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Bomba Rice Conservation with a Natural Biofilm

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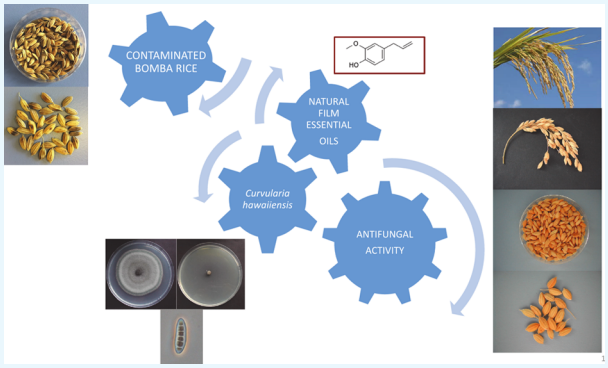
ABSTRACT: The chemical composition of commercial *Syzygium aromaticum*, *Cinnamomum verum*, and *Laurus nobilis* essential oils as well as their antifungal activity against four pathogenic fungi isolated from Mediterranean rice grains has been investigated. Eighty nine compounds accounting for between 98.5 and 99.4% of the total essential oil were identified. The phenylpropanoids eugenol (89.37 ± 0.29%) and eugenol (56.34 ± 0.41%), followed by eugenol acetate (19.48 ± 0.13%) were, respectively, the main compounds in clove and cinnamon essential oils, whereas large amounts of the oxygenated monoterpenes 1,8-cineole (58.07 ± 0.83%) and α-terpinyl acetate (13.05 ± 0.44%) were found in bay leaf essential oil. Clove and cinnamon oils showed the best antifungal activity results against all tested fungi. Against *Alternaria alternata*, clove essential oil displayed the best antifungal effect, whereas against *Curvularia hawaiiensis*, cinnamon essential oil was more active. Both essential oils showed a similar antifungal effect towards *Fusarium proliferatum* and *Fusarium oxysporum*. In vitro studies in inoculated rice grains showed that clove and cinnamon totally inhibited pathogenic fungal development after 30 days of incubation. In vivo studies showed that eugenol used with a polysaccharide such as agar−agar formed a fine coat which wraps the inoculated rice grains, creating a natural biofilm and reducing the development of all pathogenic fungi (80−95%) for 30 days.



INTRODUCTION

Cereal fungal contamination causes both economic and human health problems. Economically important diseases, such as smuts, leaf spots, crown rots, and root rots are usually caused by *Bipolaris*, *Curvularia*, *Fusarium*, and *Alternaria* species.[1] Infection of cereal seeds is a serious problem because these pathogens can remain viable for 10 years and are subsequently capable of propagating across other geographical areas, infecting further crops and achieving global dissemination.[2,3] *Bipolaris* and *Curvularia* are closely related genera of plant pathogens as well as emerging opportunistic human pathogens.[4−6] Several species, according to the method of infection and immune status, have been reported, from mild skin and nail infections to severe invasive human diseases. *Curvularia* is an important dematiaceous fungus involved in phaeohyphomycosis; *Curvularia australiensis*, *Ctenochaetus hawaiiensis*, and *Curvularia spicifera* have frequently been isolated from human phaeohyphomycoses.[7−9] These three species were formerly classified as members of the genus *Bipolaris*; however, phylogenetic studies have demonstrated that species previously placed in *Bipolaris*, especially those known as human pathogens, actually belong to the *Curvularia* genus.[4]

The genus *Fusarium* includes plant pathogens of agricultural crops, as well as mycotoxin-producing species.[10] Several species of *Fusarium*, such as *Fusarium verticillioides*, *Fusarium proliferatum*, and *Fusarium oxysporum* are responsible for the higher fumonisin levels observed in cereals. It is well known that these mycotoxins constitute a principal health risk for domesticated animals, being also associated with a number of human health problems, probably due to the consumption of large amounts of cereal-based products.[11] These emerging problems need increasing attention because of their toxic effects. Particularly worrying is the recently found high contamination levels of breakfast and infant cereals, such as muesli and cornflakes, which usually contain favorable ingredients for fungi colonization, with *Fusarium* mycotoxins such as ochratoxin A that has a studied impact on human health.[12]

Fusarium, together with *Alternaria*, *Cladosporium*, and *Trichoderma* genera, belongs to the allergenic fungi, important producers of outdoor airborne allergens which have been found in plant and soil samples from agricultural fields in which cereals are grown.[13]

On the other hand, *Alternaria* is the most common genus of endophytes in plants. Pathogenic *Alternaria* such as *Alternaria alternata* are used to control the host weed. A suspension of

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Table 1. Chemical Composition of Commercial Clove, Cinnamon, and Bay Leaf Essential Oils[a]

compound	RI	clove	cinnamon	bay
monoterpene hydrocarbons			3.84 ± 0.11	12.63 ± 0.22
tricyclene	926			0.04 ± 0.00
α-thujene	930		0.06 ± 0.00	0.10 ± 0.00
α-pinene	939		0.77 ± 0.03	2.93 ± 0.05
camphene	952		0.37 ± 0.01	0.33 ± 0.00
sabinene	976			5.56 ± 0.09
β-pinene	979		0.29 ± 0.01	2.44 ± 0.10
myrcene	991		0.05 ± 0.00	0.38 ± 0.00
α-phellandrene	1005		0.26 ± 0.01	0.10 ± 0.00
δ-3-carene	1011		0.03 ± 0.00	0.04 ± 0.00
α-terpinene	1019		0.10 ± 0.00	0.07 ± 0.00
p-cymene	1027		0.89 ± 0.02	0.50 ± 0.07
limonene	1029		0.01 ± 0.00	
β-phellandrene	1030		1.00 ± 0.03	
cis-ocimene	1039			0.04 ± 0.00
trans-ocimene	1052			0.03 ± 0.00
γ-terpinene	1061			0.06 ± 0.00
terpinolene	1089		0.03 ± 0.00	0.02 ± 0.00
oxygenated monoterpenes			1.14 ± 0.04	81.76 ± 0.24
1,8-cineole	1034			58.07 ± 0.83
cis-sabinene hydrate	1070		0.01 ± 0.00	0.13 ± 0.00
cis-linalool oxide	1072			0.05 ± 0.00
trans-linalool oxide	1086			0.03 ± 0.00
linalool	1098		0.57 ± 0.01	3.82 ± 0.04
trans-sabinene hydrate	1101			0.11 ± 0.00
cis-*p*-menth-2-en-1-ol	1121		0.02 ± 0.00	0.06 ± 0.00
trans-pinocarveol	1140			0.04 ± 0.00
trans-*p*-menth-2-en-1-ol	1141		0.03 ± 0.00	0.04 ± 0.00
camphor	1146		0.02 ± 0.00	0.01 ± 0.00
sabina ketone	1157			0.03 ± 0.00
pinocarvone	1162			0.02 ± 0.00
δ-terpineol	1165			0.12 ± 0.01
borneol	1169		0.12 ± 0.00	0.05 ± 0.00
terpinen-4-ol	1179		0.13 ± 0.00	2.00 ± 0.04
p-cymen-8-ol	1185		0.03 ± 0.00	0.04 ± 0.00
α-terpineol	1190		0.17 ± 0.00	2.26 ± 0.06
myrtenol	1194			0.15 ± 0.01
α-fenchyl acetate	1219			0.18 ± 0.01
nerol	1229			0.31 ± 0.01
linalyl acetate	1258			0.28 ± 0.01
p-menth-2-en-1,4-diol	1268			0.02 ± 0.00
bornyl acetate	1288			0.52 ± 0.00
carvacrol	1299		0.05 ± 0.01	
δ-terpinyl acetate	1315			0.25 ± 0.01
α-terpinyl acetate	1349			13.05 ± 0.44
neryl acetate	1361			0.15 ± 0.00
sesquiterpene hydrocarbons		8.06 ± 0.50	2.56 ± 0.02	0.29 ± 0.02
α-copaene	1376		0.36 ± 0.01	
β-elemene	1390			0.10 ± 0.01
β-caryophyllene	1419	6.02 ± 0.41	1.81 ± 0.01	0.10 ± 0.00
α-humulene	1454	1.70 ± 0.09	0.38 ± 0.00	
allo-aromadendrene	1460	0.02 ± 0.00		
γ-muurolene	1479	0.02 ± 0.00		
β-selinene	1490		0.02 ± 0.00	
α-muurolene	1498	0.02 ± 0.00		
γ-cadinene	1512			0.04 ± 0.01
trans-calamenene	1521	0.14 ± 0.01		
δ-cadinene	1521	0.12 ± 0.01		0.06 ± 0.01
α-calacorene	1544	0.02 ± 0.00		
oxygenated sesquiterpenes		0.93 ± 0.03	1.40 ± 0.03	0.16 ± 0.01
spathulenol	1578		0.18 ± 0.00	0.04 ± 0.00

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Table 1. continued

compound	RI	clove	cinnamon	bay
caryophyllene oxide	1583	0.77 ± 0.04	1.05 ± 0.03	0.12 ± 0.01
humulene epoxide II	1608	0.15 ± 0.01	0.15 ± 0.00	
cubenol	1646		0.01 ± 0.00	
aromatic compounds (C_6–C_3; C_6–C_1)		89.54 ± 0.16	89.91 ± 0.54	4.24 ± 0.24
benzyl acetate	1162		0.05 ± 0.00	
methyl chavicol	1196			0.21 ± 0.01
chavicol	1250		0.04 ± 0.00	
trans-cinnamaldehyde	1270		2.08 ± 0.00	
safrole	1287		0.12 ± 0.03	
cinnamyl alcohol	1304		0.08 ± 0.01	
eugenol	1359	89.37 ± 0.29	56.34 ± 0.41	0.91 ± 0.07
hydrocinnamyl acetate	1368		0.34 ± 0.00	
dihydro eugenol	1369	0.02 ± 0.01		
vanillin	1396	0.08 ± 0.02	0.06 ± 0.00	
methyl eugenol	1405	0.02 ± 0.00		3.00 ± 0.15
trans-cinnamyl acetate	1446		6.52 ± 0.03	
trans-methyl isoeugenol	1494			0.12 ± 0.01
eugenol acetate	1522		19.48 ± 0.13	
4-hydroxy-3-methoxy-cinnamaldehyde	1729	0.06 ± 0.01		
benzyl benzoate	1760		4.81 ± 0.03	
others		0.52 ± 0.15	0.02 ± 0.00	0.31 ± 0.01
isopropyl-isobutyrate	793			0.01 ± 0.00
2-methyl-ethyl-butanoate	849			0.02 ± 0.00
3-hexen-1-ol	853			0.06 ± 0.00
3-methyl-ethyl-butanoate	858			0.02 ± 0.00
2-methyl-1-methylethyl-butanoate	885			0.01 ± 0.00
2-heptanol	896			0.03 ± 0.00
2-methyl-butyl-propanoate	917			0.04 ± 0.00
2-nonanone	1090			0.03 ± 0.00
2-methyl-2-methylbutyl-butanoate	1100		0.02 ± 0.00	
monoacetin	1263	0.04 ± 0.00		
diacetin	1268	0.02 ± 0.00		
2-undecanone	1293			0.11 ± 0.00
triacetin	1376	0.47 ± 0.15		
total identified		99.04 ± 0.08	98.55 ± 0.06	99.40 ± 0.06

[a]RI: retention index relative to C_8–C_{32} *n*-alkanes on an HP-5MS column; t: trace amount ≤0.01. Values are means ± standard deviation of three samples.

Alternaria J46 mycelial segments and culture filtrates of the fungi display marked seed germination inhibition against different species, including the most important cereal crops worldwide for human consumption, wheat and rice.[14] This represents important economic losses as well as health problems due to *Alternaria*, which is also an important allergenic fungi and opportunistic human pathogen in immunocompromised patients.

Previous studies carried out by our research team showed significant antifungal effects of commercial oregano and thyme essential oils against phytopathogenic fungi.[15,16] The aims of this work were to (1) analyze the chemical composition of commercial essential oils of bay leaf, cinnamon, and clove, (2) determine the most important isolates and to identify four pathogens from rice seeds by morphological and molecular techniques, (3) evaluate in vitro and in vivo antifungal activity of essential oils, and (4) evaluate a natural biofilm we have created as an antifungal product for rice conservation.

RESULTS

Chemical Composition of Commercial Essential Oils. The identified components of commercial cinnamon, clove, and bay leaf essential oils are shown in Table 1. The individual compounds were identified by MS, and their identity was confirmed by comparison of their retention indices (RIs) and mass spectra with authentic samples or with data already available in the NIST 2005 Mass Spectral Library.[17]

Eighty nine compounds accounting for between 98.5 and 99.4% of the total essential oil were identified. In clove and cinnamon essential oils, more than 89% of aromatic compounds biosynthesized by the shikimic acid pathway were found: the phenylpropanoid eugenol (89.37 ± 0.29%) and eugenol (56.34 ± 0.41%), followed by eugenol acetate (19.48 ± 0.13%) were, respectively, the main compounds, whereas large amounts of the oxygenated monoterpenes 1,8-cineole (58.07 ± 0.83%) and α-terpinyl acetate (13.05 ± 0.44%) were found in bay leaf essential oil.

Antifungal Activity in Solid Media. Clove essential oil was more effective against *A. alternata*, whereas cinnamon essential oil was more active against *C. hawaiiensis* (Table 2, Figures 1–3). Both essential oils displayed a similar antifungal effect against *F. proliferatum* and *F. oxysporum*. Cinnamon and clove completely inhibited the growth of *C. hawaiiensis* to days 7 and 8 (Figures 1 and 2). In model conditions assayed at 300

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Table 2. Effects of Clove (Cl), Cinnamon (C), and Bay Leaf (BL) essential oils (300 μg/mL) on Radial Growth and Growth Rates of *A. alternata*, *C. hawaiiensis*, *F. proliferatum*, and *F. oxysporum*. Confidence Intervals with a Probability of 0.95[a]

species-treatment	mean	lower limit	upper limit	GR
A. alternata-PDA	25.11 ± 2.24	20.71	29.51	5.44 (0.99)
A. alternata-Cl	7.88 ± 1.26	5.40	10.36	2.53 (0.97)
A. alternata-C	11.85 ± 1.29	9.32	14.41	3.39 (0.99)
A. alternata-BL	19.67 ± 1.52	16.69	22.65	4.80 (0.99)
C. hawaiiensis-PDA	25.61 ± 1.89	21.90	29.32	7.00 (0.99)
C. hawaiiensis-CL	4.61 ± 0.82	2.99	6.22	1.10 (0.96)
C. hawaiiensis-C	2.72 ± 0.78	1.19	4.24	0.97 (0.94)
C. hawaiiensis-BL	22.83 ± 1.66	19.57	26.09	6.20 (0.99)
F. proliferatum -PDA	17.56 ± 1.40	14.80	20.31	5.21 (0.99)
F. proliferatum-CL	6.22 ± 1.09	4.08	8.37	2.49 (0.99)
F. proliferatum-C	7.03 ± 1.03	5.00	9.07	2.71 (0.99)
F. proliferatum-BL	15.88 ± 1.40	13.12	18.64	5.13 (0.99)
F. oxysporum-PDA	20.82 ± 1.40	18.06	23.58	5.74 (0.99)
F. oxysporum-CL	5.98 ± 1.09	3.84	8.13	2.46 (0.99)
F. oxysporum-C	5.85 ± 1.03	3.81	7.88	2.44 (0.98)
F. oxysporum-BL	18.77 ± 1.40	16.01	21.53	5.26 (0.99)

[a]Mean: mean radius ± standard error; GR: growth rate (R^2).

μg/mL, bay leaf essential oil showed no significant antifungal activity against the tested phytopathogenic fungi, whereas clove and cinnamon showed significant antifungal activity against all tested fungi, with a similar behavior pattern (Figure 3).

For the measured mycelial growth inhibition (MGI), the antifungal effect of cinnamon at doses of 100 and 200 μg/mL against *C. hawaiiensis* is noteworthy (Table 3). At the highest concentration (300 μg/mL), pure eugenol showed the best antifungal activity results against all tested phytopathogenic fungi (Table 3).

Essential Oils on Rice Storage. In vitro studies showed that the disease produced in rice grains inoculated with all tested fungi (*A. alternata*, *C. hawaiiensis*, *F. proliferatum*, and *F. oxysporum*) was totally inhibited when the kernels were placed into PDA-clove and PDA-cinnamon (Figure 4). Both essential oils completely inhibited the growth of pathogenic fungi in the caryopsis rice at 300 μg/mL after 30 days of incubation.

In vivo studies showed that eugenol used with a polysaccharide such as agar−agar (0.25%) formed a fine coat which wraps the inoculated rice grains, creating a natural biofilm and reducing the development of all pathogenic fungi at 300 and 600 μg/mL (Figure 4). Eugenol significantly ($P < 0.05$) reduced fungal growth in stored rice, depending on the dose used after 30 days of incubation at 28 °C. At 300 μg/mL, eugenol showed high antifungal activity, reducing *C. hawaiiensis*, *F. proliferatum*, and *F. oxysporum* by between 85 and 82%. At 600 μg/mL, *A. alternata* was reduced by 80% and the highest antifungal activity was 95 and 92% in *C. hawaiiensis*, *F. proliferatum*, and *F. oxysporum*, showing an antifungal effect after 30 days of incubation at 28 °C (Figure 5).

DISCUSSION

Eugenol, the main compound in both essential oils, is a natural phenolic compound characterized, among a wide range of

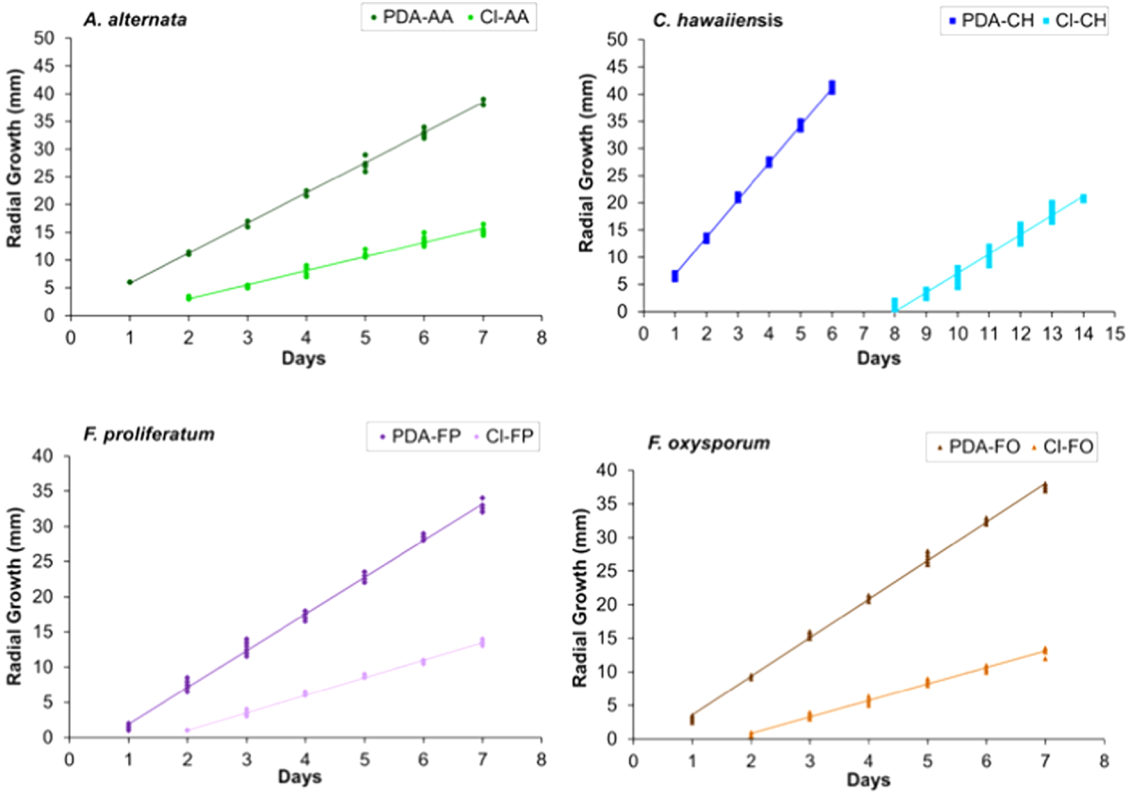

Figure 1. Growth rate (mm/day) of fungi on potato-dextrose agar (PDA) and clove (Cl) essential oil (300 μg/mL). *A. alternata* (PDA-AA: control; Cl-AA: clove), *C. hawaiiensis* (PDA-CH: control; Cl-CH: clove), *F. proliferatum* (PDA-FP: control; Cl-FP: clove), and *F. oxysporum* (PDA-FO: control; Cl-FO: clove)

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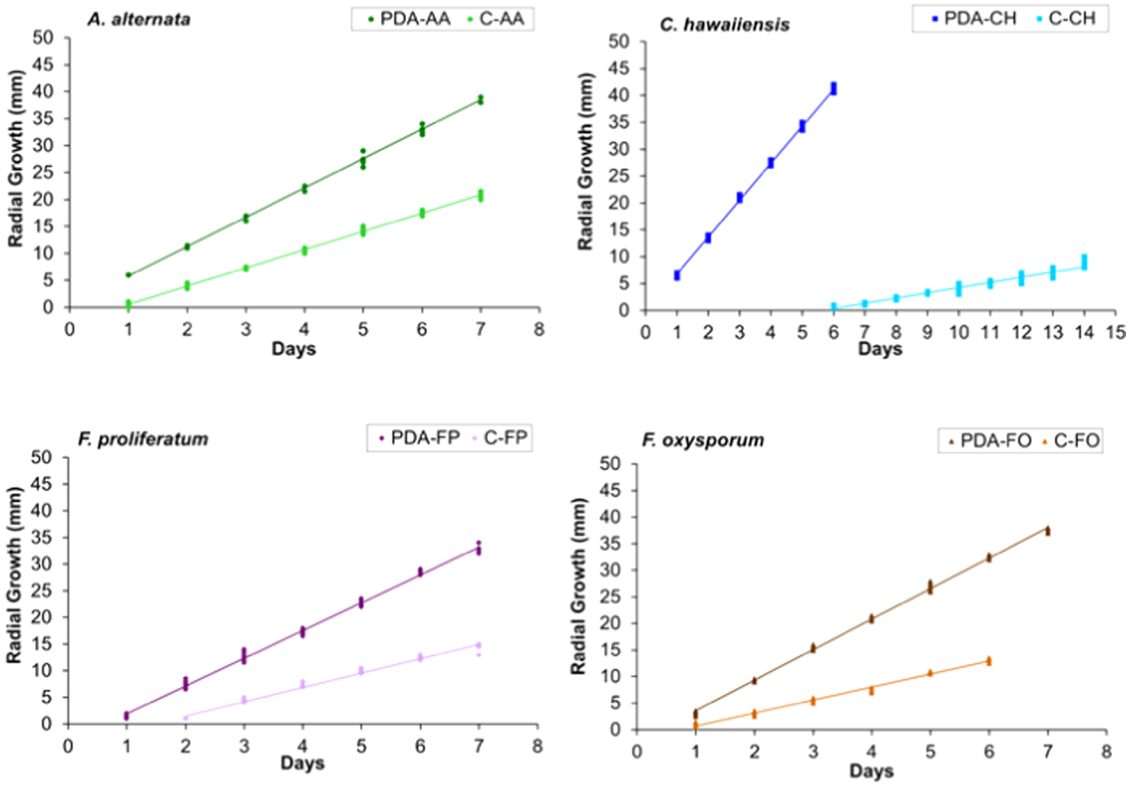

Figure 2. Growth rate (mm/day) of fungi on PDA and cinnamon (C) essential oil (300 μg/mL). *A. alternata* (PDA-AA: control; C-AA: cinnamon), *C. hawaiiensis* (PDA-CH: control; C-CH: cinnamon), *F. proliferatum* (PDA-FP: control; C-FP: cinnamon), and *F. oxysporum* (PDA-FO: control; C-FO: cinnamon).

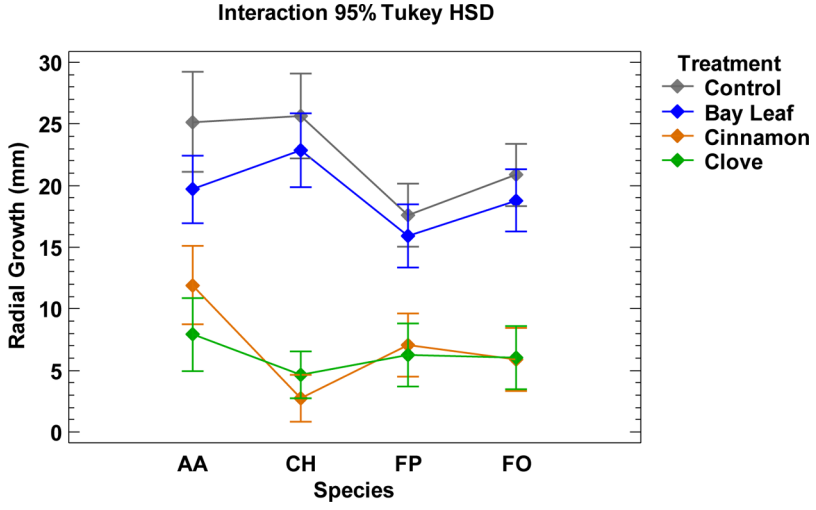

Figure 3. Interaction plot (mean radius, species, and treatment) at 300 μg/mL of bay leaf, cinnamon, and clove against *A. alternata* (AA), *Curvularia hawaiiensis* (CH), *F. proliferatum* (FP), and *F. oxysporum* (FO).

Table 3. Mycelial Growth Inhibition (MGI) of *A. alternata* (AA), *C. hawaiiensis* (CH), *F. proliferatum* (FP) and *F. oxysporum* (FO) with Cinnamon (C), Clove (Cl), and Eugenol (E)[a]

	AA			CH			FP			FO		
concentration (μg/mL)	C	Cl	E	C	Cl	E	C	Cl	E	C	Cl	E
100	19.81	33.61	39.97	77.85	26.96	49.59	22.25	17.53	35.38	23.59	21.04	31.46
200	29.72	50.12	51.65	82.55	62.85	93.14	33.97	31.68	43.67	42.63	40.43	67.77
300	55.73	62.67	66.28	100	100	100	55.64	54.80	60.06	65.28	64.94	95.02

[a]MGI: percentage inhibition.

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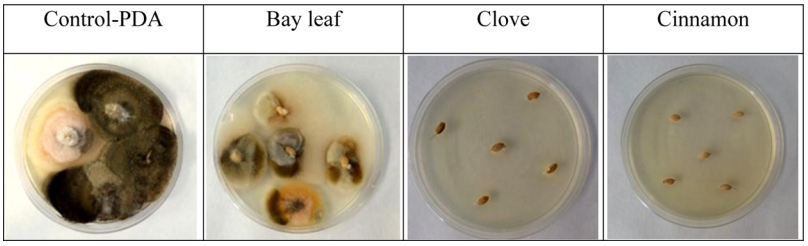

Figure 4. Experiment 1. Effect of essential oils on rice grain conservation after 30 days. From left to right: *A. alternata* on PDA, PDA-bay leaf, PDA-clove, and PDA-cinnamon (300 μg/mL). The PDA and PDA-bay leaf plates show the development of the inoculated fungus *A. alternata* plus endophytic mycobiota, whereas the PDA-cinnamon and PDA-clove plates show total inhibition of the development of *A. alternata* and endophytic mycobiota.

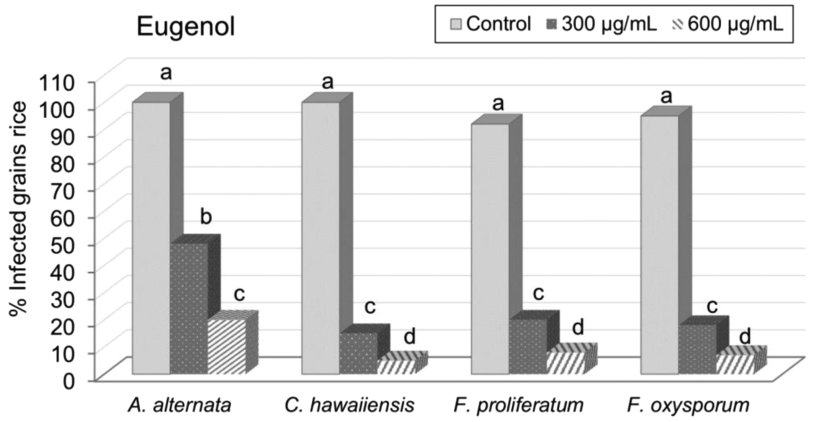

Figure 5. Efficacy of different concentrations of eugenol (300 and 600 μg/mL) on fungal development of *A. alternata*, *C. hawaiiensis*, *F. proliferatum*, and *F. oxysporum* in inoculated rice grains after 30 days. Significant difference at 95% level probability using Fisher's least significant difference.

biological properties, by its antifungal activity;[16] in the year 2013 it was approved as a fungicide by the European Food Safety Authority (EFSA), (Reg. EU No. 546/2013). Also, the use of eugenol coated with polysaccharides such as agar−agar enhances the shelf life of rice during the storage period.[18]

The chemical versatility of its structure has led to the use of this compound as a starting biological material for the synthesis of new antifungal eugenol derivative agents to reduce nosocomial infections caused by *Candida* spp., especially in patients admitted to an intensive care unit.[19] The antifungal activity of eugenol against clinically relevant fungi, including fluconazole-resistant strains, has also been observed for clove essential oil, which is able to inhibit *Aspergillus* and *Candida* species (such as *C. albicans*, *C. tropicalis*, and *Candida parapsilosis*) and fluconazole-resistant *C. albicans* isolates, as well as clinical dermatophyte strains.[20]

According to samples of *Syzygium aromaticum* leaf essential oil grown in Madagascar[21] and in the commercial clove oil analyzed here, the main compound is the phenylpropanoid eugenol (89.37 ± 0.29%), followed by the sesquiterpene hydrocarbons β-caryophyllene (6.02 ± 0.41%) and α-humulene (1.70 ± 0.09%). Only two compounds, caryophyllene oxide (0.77 ± 0.04%) and humulene epoxide (0.15 ± 0.01%), were detected in the oxygenated sesquiterpene fraction. In addition, neither hydrocarbons nor oxygenated monoterpenes were present in the commercial *S. aromaticum* essential oil analyzed here. However, the main compounds found in *Cinnamomum verum* were the phenylpropanoid eugenol (56.34 ± 0.41%), eugenol acetate (19.48 ± 0.13%), *E*-cinnamyl acetate (6.52 ± 0.03%), and *E*-cinnamaldehyde (2.08 ± 0.00%), along with a large amount of the aromatic compound benzyl benzoate (4.81 ± 0.03%). On the other hand, in this essential oil, 30 compounds biosynthesized from the mevalonic acid pathway were identified but only the monoterpene hydrocarbon β-phellandrene (1.00 ± 0.03%), the sesquiterpene hydrocarbon β-caryophyllene (1.81 ± 0.01%), and the oxygenated sesquiterpene caryophyllene oxide (1.05 ± 0.03%) reached percentages close to or higher than 1%. Finally, oxygenated monoterpenes were quantitatively the minor fraction (1.14 ± 0.04%) in cinnamon essential oil, with linalool (0.57 ± 0.01%), α-terpineol (0.17 ± 0.01%), terpinen-4-ol (0.1 3± 0.00%), and borneol (0.12 ± 0.00%) as the main compounds. Although both essential oils have the same main compound, eugenol (89.37 ± 0.29 vs 56.34 ± 0.41%), commercial clove essential oil contains a higher quantity of sesquiterpene hydrocarbons (8.06 ± 0.50 vs 2.56 ± 0.02%), whereas commercial cinnamon essential oil has more oxygenated sesquiterpenes (1.40 ± 0.03 vs 0.90 ± 0.03%) and both hydrocarbons (3.84 ± 0.13%) and oxygenated monoterpenes (1.14 ± 0.04%) that were not detected in clove essential oil. *S. aromaticum* and *Cinnamomum zeylanicum* were the most active tested essential oils against stem and ear rot caused by *Stenocarpella maydis* responsible for severe losses in maize, reducing the pathogen incidence in the seeds by 39.0 and 28.0%, respectively.[22] Although *C. zeylanicum* has a lower content of eugenol than *S. aromaticum*, the presence of eugenol acetate (19.48 ± 0.13%), *trans*-cinnamyl acetate (6.52 ± 0.03%), benzyl benzoate (4.81 ± 0.03%), and *trans*-cinnamaldehyde (2.08 ± 0.00%) in the commercial cinnamon essential oil here analyzed may contribute to the antifungal activity.[23,24] In fact, cinnamaldehyde was found to have more antifungal activity than eugenol against *Aspergillus fumigatus* and *Trichophyton rubrum*[25] and a study about the antifungal effects

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against *Fusarium* spp. and *C. zeylanicum* with *trans*-cinnamaldehyde as the main compound showed more anti-*Fusarium* activity than with *Citrus limon*, *Juniperus communis*, *Eucalyptus citriodora*, *Gaultheria procumbens*, *Melaleuca alternifolia*, *Origanum majorana*, *Salvia sclarea*, and *Thymus vulgaris* essential oils.[26]

On the other hand, eugenol (57.0%) has been described as the main compound in commercial bay leaf essential oil[27] as well as in samples of *Laurus nobilis* essential oil (eugenol 44.13%), followed by a large amount of cinnamaldehyde (30.28%)[28] that has antifungal effects against fungi (*Eurotium*, *Aspergillus*, and *Penicillium*) commonly responsible for spoilage of bakery products or postharvest diseases produced by *A. alternata* in cherry tomatoes, respectively. However, the commercial bay leaf essential oil analyzed here is characterized by a high monoterpene fraction content (94.39%), mainly oxygenated monoterpenes (81.76 ± 0.24%), with 1,8-cineol (58.07 ± 0.83%) and α-terpinyl acetate (13.05 ± 0.44%) as the main compounds. A similar composition was found in commercial *L. nobilis* essential oil from Spain with medicinal items 1,8-cineole (51%) and α-terpinyl acetate (10%),[29] with *L. nobilis* essential oil from Brazil 1,8-cineole (35.50%), linalool (14.10%), sabinene (9.45%), and terpinyl acetate (9.65%)[30] or with dried bay leaves purchased from a local market in Tunisia, with 1,8-cineole (39.76%) and α-terpinyl acetate (13.35%)[31] as the main compounds. Bay leaf essential oils with a high content of 1,8-cineole, linalool and terpinyl acetate were shown to have antibacterial activity toward foodborne pathogens, such as *Escherichia coli* and *Yersinia enterocolitica* in fresh Tuscan sausage,[30] and also antifungal activity against *Botrytis cinerea*, *Monilinia laxa*, and *Penicillium digitatum*.[32] However, the tested bay leaf essential oil with a high content of the oxygenated monoterpene 1,8-cineol did not show a significant antifungal effect against the four pathogenic fungi isolated from rice grains. These results are in accordance with the potency level of antifungal activity measured as in vitro mycelial growth of the main compounds (thymol > eugenol > carvone > terpinen-4-ol > 1,8-cineole) of essential oils.[33]

CONCLUSIONS

Clove and cinnamon essential oils with the highest amounts (89.37 and 56.34%, respectively) of eugenol, contrary to bay leaf essential oil containing 58.07% of the oxygenated monoterpene 1,8-cineole, have a significant antifungal effect against the four pathogenic fungi isolated from rice grains. In vivo studies showed that eugenol used with a polysaccharide such as agar−agar forms a fine coat that wraps the inoculated rice grains, creating a natural biofilm and reducing the development of all pathogenic fungi (80−95%) for 30 days. Eugenol could be used as an effective nontoxic preservative in stored rice grains against *A. alternata*, *C. hawaiiensis*, *F. proliferatum*, and *F. oxysporum* contamination, increasing their shelf life.

MATERIALS AND METHODS

Plant Material. Commercial samples of clove leaf (*Syzygium aromaticum* L.) were supplied by Guinama and cinnamon leaf (*C. verum* J. Presl) and bay leaf (*Laurus nobilis* L.) essential oils were supplied by Essential Arôms. The essential oils were stored at 4 °C until chemical analysis and antifungal studies were done.

Gas Chromatography (GC)−Mass Spectrometry (MS). A gas chromatography−mass spectrometry (GC/MS) analysis was carried out with Agilent 5973N apparatus, equipped with a capillary column (95 dimethylpolysiloxane-5% diphenyl) and an HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 μm film thickness). The column temperature program was 60 °C for 5 min, with 3 °C/min increases to 180 °C and then 20 °C/min increases to 280 °C. This program was maintained for 10 min. Helium was the carrier gas used at a flow rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the m/z 30−500 range, with an ionizing voltage of 70 eV. Kovat's retention index (RI) was calculated using cochromatographed standard hydrocarbons. The individual compounds were identified by MS, and their identity was confirmed by comparison of their RIs, relative to C_8−C_{32} *n*-alkanes, and mass spectra with authentic samples or with data already available in the NIST 2005 Mass Spectral Library and in the literature.[17]

Fungal Species. Four phytopathogenic fungi, *A. alternata* (Fr.) Keissler CECT 20943 (LBEA 2103), *Curvularia hawaiiensis* Manamgoda, Cai & Hyde CECT 20934 (LBEA 2105) *F. proliferatum* (Matsush.) Nirenberg CECT 20944 (LBEA 2170), and *F. oxysporum* (Sacc.) Snyder & Hansen CECT 2715 (LBEA 2004) were isolated in the Laboratorio Botánica of the Departament of Ecosistemas Agroforestales (LBEA) Universitat Politècnica de València from Bomba rice samples collected in a Mediterranean region producing rice (Valencia, Spain). The fungal species were morphologically and molecularly identified and then deposited in the Spanish Type Culture Collection (CECT).

Fungal Strain Identification. Morphological analysis consisted of inoculation, incubation, and validation of culture characteristics, as well as microscopic observation, growing data, or colony morphology. Molecular analysis was based on amplification, sequencing, and BLAST alignment comparison of target regions in fungal DNA for forward and reverse directions: internal transcribed spacer regions ITS1 and ITS2 of ribosomic DNA, including the 5.8S rRNA gene, using the primers its1 and its4;[34,35] D1/D2 domains on the 5′ end of the gene which codifies 28S rRNA, using nl1 and nl4 primers;[36,37] a 0.3 kb fragment of the EF-1α gene (transcription elongation factor), with EF-1728F and EF-986R primers[38] and, finally, partial sequencing of the β-tubulin gene using Bt2a and Bt2b primers.[39]

Sequence comparison between the amplified regions and those available in the NCBI Taxonomy Database (http://www.ncbi.nlm.nih.gov/taxonomy) and BLAST analysis of the sequences against assembling the fungal tree of life and MycoBank/CBS-KNAW Fungal Biodiversity Center (BioloMICSNet Software) databases showed that the isolate LBEA 2103 (CECT 20943) showed 99% identity for ITS regions and 100% identity for 28S rRNA 5′ domains with *C. hawaiiensis* (synonymous with *Curvularia oryzae*); the isolate LBEA 2105 (CECT 20923) showed 100% identity for ITS regions, 99% identity for EF-1α elongation factor, and 100% identity for the β-tubulin gene with the complex *Alternaria* sp. aff. *A. alternata*; the isolate LBEA 2170 (CECT 20944) showed 100% identity for ITS regions and 99.67% identity for β-tubulin gene with the species *F. proliferatum* (teleomorph *Gibberella intermedia*), and finally, the isolate LBEA 2004 (CECT 2715) showed 100% identity for ITS regions and 99.65% identity for EF-1α elongation factor with *F. oxysporum*.

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Antifungal Activity in Solid Media. *Growth Rate and Mycelial Growth Inhibition (MGI).* Essential oils were dissolved, mixed, and homogenized in previously sterilized and still liquid PDA/Tween 20 (0.1%) at 300 μg/mL. Then, it was distributed in 90 × 15 and 150 × 15 mm^2 Petri dishes. Fungi were sowed in the center of each Petri dish with 8 mm discoid explants from a 7 day culture. Petri dish plates were incubated in the dark at 25 °C for 7 and 14 days. Control Petri dishes only had PDA/Tween 20 (0.1%). Fungi growth was evaluated by measuring the daily diameter of the colony in two perpendicular directions, calculating the speed of growth. Six repetitions were made per treatment. MGI also was calculated according to the following formula at 100, 200, and 300 μg/mL

$$\mathrm{MGI} = [(\mathrm{CD} - \mathrm{OD})/\mathrm{CD}] \times 100$$

CD: average diameter of colonies in nontreated dishes (without essential oil); OD: average diameter of colonies in treated dishes (with essential oil).

Essential Oils on Rice Storage. Valencian rice healthy grains were washed with sodium hypochlorite (20%) for 5 min, rinsed twice with distilled water, and air-dried at room temperature (25 ± 2 °C). Then, 150 seeds of rice for each tested fungus were dipped into a flask containing 50 mL of a spore suspension of 5 × 10^5 conidia/mL prepared in water−Tween 20 (0.1%) for 30 min; finally, they were air-dried to complete dryness. Two experiments were carried out.

In Vitro Study of Essential Oil Antifungal Effect on Rice Caryopsis. The eugenol (98%) used in this study was supplied by Sigma-Aldrich. Inoculated rice caryopsis were placed into Petri dishes containing PDA-bay leaf, PDA-clove, and PDA-cinnamon, 300 μg/mL (5 seeds per plate). For each fungus, six replicate dishes were used. Plates were incubated in the dark at 25 °C and high relative humidity (90−95%) for 30 days. Control Petri dishes contained equal amounts of sterilized water/Tween 20 (0.1%) on PDA but without essential oils. Fungal growth was evaluated through observation for 30 days.

In Vivo Study of Eugenol Antifungal Effect on Rice Caryopsis. Rice caryopsis inoculated with the molds were placed inside 150 × 150 mm^2 plastic boxes, 100 seeds per box. Two concentrations (300 and 600 μg/mL) of eugenol were prepared in Tween 20 (0.1%)−agar 0.25%. Then, 5 mL of each solution was sprayed into the boxes. The seeds were wetted with the prepared solutions and dried to complete dryness, forming a fine coating. Controls were prepared similarly for volatile treatment with equal amounts of sterilized water/Tween 20 (0.1%)−agar 0.25% but without eugenol. All boxes were then transferred to storage at 28 °C and high relative humidity (90−95%) for 30 days. The percentage of infected rice grains was recorded after 30 days of incubation with an Olympus SZX10 magnifying glass.

Statistical Analysis. The fungal growth results were submitted to an analysis of variance (ANOVA). Furthermore, HSD Tukey intervals were represented with significant values at $P < 0.05$. Data analysis was performed using Statgraphics Centurion XVI.

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Notes

The authors declare no competing financial interest.

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