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Commercial *Laurus nobilis* L. and *Syzygium aromaticum* L. Merr. & Perry essential oils against post-harvest phytopathogenic fungi on rice

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ABSTRACT

Rice is exposed in the field and in stored conditions to a great variety of fungi that can cause a lot of diseases with potential risk to consumers. In the present study, the chemical composition of commercial *Laurus nobilis* L. and *Syzygium aromaticum* L. Merr. & Perry essential oils and antifungal activity against five pathogenic fungi isolated from Mediterranean rice grains has been investigated. Thirty-seven compounds accounting for more than 99.5% of the total essential oil were identified by GC and GC/MS. 1,8-Cineole (51.95%), α-terpinyl acetate (12.93%) and the monoterpene hydrocarbon sabinene (9.56%) were the main compounds in bay leaf essential oil, while the phenylpropanoid eugenol (88.58%), and the sesquiterpene hydrocarbons β-caryophyllene (8.13%) and α-humulene (2.35%) were found in clove essential oil. Clove essential oils at 300 μg/mL showed more antifungal effect than bay leaf essential oil against all tested strains. *S. aromaticum* essential oil showed the best antifungal activity towards *Fusarium graminearum* and similar antifungal activity compared to pure eugenol against all tested phytopathogenic fungi. In inoculated rice grain, clove essential oil significantly reduced the fungal infection in food so *S. aromaticum* essential oil could be a good alternative as preservative in stored rice grain.

1. Introduction

Spices mainly used to enhance the flavour and taste of foods, have been employed since ancient times for their preservative and medicinal properties. Several researches about the antioxidant, insecticidal, antimicrobial or antifungal properties of their essential oils have been carried out in order to applied theses complex mixtures as natural food preservatives. Reducing or eliminating food-related microorganisms without negative effects on food quality can extend the shelf life of food and become an attractive option to fight against foodborne diseases (Blázquez, 2014).

Dried bay leaf (*Laurus nobilis* L.), a spice used in traditional culinary practices as a flavouring agent in soups, meats, fish, vinegars and beverages, plays an important role in the human Mediterranean diet, and its essential oil is commonly employed by the pharmaceutical industry. Bay leaf essential oil shows chemical polymorphism according to origin or food and pharmaceutical items (Da Silveira et al., 2014; Peris & Blázquez, 2015; Xu et al., 2014). The high content in oxygenated monoterpenes, such as 1,8-cineole, linalool and terpinyl acetate, in bay leaf essential oils showed, in model conditions, antimicrobial activity towards foodborne pathogens *Escherichia coli* and *Yersinia enterocolitica* (Da Silveira et al., 2014) and also antifungal activity against *Botrytis cinerea*, *Monilinia laxa* and *Penicillium digitatum*. *M. laxa* was the most sensitive fungus to bay leaf essential oil, followed by *B. cinerea*, whereas the mycelial growth of *P. digitatum* was only partially inhibited by all concentrations applied (De Corato, Maccioni, Trupo, & Di Sanzo, 2010). The antimicrobial and antifungal activities were corroborated in food, since *L. nobilis* essential oil was able to improve safety and to extend the product shelf life of fresh sausages stored at 7 °C, as well as to control fruit post-harvest diseases, mainly on kiwi fruits and peaches (Da Silveira et al., 2014; De Corato et al., 2010). On the other hand *L. nobilis* essential oil with its high content of the phenylpropanoids, eugenol (44.13%) and cinnamaldehyde (30.28%), has also, in model conditions, demonstrated significant antifungal effects against *Alternaria alternata*, being able to decrease efficiently the infection ratio of *Alternaria* rot disease on cherry tomatoes. So, different compositions of *L. nobilis* essential oils can be applied as a natural and environmentally friendly

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fungicide to control the post-harvest disease of fruits (Xu et al., 2014).

Cloves (*Syzygium aromaticum* (L.) Merr. and Perry), like other spices with an intense flavour, are recommended as a preservative in processed and ready-to-eat foods. The essential oil's high content of the phenylpropanoids eugenol and eugenyl acetate is responsible for a wide range of biological activities and uses. Eugenol is a fast-acting contact insecticide effective against a broad variety of insects and mites (Dayan, Cantrell, & Duke, 2009). Clove essential oil (90% eugenol) is a desirable tool, instead of conventional insecticides, for protecting *Phaseolus vulgaris* L. against *Acanthoscelides obtectus* Say, which is responsible for severe post-harvest losses in the common bean (Viteri Jumbo, Faroni, Oliveira, Pimentel, & Silva, 2014). A lower content of eugenol still produces an interesting natural product since clove essential oil with only 59.75% eugenol and 29.24% eugenyl acetate showed significant activity against the most devastating visceral leishmaniasis or kala-azar caused by *Leishmania donovani*, with IC50 of 21 ± 0.16 mg/mL and 15.24 ± 0.14 mg/mL against promastigotes and intracellular amastigotes respectively, which could be an alternative to more expensive drugs of choice that also have multiple side effects (Islamuddin, Sahal, & Afrin, 2014). With regard to the antifungal effect, clove inhibited in a dose-dependent manner the growth of *Penicillium citrinum*, although it required higher concentrations to significantly inhibit citrinin production, a nephrotoxin found as a common contaminant in rice, wheat and red yeast rice, in culture medium. The growth of *P. citrinum* as well as citrinin production on rice was delayed by up to 3 days by clove (Aiko & Mehta, 2013).

The antifungal activity shown by *L. nobilis* essential oil (eugenol type) against *A. alternata* and *S. aromaticum* on rice towards *P. citrinum* leads us to continue with these spices, testing the antifungal activity of bay leaf essential oil (1,8-cineol type), used in the pharmaceutical industry, and clove essential oil against five post-harvest phytopathogenic fungi (*A. alternata*, *Bipolaris oryzae*, *Fusarium graminearum*, *Fusarium equiseti* and *Fusarium verticillioides*), isolated from rice populations from Valencia in the Mediterranean area (Santamarina, Roselló, Sempere, Giménez, & Blázquez, 2015), as well as to compare, using the same doses, the more antifungal essential oils with those of eugenol, since this phenolic compound has been recently approved (Reg. (EU) No 546/2013) by the European Food Safety Authority (EFSA) as a fungicide (2007/442/EC, Dossier complete 2011/266/EU), in order to find cheaper natural products to improve the safety and shelf life of stored rice grains.

2. Materials and methods

2.1. Plant material

Commercial samples of bay leaf (*Laurus nobilis* L.) essential oil, lot 719B032807, supplied by Essential Arôms (Lleida, Spain), and clove leaf (*Syzygium aromaticum* (L.) Merr. & Perry) essential oil, lot 9449600032, purchased from Guinama (Valencia, Spain), were stored at 4 °C until chemical analysis and antifungal studies. Other materials and chemicals used were of analytical grade and purchased from local suppliers.

2.2. Chemical composition of the essential oils

2.2.1. Gas chromatography (GC/FID)

Gas chromatography was performed using a Perkin–Elmer Clarus 500 GC apparatus equipped with a flame ionization detector (FID), and a Hewlett–Packard HP-1 (cross-linked methyl silicone) capillary column (30 m long and 0.2 mm i.d., with 0.33 μm film thickness). The column temperature programme was 60 °C during 5 min, with 3 °C/min increases to 180 °C, then 20 °C/min increases to 280 °C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Both the FID detector and injector port temperature were maintained at 250 and 220 °C, respectively.

2.2.2. Gas chromatography and mass spectrometry (GC/MS)

Gas chromatography and mass spectrometry analyses were carried out with a Varian Saturn 2000 equipped with a Varian C.S VA-5MS capillary column (30 m long and 0.25 mm i.d. with 0.25 μm film thickness). The same working conditions used for GC and split mode injection (ratio 1:25) were employed. Mass spectra were taken over the *m*/*z* 28–400 range with an ionizing voltage of 70 eV. Kováts retention index was calculated using co-chromatographed 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 by comparing their mass spectra and retention times with those of authentic samples or with data already available in the NIST 2005 Mass Spectral Library and in the literature (Adams, 2007).

2.3. Antifungal activity

2.3.1. Fungal species

Five phytopathogenic fungi, *A. alternata* (Fr.) Keissler CECT 20923 (LBEA 2105), *Bipolaris oryzae* (Breda de Haan) Shoemaker CECT 2776 (LBEA 2100), *F. graminearum* Schwabe CECT 20924 (LBEA 2165), *F. equiseti* (Corda) Saccardo CECT 20925 (LBEA 2166), *F. verticillioides* (Sacc.) Nirenberg CECT 20926 (LBEA 2167), were isolated in the Botany Laboratory of the Department of Agroforest Ecosystems (LBEA) from rice samples collected in the Albufera rice-producing area in the Mediterranean (Valencia, Spain). The fungal species were identified and then deposited in the Spanish Type Culture Collection (CECT).

2.3.2. Fungal strains identification

The fungal strains were determined at the molecular level by the analysis of two different regions of ribosomal DNA genes: the nuclear ribosomal internal transcribed spacer 'ITS region' and D1/D2 domains of the 28S rRNA. The ITS region, considered as the formal fungal barcode, includes the contiguous region of ITS1, the 5.8S gene and ITS2, and is in most cases the marker of choice for the exploration of fungal diversity in environmental samples (Schoch et al., 2012).

A third genetic marker, the translation elongation factor 1-alpha (EF-1α) gene region, was used for species-level identification of the isolates belonging to the genus *Fusarium*.

The primers used for the amplification were ITS1 and ITS4 (White, Bruns, Lee, & Taylor, 1990) for the ITS region, NL1 and NL4 (Kurtzman & Robnett, 1998) for the D1/D2 LSU region and EF1-728F and EF1-986R (Carbone & Kohn, 1999) for the EF-1α gene.

Sequences comparison between the amplified regions and those available in the NCBI Taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy) showed that isolate LBEA 2105 (CECT 20923) showed 100% identity with genus *Alternaria* section *Alternata*, and the isolate LBEA 2100 (CECT 2776) showed 99% identity with *Curvularia spicifera (Bipolaris oryzae)*, the genus synonymous with *Coclhiobolus*, *Pseudocochliobolus*.

The BLAST analysis of the sequences against AFTOL (Assembling the Fungal Tree of Life) and MycoBank/CBS-KNAW Fungal Biodiversity Centre (BioloMICSNet Software) databases for the isolated LBEA 2105 (CECT 20923) showed a 99% identity and 100% identity with the species *A. alternata*, and for the isolate LBEA 2100 (CECT 2776) showed a 99% identity for *Curvularia spicifera (Bipolaris oryzae)*.

The BLAST analysis of the EF-1α gene sequences with those

available in the NCBI Taxonomy database (Fusarium taxid 5506) and the Fusarium-ID database (Geiser et al., 2004) showed 100% identity with the species *F. graminearum* (Teleomorph *Gibberella zeae*) for the strain LBEA 2165 (CECT 20924).

Finally, regarding the strain LBEA 2166 (CECT 20925), 99% identity with the species *F. equiseti* (Teleomorph *Gibberella intricans*) was found, and the strain LBEA 2167 (CECT 20926) showed 100% identity with the species *F. verticillioides* (Teleomorph *Gibberella moniliformis*).

2.3.3. Antifungal activity in solid media (potato dextrose agar)

The bioassay was performed in a Petri dishes (90 × 15 mm and 150 × 20 mm), dissolving 300 μg/mL (Tween 20, 0.1%) of commercial essential oils in previously sterilized potato dextrose agar (PDA) growth medium flasks at 45–50 °C while the medium was still in a liquid form and distributed into Petri dishes. Petri dishes were inoculated with an 8 mm diameter disk of a 7-day-old colony on PDA of each tested fungi. Plates were incubated in the dark at 25 °C for 7 and 14 days. Petri dishes control contained equal amounts of sterilized water/Tween 20 (0.1%) on PDA were employed. Fungal growth was evaluated by measuring daily the diameter of the colony in two perpendicular directions and speed of growth was calculated. The growth was measured during the 7 and 14 days at intervals of 24 h. To calculate the growth rates ($mm.day^{-1}$) a linear regression of the radius (mm) as opposed to the time (days) was carried out. The computer software used was Microsoft Excel 2013. For each essential oil and fungi, six replicate dishes were used. Also, mycelial growth inhibition (MGI) was calculated at day 7, using the following formula (Albuquerque, Camara, Willadino, & Ulises, 2006).

$$MGI = [(DC - DO)/DC] \times 100$$

where DC is average of colonies diameter in untreated plates, DO is the average of colonies diameter in plates treated with oil.

2.3.4. Effect of clove essential oil on rice grain conservation

Healthy Valencia rice grain, was collected from the Albufera, a Mediterranean rice-producing area. Kernels were washed with sodium hypochlorite (0.2%) for 5 min, rinsed twice with distilled water and air-dried at room temperature (25 ± 2 °C). Rice grains were placed into 150 × 150 mm polystyrene containers (100 grains per container). The containers with rice grain were sprayed with 5 mL of a spore suspension of 5×10^5 conidia ml^{-1} of each fungus tested, and were air-dried to completely dry.

Two concentrations (300 and 600 μg/mL) of clove essential oil 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 period. All the containers were then transferred to storage at 28 °C for 20 days. The percentage of infected rice grains was recorded after 15 days of incubation with a magnifying glass model Olympus SZX10. Five replicates per treatment were used.

2.4. Statistical analysis

The fungal growth results were submitted to variance analysis (ANOVA) using Fisher's test of least significant difference (LSD) with significant values at $P < 0.05$. Data analysis was performed using Stat Graphics Plus 5.0 software (Stat Point, Inc., Herndon, Virginia, USA).

3. Results and discussion

3.1. Chemical composition of the essential oils

The chemical composition of commercial bay and clove leaves essential oils was determined by GC and GC/MS analysis. Thirty-seven compounds accounting for more than 99.5% of the total essential oil were identified. Components are clustered (Table 1) in homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, phenylpropanoids and others and listed according to Kováts retention index calculated in GC on apolar HP-1 column. In *Laurus nobilis* essential oil, the highest quantities of monoterpene compounds (95.83%) were found. Both hydrocarbons (21.49) and oxygenated monoterpenes (74.34%) with 12 and 11 identified compounds, respectively, were also qualitatively the principal phytochemical group. The main compounds were 1,8-cineole (51.95%), α-terpinyl acetate (12.93%) followed by the monoterpene hydrocarbon sabinene (9.56%). Among the sesquiterpene fraction, only β-caryophyllene (0.10%), β-elemene and caryophyllene oxide were detected as trace amounts. Between the phenylpropanoid compounds biosynthesized by the shikimic acid pathway, relatively large amounts of methyl eugenol (2.89%) followed by eugenol (0.62%) were identified.

Several studies *in vitro* have been conducted regarding the antifungal activity of *L. nobilis* essential oils in order to obtain a natural food preservative. The inhibitory effect has been attributed to the main compounds, however the chemical composition is highly influenced by many factors, including the genotype of the plant species, seasonality, geographic and weather conditions. The main compounds of bay essential oil against the fungi commonly causing spoilage of bakery products, namely *Eurotium amstelodami*, *E. herbariorum*, *E. repens*, *E. rubrum*, *Aspergillus flavus*, *Aspergillus niger* and *Penicillium corylophilum*, were eugenol (57.0%), myrcene (12.4%) and linalool (2.6%) (Guynot et al., 2003). In addition, eugenol (44.13%), cinnamaldehyde (30.28%) and linalool (8.49%) were the principal components of *L. nobilis* essential oil of Chinese origin responsible for the fungicide effect against the post-harvest disease caused by *A. alternata* in cherry tomato (Xu et al., 2014).

A high content of 1,8-cineole (39.81%), 2-carene (13.08%), *trans*-ocimene (7.05%), sabinene (6.17%) and *cis*-ocimene (3.06%) were the main constituents of *L. nobilis* essential oil with antimicrobial activity against foodborne pathogens (Cherrat et al., 2014). Also 1,8-cineole, followed by linalool and terpinyl acetate, was found in *L. nobilis* essential oil against *B. cinerea*, *Monilinia laxa* and *P. digitatum* (De Corato et al., 2010) or towards foodborne pathogens in fresh Tuscan sausage (Da Silveira et al., 2014). Similar quantitative results of the main compounds were recently found in commercial *Laurus nobilis* essential oil (Peris & Blázquez, 2015) purchased in pharmacies for medicinal use, as well as in plants from Brazil and Argentina (Da Silveira et al., 2014; Di Leo Lira et al., 2009) or with essential oil obtained using different extraction processes (De Corato et al., 2010; Flamini et al., 2007).

On the other hand, phenylpropanoids (88.58%) with only eugenol identified, are by far the main fraction of *Syzygium aromaticum* essential oil. Although, qualitatively, sesquiterpene hydrocarbons with five compounds identified are the principal fraction, they only reached 10.70% of the total essential oil. β-caryophyllene (8.13%) followed by α-humulene (2.35%) were the main compounds of this fraction. These results were also found (Srivastava, Srivastava, & Syamsundar, 2005) with samples of *Syzygium aromaticum* leaf essential oil, grown in Madagascar (eugenol 82%, β-caryophyllene 13% and α-humulene 1.5%). In addition, eugenol (78.1%) and β-caryophyllene (20.5%) were the main compounds in clove leaves essential oil from Cuba (Pino,

Table 1
Chemical composition of commercial *Laurus nobilis* L. and *Syzygium aromaticum* (L.) Merr. & Perry essential oils.

Compound	RI	GC peak area (%) bay	GC peak area (%) clove
Monoterpene hydrocarbons		**21.49**	–
Tricyclene	925	t	–
α-Thujene	930	0.17	–
α-Pinene	938	4.93	–
Camphene	955	0.50	–
Sabinene	976	9.56	–
β-Pinene	980	4.80	–
Myrcene	988	0.66	–
α-Phellandrene	1003	0.13	–
α-Terpinene	1016	0.21	–
cis-Ocimene	1048	0.12	–
γ-Terpinene	1061	0.41	–
Terpinolene	1088	t	–
Oxygenated monoterpenes		**74.34**	–
1,8-Cineole	1039	51.95	–
Linalool	1100	5.27	–
δ-Terpineol	1166	0.08	–
Terpinen-4-ol	1179	2.84	–
α-Terpineol	1193	0.10	–
α-Fenchyl acetate	1213	0.18	–
Linalyl acetate	1249	0.24	–
Bornyl acetate	1286	0.51	–
δ-Terpinyl acetate	1314	0.24	–
α-Terpinyl acetate	1347	12.93	–
Neryl acetate	1361	t	–
Sesquiterpene hydrocarbons		**0.10**	**10.70**
β-Elemene	1383	t	–
β-Caryophyllene	1419	0.10	8.13
α-Humulene	1456	–	2.35
trans-calamenene	1522	–	t
δ-cadinene	1523	–	0.14
cis-calamenene	1529	–	0.08
Oxygenated sesquiterpenes		**t**	**0.67**
Caryophyllene oxide	1584	t	0.67
Humulene epoxide II	1609	–	t
Phenylpropanoids		**3.51**	**88.58**
Eugenol	1363	0.62	88.58
Methyl eugenol	1397	2.89	–
trans-Methylisoeugenol	1489	t	–
Others		**0.07**	–
Isobutyl isobutyrate	912	t	–
2-Nonanone	1089	t	–
2-Undecanone	1296	0.07	–
Total identified		**99.51**	**99.95**

Compounds listed in order of elution in the HP-1 column. RI: retention index relative to C_8–C_{32} *n*-alkanes on the HP-1 column. Peak area percentages are calculated in GC on apolar HP-1 column. t = trace amounts <0.05.

Marbot, Aguero, & Fuentes, 2001), showing lower percentages in clove buds essential oil, characterized also by a large amount of eugenyl acetate (eugenol 69.8%, β-caryophyllene 13.0% and eugenyl acetate 16.1%). The oxygenated sesquiterpene fraction was only represented (0.67%) by epoxide derivatives of caryophyllene (0.67%) and humulene (trace amount <0.05%). Finally, monoterpene compounds were not detected in the commercial *Syzygium aromaticum* essential oil analysed here.

3.2. Antifungal activity

3.2.1. Antifungal effects of essential oils on mycelial growth and growth rates

Clove essential oil at 300 μg/mL displayed more antifungal potential as a mycelia growth inhibitor than bay leaf essential oil (Table 2, Figs. 1 and 2) against all tested phytopathogenic fungi isolated from rice. In model conditions at the higher doses assayed, the most susceptible fungi against clove essential oil were *F. graminearum* and *Bipolaris oryzae*, with growth values of 1.49 and 1.15 mm/d, a reduction of 88% and 83%, respectively, compared to the control. Clove essential oil also inhibited the growth of *Bipolaris*

Table 2
Effects of clove and bay leaf essential oils (300 μg/mL) on radial growth and growth rates of *A. alternata*, *B. oryzae*, *F. graminearum*, *F. equiseti* and *F. verticillioides*. Confidence intervals with probability of 0.95.

Species-treatment	Mean	Lower limit	Upper limit	GR
A. alternata-PDA	26.27 ± 1.79	22.76	29.78	5.27 (0.98)
A. alternata-C	11.78 ± 1.36	9.11	14.46	2.51 (0.97)
A. alternata-BL	23.61 ± 1.66	20.34	26.88	4.61 (0.99)
B. oryzae-PDA	27.51 ± 1.58	24.40	30.63	6.84 (0.99)
B. oryzae-C	5.35 ± 0.88	3.61	7.08	1.15 (0.89)
B. oryzae-BL	28.32 ± 1.82	24.73	31.91	6.63 (0.98)
F. graminearum-PDA	39.00 ± 1.71	35.63	42.36	12.27 (0.99)
F. graminearum-C	6.51 ± 1.48	3.60	9.42	1.49 (0.92)
F. graminearum-BL	34.91 ± 2.72	29.57	40.26	12.49 (0.99)
F. equiseti-PDA	26.82 ± 1.32	24.22	29.42	6.60 (0.98)
F. equiseti-C	7.00 ± 1.12	4.80	9.21	1.91 (0.87)
F. equiseti-BL	24.88 ± 1.66	21.61	28.15	4.85 (0.98)
F. verticillioides-PDA	22.96 ± 1.12	20.76	25.16	5.44 (0.99)
F. verticillioides-C	9.18 ± 1.12	6.98	11.38	2.21 (0.99)
F. verticillioides-BL	20.77 ± 1.15	17.74	23.80	5.40 (0.99)

Mean: mean radius (mm) ± standard deviation, GR: growth rate (R^2).

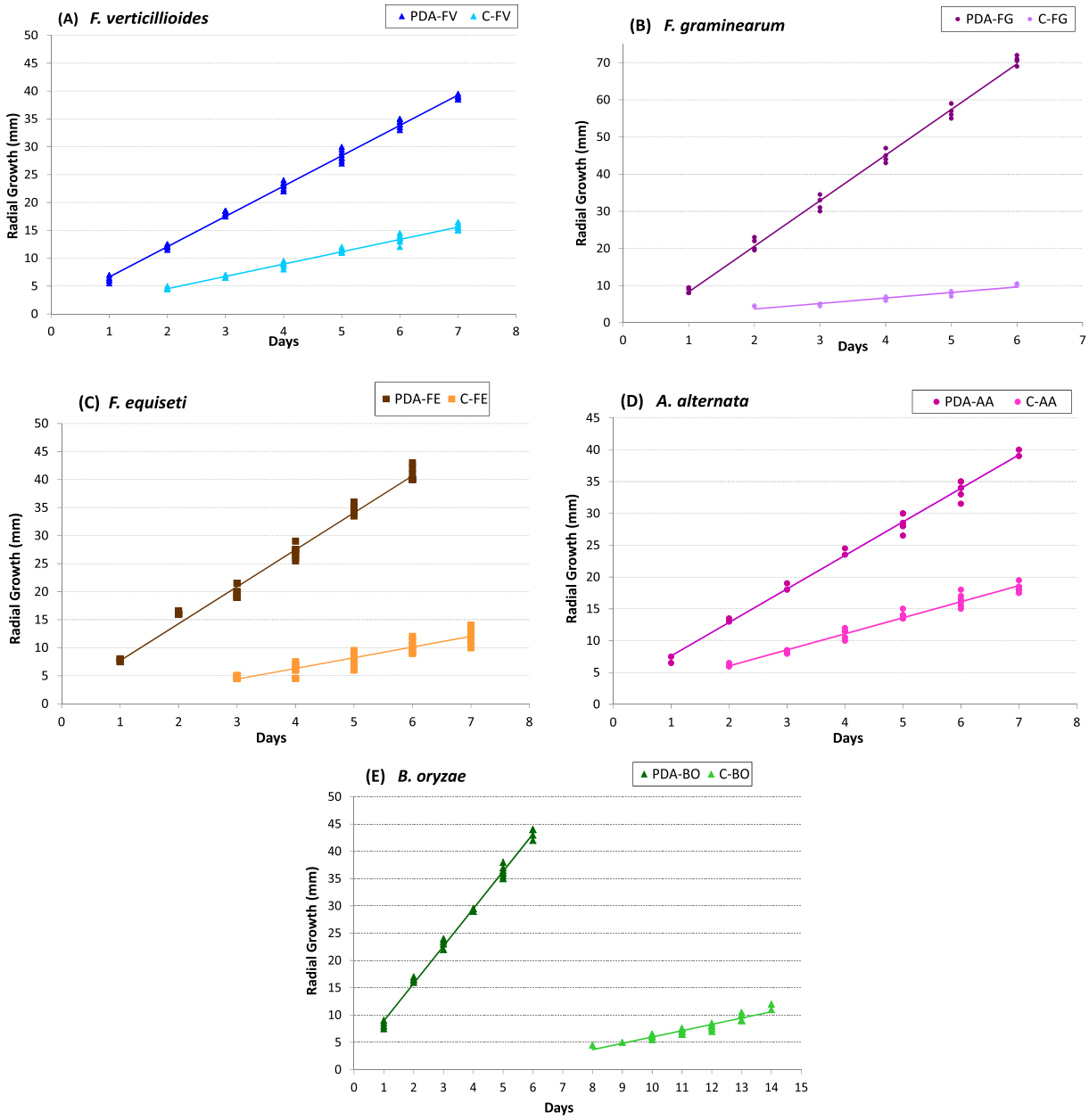

Fig. 1. Growth rate (mm/d) of fungi on PDA and clove (C) essential oil (300 μg/mL). (A) *F. verticillioides* (FV): ▲ PDA-FV (control), ▲ L-FV; (B) *F. graminearum* (FG): ● PDA-FG (control), ● L-FG; (C) *F. equiseti* (FE): ■ PDA-FE: (control), ■ L-FE; (D) *A. alternata* (AA): ● PDA-AA (control), ● L-AA; (E) *B. oryzae*: ▲ PDABO:(control), ▲ L-BO.

until 18th day. The most resistant fungi were *A. alternata* and *F. verticillioides* with growth values of 2.5 and 2.21 mm/d, a reduction of 52% and 59%, respectively, compared to the control. *A. alternata* was also the most resistant fungus by disk diffusion methods, with higher concentrations (400 and 800 μg/mL) of *Echinophora platyloba* essential oil (Moghaddam, Taheri, Pirbalouti, & Mehdizadeh, 2015), rich in the monoterpene hydrocarbons *p*-cymene (22.15%), α-pinene (18.52%), β-phellandrene (14.50%) and α-phellandrene (9.68%) and also with a relatively large amount of carvacrol (3.49%). Our results corroborated the fact that monoterpene hydrocarbons are less active than oxygenated monoterpenes and between them essential oils rich in eugenol (clove 88.58%) are more effective against *A. alternata* than essential oils rich in 1,8-cineol (bay leaf 51.95%). In this context *Eucalyptus globulus* and *E. radiata* essential oils with high levels of 1,8-cineole 80.8% and 69.8%, respectively, exhibited poor activities against *Lasiodiplodia theobromae*, *Colletotrichum gloeosporioides*, *Alternaria citrii* and *B. cinerea*, being effective against *P. digitatum* from citrus and cactus pear, whereas *Syzygium aromaticum* and *Cinnamonum zeylanicum* essential oils containing 88.3% and 81.2% of eugenol, respectively, were more effective against the same pathogens (Combrinck, Regnier, & Kamatou, 2011). Regarding *F. verticillioides*, this is more sensitive to essential oils rich in the phenolic compound cinnamaldehyde. The antifungal activity of cinnamon essential oil was proportional to its cinnamaldehyde concentration, the minimal inhibitory concentrations (MICs) of cinnamon essential oil (85% cinnamaldehyde), natural cinnamaldehyde (95%), and synthetic cinnamaldehyde (99%) being 60, 50, and 45 μL/L, respectively. The *F. verticillioides* diameter treated with cinnamon essential oil did not increase and was maintained at 1.1 ± 0.1 cm from 6 to 20 days.

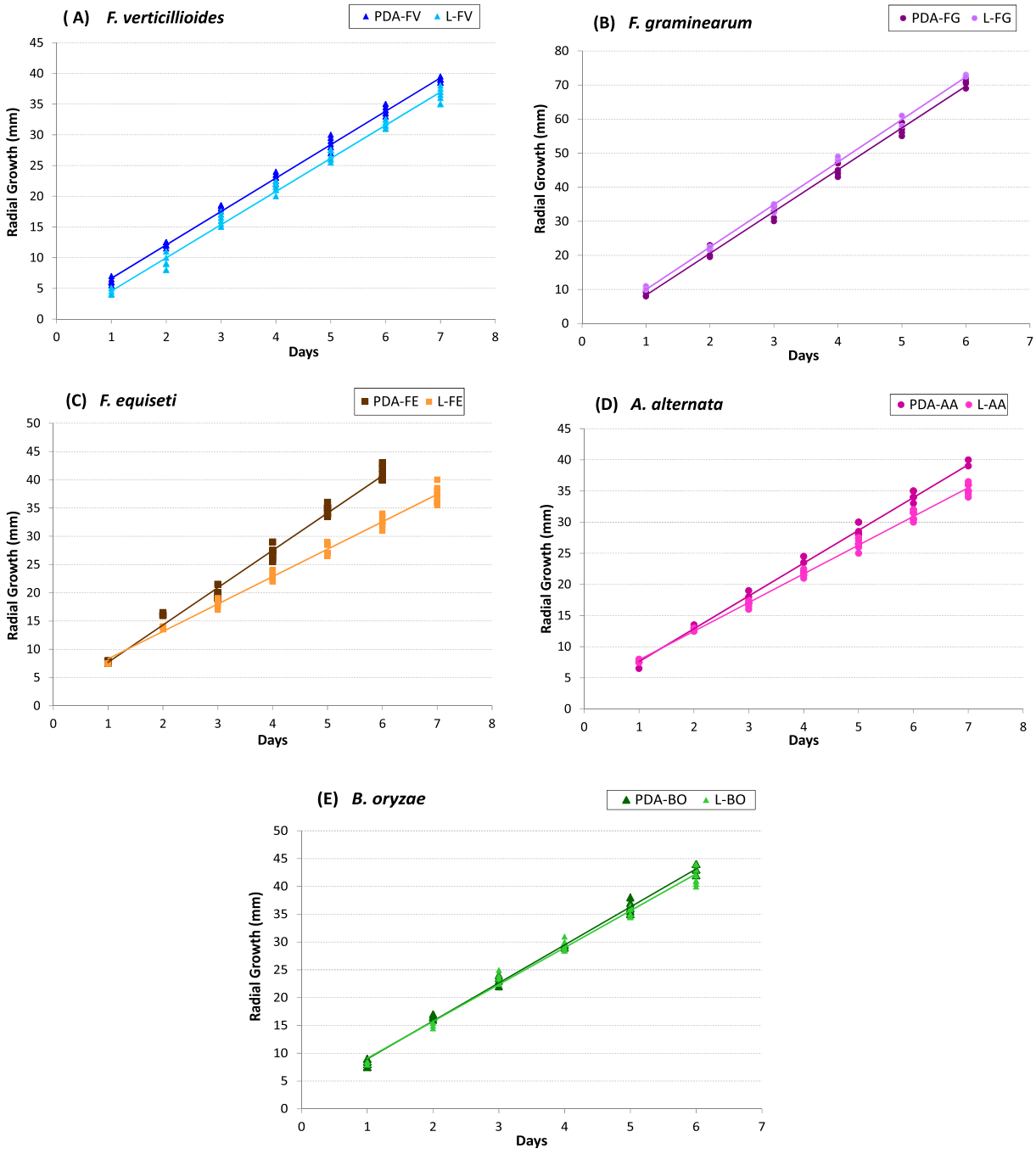

Fig. 2. Growth rate (mm/d) of fungi on PDA and bay leaf (L) essential oil (300 μg/mL). (A) *F. verticillioides* (FV): ▲ PDA-FV (control), ▲ L-FV; (B) *F. graminearum* (FG): ● PDA-FG (control), ● L-FG; (C) *F. equiseti* (FE): ■ PDA-FE: (control), ■ L-FE; (D) *A. alternata* (AA): ● PDA-AA (control), ● L-AA; (E) *B. oryzae*: ▲ PDABO: (control), ▲ L-BO.

Cinnamon essential oil was the most effective against *F. verticillioides*, followed by peppermint essential oil (50% menthol), eugenol (99%), camphor essential oil (55% borneol), anise essential oil (92% anethole), and eucalyptus essential oil with 80% of 1,8-cineole (Xing et al., 2014). The use of essential oils rich in eugenol and cinnamaldehyde is interesting because recently it has been found that antifungal activity after 14 days against *A. niger*, was confirmed in five (thymol, thymoquinone, eugenol, carvacrol, and cinnamaldehyde) of the seven tested compounds, and furthermore, when these substances were encapsulated into mesoporous silica material MCM-41, thymol, thymoquinone, and eugenol were even more effective in a state of encapsulation than in the pure state (Janatova et al., 2015).

On the other hand, the growth speed until the sixth (*F. graminearum* and *B. oryzae*) or seventh (*F. equiseti*, *Fusarium verticillioide* and *A. alternata*) day (Fig. 2) was not significantly affected by the treatment with bay leaf essential oil. In the same conditions, clove essential oil produced, between the second and seventh days, a very low growth speed on *Fusarium* sp. and *A. alternata* (2.517 mmd^{-1} *vs* 5.2657 mmd^{-1}) as well as total radial inhibition of *B. oryzae* until the eighth day, producing between day 8 and day 14 a very low growth speed (1.1592 mmd^{-1} *vs* 6.841 mmd^{-1}) (Fig. 1). *B. oryzae* is the most sensitive fungus to essential oils rich in phenolic compounds, such as thymol, carvacrol or eugenol, its behaviour also

Table 3
Micelial growth inhibition (MGI), of *A. alternata* (AA), *B. oryzae* (BO), *F. graminearum* (FG), *F. equiseti* (FE) *and F. verticillioides* (FV) with clove (C) and eugenol (E).

Concentration μg/mL	AA		BO		FG		FE		FV	
	C	E	C	E	C	E	C	E	C	E
100	30.19	35.91	24.65	45.32	1.45	8.53	26.11	43.56	19.47	41.24
200	45.02	46.40	57.44	85.13	24.78	34.84	45.04	58.54	39.96	58.53
300	53.60	59.54	100	100	82.15	87.38	77.03	78.72	61.49	66.05

MGI: per cent inhibition.

being proportional to eugenol content (Santamarina et al., 2015). So, the total radial inhibition of *B. oryzae* was until the tenth day after the treatment with oregano essential oil (thymol 21.64%, carvacrol 43.26%) and until the sixth or eighth day with cinnamon (eugenol 62.65%) and clove (eugenol 88.58%) essential oils, respectively.

Since eugenol, the main compound of clove essential oil, has recently been approved as a natural fungicide, the micelial growth inhibition of the five isolated phytopathogenic fungi was determined with 100, 200 and 300 μg/mL of both clove essential oil and eugenol, in order to corroborate whether the antifungal activity of clove essential oil is due to its major component or if other minor compounds may act synergistically. Clove essential oil reduced mycelium growth of *A. alternata*, *B. oryzae and F. equiseti* at 100, 200 and 300 μg/mL concentrations, with the percentage of reduction ranging from 30.19 to 53.60%, 24.65–100% and 26.11–77.03%, respectively (Table 3). At the higher concentration (300 μg/mL), clove essential oil showed similar antifungal activity to pure eugenol against all tested phytopathogenic fungi (Table 3), however at lower doses eugenol was more active towards *B. oryzae*, *F. graminearum*, *F. equiseti* and *F. verticillioides*. Similar results against *Fusarium culmorum* and *F. verticillioides* were recently found in commercial essential oils by Roselló, Chiralt, Sempere, and Santamarina (2015). It is interesting to note that the micelial growth inhibition of clove essential oil was similar at all assayed doses of eugenol against *A. alternata*, responsible for several postharvest diseases of fruit and vegetables, such as the black spot in pineapple or *Alternaria* rot in stone fruits (Antunes & Cavaco, 2010). The results obtained in the present study indicated that there was a significant antifungal effect owing to the increased concentration of eugenol, which possesses antifungal activity, and confirmed that this phenylpropanoid is the most effective component in clove essential oil. Relatively large amount of the sesquiterpene hydrocarbons β-caryophyllene (8.13%) and α-humulene (2.35%) showed no synergistic interactions against the isolated pathogenic fungi strains of rice.

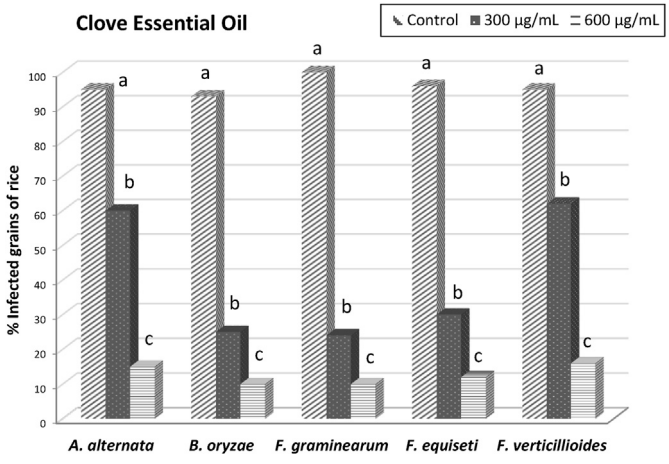

Fig. 3. Efficacy of the different concentrations of clove essential oil (300 and 600 μg/mL) on fungal development of *A. alternata*, *B. oryzae*, *F. graminearum*, *F. equiseti and F. verticillioides* in inoculated rice grains. Significant difference at 95% level of probability ($P < 0.05$) using Fisher's least significant difference test (LSD).

3.2.2. *Effects of clove essential oil and eugenol on disease development in food conditions*

The disease produced in rice grain inoculated with *A. alternata*, *B. oryzae*, *F. graminearum*, *F. equiseti* and *F. verticillioides*, with clove essential oil at 300 μg/mL and 600 μg/mL, was reduced compared with the control. Clove essential oil significantly ($P < 0.05$) inhibited pathogenic fungal development in stored rice compared with the control in a dose-dependent manner after 20 days of incubation at 28 °C. At 300 μg/mL clove essential oil showed the higher antifungal activity, reducing *B. oryzae* and *F. graminearum* by between 73 and 76%. The development of all pathogenic fungi in rice grain was significantly reduced by 85 and 90% with 600 μg/mL of clove essential oil (Fig. 3), suggesting that this essential oil could be used as an ecofriendly preservative for storing Valencia rice.

4. Conclusions

The results showed that bay leaf essential oil with a high content in the oxygenated monoterpene 1,8-cineole has no significant antifungal effect on the five pathogenic fungi isolated from rice grains. The inhibitory effect of clove essential oil on mycelia growth depends on the number of days, dose and fungus. The antifungal activity of clove essential oil is mostly due to the presence of a high amount of eugenol. At 100, 200 and 300 μg/mL, clove essential oil (eugenol 88.58%) showed similar mycelia growth inhibition to the recently approved antifungal compound eugenol against *A. alternata*. In food clove essential oil at 600 μg/mL produced a significant reduction of up to 85–90% for 20 days. The addition of clove essential oil can provide an alternative to chemical preservatives for controlling the fungi in stored rice grains, thus extending their shelf life. Further formulation and field experiments are necessary to corroborate this target and also should be extended to rice-derived products.

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