial growth and colony morphology of *Fusarium culmorum* and *Fusarium verticillioides* on PDA and different essential oils (a, PDA; b, PDA-bay leaf; c, PDA-cinnamon; d, PDA-clove; e, PDA-oregano) at 25 °C throughout the incubation time. Cryo-scanning electron micrographs showing f, hyphae and conidiophores (PDA); g, hyphae (PDA-oregano).