Santamarina Siurana, MP.; Rosello Caselles, J.; Gimenez, S.; MARIA AMPARO BLAZQUEZ FERRER (2016). Commercial Laurus nobilis L. and Syzygium aromaticum L. Men. & Perry essential oils against post-harvest phytopathogenic fungi on rice. LWT - Food Science and Technology. 65:325-332. doi:10.1016/j.lwt.2015.08.040

Elsevier Editorial System(tm) for LWT - Food Science and Technology
Manuscript Draft

Manuscript Number:

Title: In vitro and in vivo control of postharvest phytopathogenic fungi in rice by commercial Laurus nobilis L. and Syzygium aromaticum L. Merr. & Perry essential oils

Article Type: Research Article

Keywords: essential oil, Laurus nobilis, Syzygium aromaticum, antifungal activity, rice

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Abstract: ABSTRACT
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COVER LETTER

In vitro and in vivo control of postharvest phytopathogenic fungi in rice by commercial *Laurus nobilis* L. and *Syzygium aromaticum* L. Merr. & Perry essential oils

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Submitted work represents a advancement in the field of plant pathology, and is clearly demonstrated in the manuscript.

Due to the chemical polymorphism occurring in clove and bay leaf essential oils from different provenances as well as the variability observed within and between species of *Fusarium* sp. from rice, it its fundamental known the chemical composition of the samples employed and perform the isolation of the phytopathogenic strains. So, the aims of this study was determine the qualitative and quantitative composition of commercial essential oils by GC and CG-MS and investigated the antifungal potential against *Alternaria alternata Bipolaris oryzae, Fusarium sp.* isolated in rice populations of Valencia (Valencia rice) from the Mediterranean area in order to obtain potential ecofriendly substances for sustainable management in both field and stored food products.

Valencia, 2 de abril de 2015

In vitro and *in vivo* control of postharvest phytopathogenic fungi in rice by commercial *Laurus nobilis* L. and *Syzygium aromaticum* L. Merr. & Perry essential oils

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Highlights

- The essential oil composition of bay and clove leaves was determined by CG and GC/MS.
- Clove essential oil inhibited pathogenic fungi in stored rice in a dose-dependent manner.
- The antifungal activity of clove essential oil is mainly due to high content of eugenol.
- The antifungal activity of clove in rice-grains for 20 days suggests their use as preservative.

In vitro and *in vivo* control of postharvest phytopathogenic fungi in rice by commercial *Laurus nobilis* L. and *Syzygium aromaticum* L. Merr. & Perry essential oils

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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 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 have 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 300µg/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 than pure eugenol against all tested phytopathogenic fungi. In inoculated rice-grain significantly reduced the fungal infection *in vivo*, so *S. aromaticum* essential oil could be a good alternative as preservative in stored rice-grain.

Keywords: essential oil, *Laurus nobilis*, *Syzygium aromaticum*, antifungal activity, rice

Introduction

Spices mainly used to enhance 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. Reduce or eliminate food-related microorganisms without negative effects on food quality can extend shelf-life of food and become in an attractive option against foodborne diseases (Blázquez, 2015).

Dried bay leaf (*Laurus nobilis* L.), a spice used in the traditional culinary practices as flavoring agent in soups, meats, fish, vinegars and beverages, play an important role in the human Mediterranean diet, being bay leaf essential oil commonly employed by the pharmaceutical industry. The antimicrobial activity showed *in vitro* of bay leaf essential oil with 1,8-cineole (35.50%), linalool (14.10 %), terpinyl acetate (9.65 %) and sabinene (9.45 %) as the main compounds, towards foodborne pathogens (*Escherichia coli* and *Yersinia enterocolitica*) and also *in vivo* in fresh sausages (0.05 and 0.1 g/100 g concentrations) stored at 7ºC can be used to improve its safety and to extend the product shelf life for two days (Da Silveira et al., 2014). *L. nobilis* essential oil with eugenol (44.13%), cinnamaldehyde (30.28%) and linalool (8.49%) as principal components, completely inhibited the growth of *Alternaria alternata* at 800 μg/mL, showing also a significant effect (68.2%) at relatively low concentration (500 μg/mL). *In vivo L. nobilis* essential oil efficiently decreased the infection ratio of *Alternaria* rot disease on cherry tomatoes. In this case and also with different composition *L. nobilis* essential oil can be applied as a natural and environmentally friendly fungicide to control the postharvest disease of fruits (Xu et al., 2014). The essential oil of *L. nobilis* with 1,8-cineole (24.84%), linalool (14.46%), terpinyl acetate (12.36%) and methyl eugenol (10.09%), obtained by supercritical carbon dioxide technique showed *in vitro* and *in vivo* antifungal activity against the serious fruit postharvest diseases caused by *Botrytis cinerea*, *Monilinia laxa* and *Penicillium digitatum*. *M. laxa* was totally inhibited *in vitro* at all the concentrations (1000, 800, 600, 400 and 200 μg/mL) applied. The mycelial growth inhibition of *B. cinerea* resulted 100, 90, 84, 68 and 54% respectively, whereas *P. digitatum* was only partially inhibited (71, 53, 31, 34 and 23%) at all the concentrations assayed. *In vivo* the best antifungal activity (3 mg/mL)

was found on kiwifruits and peaches (68 and 91% of decay inhibition respectively) (De Corato, Maccioni, Trupo, & Di Sanzo, 2010).

Clove (*Syzygium aromaticum* (L.) Merr. and Perr.), like others spices with an intense flavor, is recommended in processed and ready-to-eat foods as a preservative. The essential oil contents high percentages of phenylpropanoids such as eugenol, responsible for a wide range of activities and uses. It 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, liable of severe postharvest losses in the common bean (Viteri Jumbo, Faroni, Oliveira, Pimentel, & Silva, 2014). Recently the clove essential oil (eugenol 59.75% and eugenyl acetate 29.24%) 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. So, this essential oil could be an alternative to drugs of choice more expensive and with multiple side effects (Islamuddin, Sahal, & Afrin, 2014). In concerning to the effect of clove on the growth of *Penicillium citrinum* and citrinin production in culture medium and rice, all the concentrations (0.2, 0.5, 0.8, 1.6, 1.8 mg/mL) assayed, inhibited the fungal growth in a dose dependent manner, however citrinin production, a nephrotoxin found as a common contaminant in rice, wheat and red yeast rice, was inhibited significantly only at higher concentration. In rice, clove inhibited the fungal growth as well as citrinin production up to 3 days (Aiko & Mehta, 2013).

The antifungal activity showed by *L. nobilis* essential oils (eugenol type) againts *Alternaria alternata* and *S. aromaticum* essential oil on rice towards *Penicillium citrinum* leads us to continue with these spices testing the antifungal activity of bay leaf essential oil (1,8-cineol type) employed in pharmaceutical industry and clove essential oil against five postharvest phytopathogenic fungi (*Alternaria alternata*, *Bipolaris oryzae*, *Fusarium graminearum*, *Fusarium equiseti* and *Fusarium verticillioides*) isolated from rice populations of Valencia from Mediterranean area (Santamarina, Roselló, Sempere, Giménez, & Blázquez, 2015) and wells as to compare at 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 fungicide (2007/442/EC, Dossier complete 2011/266/EU), in order to find cheaper natural products to improve safety and shelf life of stored rice-grains.

1. Materials and Methods

2.1 Plant material

Commercial samples of bay leaf (*Laurus nobilis* L.) essential oil lot 719B032807supplied 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 4ºC until chemical analysis and antifungal studies. Other materials and chemical used were of analytical grade and purchased from local suppliers.

1.2 Chemical composition of the essential oils

2.2.1 Gas chromatography (GC/FID)

GC was performed using a Perkin-Elmer Clarus 500GC 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 program 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 gas chromatography/mass spectrometry (GC/MS)

GC-MS analysis 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. Kovat's 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, *Alternaria alternata* (Fr.) Keissler CECT 20923 (LBEA 2105), *Bipolaris oryzae* (Breda de Haan) Shoemaker CECT 2776 (LBEA 2100), *Fusarium graminearum* Schwabe CECT 20924 (LBEA 2165), *Fusarium equiseti* (Corda) Saccardo CECT 20925 (LBEA 2166), *Fusarium 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 of the Mediterranean area (Valencia, Spain) and deposited in Spanish Culture Tipe 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.

2.3.3 Antifungal activity in solid media (Potato Dextrose Agar)

The bioassay was performed in Petri dish (90x15mm and 150x20mm), dissolving 300 µg/mL (Tween 20, 0.1%) of commercial essential oils in previously sterilized Potato Dextrose Agar (PDA) growth medium flasks at 45-50ºC 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 7-day old colony on PDA of each tested fungi. Plates were incubated in the dark at 25ºC during 7 and 14 days. Petri dish control contained equal amounts of sterilized water/Tween 20 (0.1%) on PDA was employed. Fungal growth was evaluated by measuring daily the diameter of the colony in two perpendicular directions and speed of growth was calculated. 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).

147 MGI = [(DC-DO)/DC]×100

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

150 *2.3.4. Effect of clove essential oil on rice grain conservation*

151 Healthy Valencia rice-grain, were collected from the Albufera rice-producing Mediterranean area.
152 Kernels were washed with sodium hypochlorite (0.2%) for 5 min, rinsed twice with distilled water and
153 air-dried at room temperature (25±2ºC). Rice grains were placed into 150x150mm polystyrene containers
154 (100 grains per container). The containers with rice-grain were sprayed with 5 mL of a spore suspension
155 of $5x10^5$ conidia ml^{-1} of each fungus tested, and were air-dried to completely dry.

156 Two concentrations (300 and 600 µg/mL) of clove essential oil were prepared in Tween 20 (0.1%).
157 Then 2 mL of each solution was vaporized (Sprayed) into the containers spontaneously at 20ºC. Controls
158 were prepared similarly except for the volatile treatment. Filter paper, moistened with 0.5 mL sterilized
159 water, was placed into each container and high relative humidity (90-95%) was maintained during the
160 storage period. All the containers were then transferred to storage at 28ºC for 20 days. The percentage of
161 infected rice grains was recorded after 15 days of incubation with a magnifying glass model Olympus
162 SZX10. Five replicates per treatment were used.

163

164 *2.4 Statistical analysis*

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

168

169 **2. Results and discussion**

170 *3.1 Chemical composition of the essential oils*

171 The chemical composition of commercial bay and clove leaves essential oils was determined by GC and
172 GC/MS analysis. Thirty seven compounds accounting for more than 99.5% of the total essential oil were
173 identified. Components are clustered (Table 1) in homologous series of monoterpene hydrocarbons,
174 oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, phenylpropanoids

and others and listed according to Kovat's retention index calculated in GC on apolar HP-1 column. In *Laurus nobilis* essential oil, highest quantities of monoterpene compounds (95.83%) was found. Both hydrocarbons (21.49) and oxygenated monoterpenes (74.34%) with 12 and 11 identified compounds respectively, were also qualitatively the principal phytochemical group. 1,8-cineole (51.95%), α-terpinyl acetate (12.93%) followed of the monoterpene hydrocarbon sabinene (9.56%) were the main compounds. Among the sesquiterpene fraction, only β-caryophyllene (0.10%), β-elemene and caryophyllene oxide as trace amounts were detected. Between phenylpropanoid compounds biosynthesized by shikimic pathway, relative large amount of methyl eugenol (2.89%) followed by eugenol (0.62%) were identified.

Several studies *in vitro* have been conducted about de antifungal activity of *L. nobilis* essential oils in order to obtain 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 *Eurotium amstelodami*, *E. herbariorum*, *E. repens, E. rubrum, Aspergillus flavus, A. niger* and *Penicillium corylophilum* were eugenol (57.0%), myrcene (12.4%) and linalool (2.6%) (Guynot et al., 2003). Also eugenol (44.13%) but followed of cinnamaldehyde (30.28%) and linalool (8.49%) were the principal components of *L. nobilis* essential oil of Chinese origin responsible of the fungicide effect against the postharvest disease caused by *Alternaria alternata* in cherry tomato (Xu et al., 2014).

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., 2013). Also 1,8-cineole, but followed of linalool and terpinyl acetate was found in *L. nobilis* essential oil against *Botrytis cinerea*, *Monilinia laxa* and *Penicillium digitatum* (De Corato, Maccioni, Trupo, & Di Sanzo, 2010) or towards foodborne pathogens in fresh Tuscan sausage (Da Silveira et al., 2014). Quantitative similar results of the main compounds were recently found in commercial *Laurus nobilis* essential oil (Peris & Blázquez, 2015) purchased in Pharmacy for medicinal use as well as in plants from Brazil and Argentine (Di Leo Lira et al., 2009; Da Silveira et al., 2014) or with essential oil obtained by different extraction process (Flamini et al., 2007; De Corato, Maccioni, Trupo, & Di Sanzo, 2010).

On the other hand phenylpropanoids (88.58%) with only eugenol identified, is by far the main fraction of *Syzygium aromaticum* essential oil. Although qualitatively sesquiterpene hydrocarbons with 5

compounds identified, is the principal fraction, 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%). Also eugenol (78.1%) and β-caryophyllene (20.5%) were the main compounds in clove leaves essential oil from Cuba (Pino, Marbot, Aguero, & Fuentes, 2001), showing less percentages in clove buds essential oil, characterized also by 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 no detected in the commercial *Syzygium aromaticum* essential oil here analysed.

2.2 Antifungal activity

3.2.1 Fungal strains

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% of identity for both regions ITS región (570/570 pb) and D1/D2 LSU (614/614 pb) ribosomal DNA genes with genus *Alternaria* section *Alternata*.

The Blast analysis of the sequences against AFTOL (Assembling the Fungal Tree of Life) and MycoBank /CBS-KNAW Fungal Biodiversity Centre (BioloMICSNet Software) databases showed a 99% identity for D1/D2 LSU (602/603 bp) with the sequence DQ678082 (strain AFTOL 1610; CBS 916.96) and 100% identity for ITS region (570/570 pb) with the sequence KC253942, belonging to the species *Alternaria alternata*.

Sequences comparison between the amplified regions and those available in the NCBI Taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy) showed that isolate *Bipolaris oryzae* LBEA 2100 (CECT 2776) showed 99% of identity for both regions ITS región and D1/D2 LSU ribosomal DNA genes with *Curvularia spicifera (Bipolaris oryzae)* synonymous genus *Coclhiobolus, Pseudocochliobolus, Bipolaris*.

The Blast analysis of the sequences against AFTOL (Assembling the Fungal Tree of Life) and MycoBank

234 /CBS-KNAW Fungal Biodiversity Centre (BioloMICSNet Software) databases showed a 99% identity
235 for *Curvularia spicifera* CBS 198.31S15646. It also appears very close to *Curvularia australiensis*
236 species, but both species can be differentiated morphologically.
237 The Blast analysis of the EF-1α gene sequences with those available in the NCBI Taxonomy database
238 (Fusarium taxid 5506) and the Fusarium-ID database (Geiser et al., 2004) showed 100% identity (305/305
239 pb) with the sequence AF212459 (strain NRRL 28336) belonging to the species *Fusarium graminearum*
240 (Teleomorph *Gibberella zeae*) for the strain LBEA 2165 (CECT 20924).
241 Regarding to the strain LBEA2166 (CECT 20925), 99% identity (287/289 pb) with the sequence
242 JF508173 (strain HEB01, sequence equivalent to type material NRRL 26419) belonging to the species
243 *Fusarium equiseti* (Teleomorph *Gibberella intricans*) was found.
244 Finally, the strain LBEA 2167 (CECT 20926) showed 100% identity (284/284 pb) with the sequence
245 AF273317 (strain NRRL 28898) belonging to the species *Fusarium verticillioides* (Teleomorph
246 *Gibberella moniliformis*).
247

248 *3.2.2 Antifungal effects of essential oils on mycelial growth and growth rates*

249 Clove essential oil at 300µg/mL displayed more antifungal potential as a mycelia growth inhibitor
250 than bay leaf essential oil (Table 2, Figures 1,2) against all tested phytopathogenic fungi isolated from
251 rice. *In vitro* at the higher doses assayed the most susceptible fungus against clove essential oil was *F.*
252 *graminearum* (6.51±1.48), being the most resistant fungi *A. alternata* and *F. verticillioides* with
253 11.78±1.36 and 9.18±1.12 of mycelia radial growth respectively. *A. alternata* was also the most resistant
254 fungus by disk diffusion methods with higher concentrations (400 and 800 µg/mL) of *Echinophora*
255 *platyloba* essential oil (Moghaddam, Taheri, Pirbalouti & Mehdizadeh, 2015), rich in the monoterpene
256 hydrocarbons *p*-cymene (22.15%), α-pinene (18.52%), β-phellandrene (14.50%) and α-phellandrene
257 (9.68%) and also with relative large amount of carvacrol (3.49%). Our results corroborated than
258 monoterpene hydrocarbons are less active than oxygenated monoterpenes and between them essential oil
259 rich in eugenol (clove 88.58%) are more effective against *A. alternata* than essential oil rich in 1,8-cineol
260 (bay leaf 51.95%). In this sense *Eucalyptus globulus* and *E. radiata* essential oils with high levels of 1,8-
261 cineole 80.8% and 69.8% respectively exhibited poor activities against *Lasiodiplodia theobromae*,
262 *Colletotrichum gloeosporioides*, *Alternaria citrii* and *Botrytis cinerea* being effective against *Penicillium*

digitatum from citrus and cactus pear, whereas *Syzygium aromaticum* and *Cinnamonum zeylanicum* essential oils contained 88.3% and 81.2% of eugenol respectively were more effectives against the same pathogens (Combrinck et al., 2011). In concerning to *F. verticillioides* it is more sensitive with essential oils rich in the phenolic compound cinnamaldehyde. The antifungal activity of cinnamon essential oil was proportional to its cinnamaldehyde concentration, being the minimal inhibitory concentrations (MICs) of cinnamon essential oil (85% cinnamaldehyde), natural cinnamaldehyde (95%), and synthetic cinnamaldehyde (99%) of 60, 50, and 45 µL/L, respectively. *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. 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 are interesting because recently it has been found that the antifungal activity after 14 days against *Aspergillus niger*, was confirmed in five (thymol, thymoquinone, eugenol, carvacrol, and cinnamaldehyde) of the seven tested compounds and in addition 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*, *F. verticillioide* and *A. alternate*) day (Figure 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 eight day, producing between the eight and fourteen a very low growth speed (1.1592 mmd^{-1} *vs* 6.841 mmd^{-1}) (Figure 1). *B. oryzae* is the most sensitive fungus to essential oils rich in phenolic compounds such as thymol, carvacrol or eugenol, being also this behavior proportional to eugenol content (Santamarina, Roselló, Sempere, Giménez, & Blázquez, 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 eight day with cinnamon (eugenol 62.65%) and clove (eugenol 88.58%) essential oils respectively.

Since eugenol, the main compounds of clove essential oil has been recently approved as 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 if the antifungal

activity of clove essential oil is due to 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 percentage of reduction ranging from 30.19 to 53.60%, 24.65 to 100% and 26.11 to 77.03%, respectively (Table 3). At the higher concentration (300 µg/mL), clove essential oil showed similar antifungal activity than 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 *F. 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 to eugenol against *A. alternata*, responsible of 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. Relative large amount of the sesquiterpene hydrocarbons β-caryophyllene (8.13%) and α-humulene (2.35%) not showed synergistic interactions against the isolated pathogenic fungi strains of rice.

3.2.4. Effects of clove essential oil and eugenol on disease development in vivo 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 28ºC. At 300 µg/mL clove essential oil showed the higher antifungal activity, reducing *B. oryzae* and *F. graminearum* between 73-76%. The development of all pathogenic fungi in rice grain was significantly reduced between 85-90% with 600 µg/mL of clove essential (Figure 3), suggesting that this essential oil could be used as ecofriendly preservative for stored Valencia rice.

3. Conclusions

The results showed that bay leaf essential oil with high content in the oxygenated monoterpene 1,8-cineole has not significant antifungal effect towards the five pathogenic fungi isolated from rice-grains. The inhibitory effect of clove essential oil on mycelia growth depends on the day, dose and fungus. The antifungal activity of clove essential oil is mostly due to the presence of high amount of eugenol. At 100, 200 and 300 µg/mL, clove essential oil (eugenol 88.58%) showed similar mycelia growth inhibition than the recently approved antifungal compound eugenol against *A. alternate.* *In vivo* clove essential oil at 600 µg/mL produced a significant reduction 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, field experiments are necessary to corroborate this target and also extend to rice derived products.

Acknowledgments

This study has been financied by Ministerio de Economía y Competitividad. Subdirección General de Proyectos de Investigación. Convocatoria de ayudas a Proyectos de I+D+i. Research challenges. Program oriented societal challenges 2014-2017, reference number AGL2013-42989-R-AR.

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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-1column. 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.

Table 2

Table 2. Effects of clove and bay leaf essential oils (300µg/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. alternaria-PDA	26.27 ± 1.79	22.76	29.78	5.27 (0.98)
A. alternaria-C	11.78 ± 1.36	9.11	14.46	2.51 (0.97)
A. alternaria-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 radio $\pm$ standard error, GR: growth rate (R^2)

Table 3

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		**AA**		**BO**		**FG**		**FE**		**FV**
µg/mL	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: percent inhibition

Figure 1

Figure 1. Mycelial growth of *A. alternate*, *B. oryzae*, *F. graminearum*, *F. equiseti* and *F. verticillioides* on PDA and with clove *(Syzygium aromaticum*) essential oil (300µg/mL). ○ PDA-AA: *A. alternata* (control), ○ C-AA: *A. alternata* (clove), △ PDA-BO: *B. oryzae* (control), △ C-BO: *B. oryzae* (clove), ● PDA-FG: *F. graminearum* (control), ● C-FG: *F. graminearum* (clove), □ PDA-FE: *F. equiseti* (control), □ C-FE: *F. equiseti* (clove), ▲ PDA-FV*: F. verticillioides* (control), ▲ C-FV*: F. verticillioides* (clove).

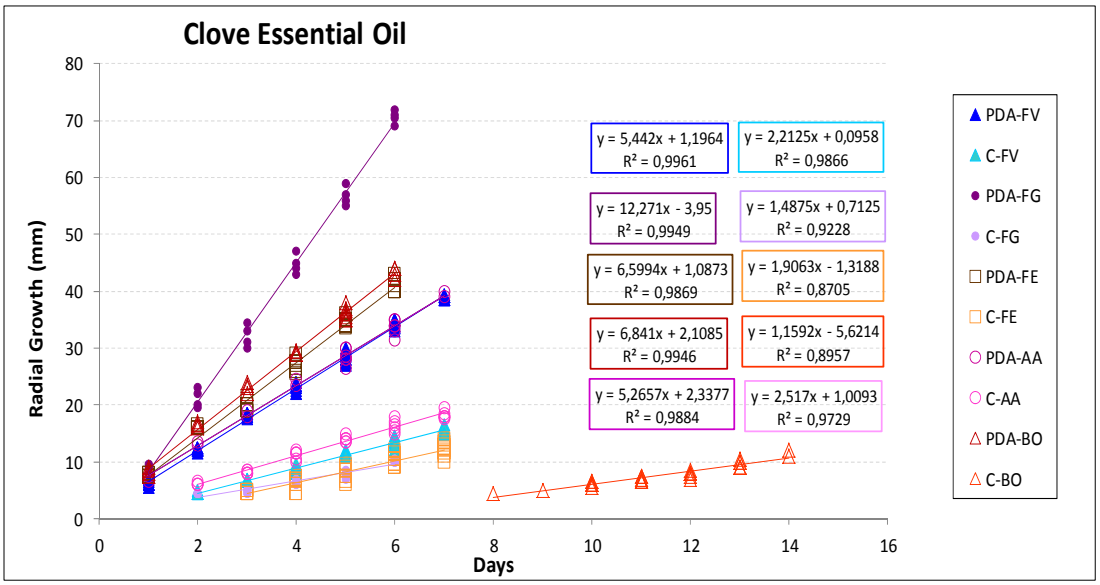

Figure 2

Figure 2. Mycelial growth of *A. alternate*, *B. oryzae*, *F. graminearum*, *F. equiseti* and *F. verticillioides* on PDA and with bay leaf *(Laurus nobilis*) essential oil (300µg/mL). ○ PDA-AA: *A. alternata* (control), ○ BL-AA: *A. alternata* (bay leaf), △ PDA-BO: *B. oryzae* (control), △ BL-BO: *B. oryzae* (bay leaf), ● PDA-FG: *F. graminearum* (control), ● BL-FG: *F. graminearum* (bay leaf), □ PDA-FE: *F. equiseti* (control), □ BL-FE: *F. equiseti* (bay leaf), ▲ PDA-FV*: F. verticillioides* (control), ▲BL-FV*: F. verticillioides* (bay leaf).

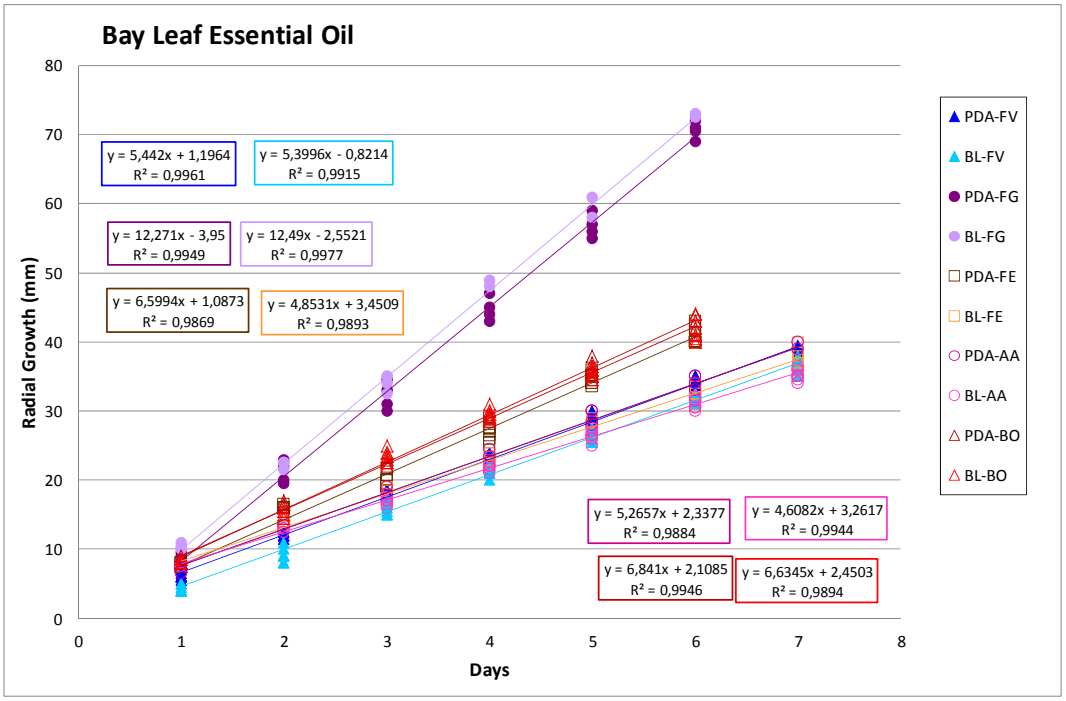

Figure 3

Figure 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).