**Impact of bioactive packaging systems based on EVOH films and essential oils
in the control of aflatoxigenic fungi and aflatoxin production in maize**

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Abstract

Aspergillus flavus and *A. parasiticus* are the most common fungal species associated with aflatoxin (AF) contamination of cereals, especially maize, and other agricultural commodities. Under appropriate conditions, *A. flavus* produces AFB₁ and AFB₂ and *A. parasiticus* produces AFB₁, AFB₂, AFG₁, and AFG₂. AFB₁, the most frequent and toxic metabolite, is a powerful hepatotoxic, teratogenic and mutagenic compound. Effective strategies to control these fungal species and AFs in food and feed are required. Active packaging films containing essential oils (EO) is one of the most innovative food packaging concepts. In this study, ethylene-vinyl alcohol (EVOH) copolymer films incorporating EO from *Origanum vulgare* (ORE), *Cinnamomum zeylanicum* (CIN) or their major active constituents, carvacrol (CAR) and cinnamaldehyde (CINHO), respectively, were developed and assayed to control growth of *A. flavus* and *A. parasiticus* and AF production in maize grains under different a_w and temperature regimes. EO doses assayed in cultures were in the range 0.25 - 4.0 mg/Petri dish. The factors a_w , temperature, type of EVOH-EO film and fungal species significantly influenced the ED₅₀ values of all assayed films. Growth rate (GR) of both species was usually higher at 0.99 than at 0.96 a_w and at 37 °C than at 25 °C. However, the contrary was found with regard to AF production. The efficacy of EVOH-EO films to control growth of both species and AF production increased in the order EVOH-CINHO > EVOH-CAR > EVOH-ORE > EVOH-CIN. The effective dose (ED₅₀) (mg EO/plate) for EVOH-CINHO and EVOH-CIN films against *A. flavus* were in the ranges of 0.125 and 2.475-3.500 and against *A. parasiticus* in the ranges of 0.121-0.133 and 2.275-3.625, respectively. Under the assayed conditions, the ED₉₀ for EVOH-CINHO film were 0.22-0.23 mg/plate for both species. It was the most effective bioactive film to control fungal growth (vapour phase) and AF production, regardless of a_w and temperature. This is the first study about the impact that interacting environmental conditions and bioactive EVOH-CINHO, EVOH-ORE, EVOH-CIN

EVOH-CAR films have on the growth of aflatoxigenic fungi and on AF production in maize grains.

Keywords: bioactive ethylene-vinyl alcohol copolymer (EVOH); *Aspergillus flavus*; *Aspergillus parasiticus*; aflatoxins; essential oils; maize.

1. Introduction

Moulds are responsible for considerable economical losses around the world. Many of them are spoilage agents of agricultural products in pre- and post-harvest, mainly in cereals, fruits, vegetables and their derivatives (Pitt and Hocking, 2009). In addition, some moulds constitute a health risk for consumers due to their potential to produce mycotoxins. Among all mycotoxins, aflatoxins (AFs) are of greatest concern in terms of incidence in food and feed and toxicity to humans and animals (European Union, 2016; Pitt, 2014). AFB₁ has been classified as a human carcinogen (group 1) by the International Agency for Research on Cancer (IARC, 2012).

Aflatoxigenic species belong to sections *Flavi*, *Nidulantes* and *Ochraceorosei* of the genus *Aspergillus* (Varga et al., 2009), although *A. flavus* and *A. parasiticus* (section *Flavi*) are the most common species associated to AF contamination of food and feed (Varga et al., 2011). *A. flavus* produces AFB₁ and AFB₂ and *A. parasiticus* produces AFB₁, AFB₂, AFG₁, and AFG₂. A strong correlation between occurrence of these aflatoxigenic fungi and AFs in cereals has been found (EFSA, 2012; Mateo et al., 2011a). Aflatoxigenic fungi are robust and competitive organisms capable of surviving, growing and producing AFs in a wide range of commodities and water activity (a_w) and temperature levels (Bhatnagar-Mathur et al., 2015; Gallo et al., 2016). AFs are present in very important food and feed with a large number of examples, such as cereals, mainly maize (EFSA, 2012; Lai et al., 2015), nuts (EFSA, 2007; Van de Perre et al., 2015), breakfast cereals (Ibáñez-Vea et al., 2011), infant foods (Kabak, 2012), cocoa (Copetti et al., 2011), legumes (Lutfullah and Hussain, 2012) and milk (Portela et al.,

2016), among others. The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by AFs. Currently this percentage might be higher in new scenarios associated to climate change, which stimulate the development of aflatoxigenic species (EFSA, 2012; Varga et al., 2009). Effective strategies and tools are required to address the prevention, control and suppression of aflatoxigenic fungi and AFs in food and feed (IARC, 2015; Zhu et al., 2016).

Essential oils (EOs) and their constituents are natural substances categorized as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration and some of them have shown antioxidant/antifungal properties (da Cruz Cabral et al., 2013; Prakash et al., 2015). They represent interesting ingredients for biodegradable food packaging films although their possible implementation must be studied carefully (film materials, environmental conditions or fungal species). The antimicrobial quality conferred to the film is caused by the specific activity of the oil compound and its release kinetics, which is governed by potential covalent links with the matrix, the temperature and humidity conditions, since often increment of these two last variables results in a release increase and can even be used as a release triggering mechanism (Balaguer et al., 2013).

Active packaging is one of the most innovative food packaging concepts. In the last decade an emerging research on active packaging films, combining the polymer good general properties (mechanical, barrier, optical and thermal) with the inclusion of additives with antioxidant/antimicrobial properties (flavours, spices and colorants) has been developed. Most of the studies show their behaviour against bacteria (Burt, 2004; Hafsa et al., 2016; Ruiz-Navajas et al., 2013; Zhang et al., 2015) or spoilage fungi (Ávila-Sosa et al., 2012; Vu et al., 2011) but very little attention has been paid to aflatoxigenic fungi and AF production (López et al., 2007; Manso et al., 2013, 2015).

The development of active antifungal packaging films with EOs is of great interest for the industry and the present study is based on this idea. The main advantages of using this technology for the application of natural antifungal agents in foods are the

controlled release of the bioactive compounds into the product during the storage time and the lower possibility of development of undesirable flavours compared to direct addition into food.

Ethylene-vinyl alcohol copolymer (EVOH) is composed of two segment chains: one, olefinic and hydrophobic, comes from ethylene, and the other, with a hydroxyl substituent, presents hydrophilic behaviour. EVOH is a packaging material used to provide high oxygen barrier properties and their hydrophilic nature makes it very sensitive to water. EVOH materials have been used as matrices for the development of active packaging systems, where the polymer protects the active agents during storage and triggers their activity on exposure to humidity (López-de-Dicastillo et al., 2010a, 2010b, 2011; Muriel-Galet et al., 2012, 2013). These properties combined with appropriated EOs, could make EVOH a highly suitable material for control of aflatoxigenic fungi (aerobic organisms) and AFs in food and feed, such as maize and by-products.

The aims of this work were to develop effective anti-aflatoxigenic fungi and anti-aflatoxin production films for food packaging applications incorporating EOs from oregano (*Origanum vulgare*) (ORE), cinnamon (*Cinnamomum zeylanicum*) (CIN) or their major active constituents, carvacrol (CAR) and cinnamaldehyde (CINHO), respectively, in EVOH copolymer with 29 % ethylene molar content. For this purpose: i) the ability of the designed active films *versus* *A. flavus* and *A. parasiticus* growth in maize grains under different environmental conditions is determined and ii) the effect of these bioactive films to control the production and accumulation of AFB₁, AFB₂, AFG₁, and AFG₂ in the medium under the assayed conditions is investigated.

2. Materials and Methods

2.1. Film preparation

Ethylene vinyl alcohol copolymer with 29% ethylene molar content (EVOH-29) was kindly supplied by The Nippon Synthetic Chemical Industry Co., Ltd. (Osaka, Japan). Oregano, 86% carvacrol (ORE) and cinnamon bark, 66.5% cinnamaldehyde (CIN) essential oils (EO) were purchased from Jarpil (Almería, Spain). Carvacrol (CAR) [PubChem CID 10364], the major component of ORE, and cinnamaldehyde (3-phenyl-2-propenal) (CINHO) [PubChem CID 637511], the major component of CIN of Kosher quality were supplied by Sigma-Aldrich (Barcelona, Spain).

In this study, films of EVOH containing ORE, CAR, CIN and CINHO, labelled as EVOH-ORE, EVOH-CAR, EVOH-CIN and EVOH-CINHO, and control films (in absence of active substances) were obtained by casting in an oven at 75 °C for 15 min. For this purpose, 13 g of EVOH-29 were initially dissolved in 100 ml of a 1:1 (v/v) mixture of 1-propanol-distilled water by heating at 75 °C under reflux. Once the copolymer was completely dissolved, the active component was added to the solution (10 % w/w dry polymer). Then, the mixture was stirred at 40 °C for 30 min. The mixture was spread on a Teflon-coated glass plate by using a 200-µm spiral bar coater providing films with a thickness of 0.013 ± 0.002 mm.

In order to know the final content of the active compounds in the resultant films, three replicates of each film were analysed by thermal desorption-gas chromatography (TD-GC) analysis, using an 890 thermal tube desorber (Dynatherm Analytical Instruments Inc., Kelton, PA, USA). It was connected in series to an HP 5890 Series II Plus gas chromatograph (Agilent Tech., Barcelona, Spain) equipped with a flame ionization detector (FID) and an Agilent HP-1 semi-capillary column of 30 m length, 0.53 mm internal diameter and 2.65 µm film thickness (Teknokroma S.C.L., Barcelona, Spain) following the procedure described in previous report (Cerisuelo et al., 2012). In brief, a portion of the tested film (about 20 mg) was placed in the desorption cell and heated at 210 °C for 7 min. The gas stream carried the desorbed gaseous compounds to the GC through a transfer line heated at 230 °C. Chromatographic conditions were as follows: He was the carrier gas, detector temperature was 260 °C, oven temperature

programme was 7 min at 45 °C, heating ramp to 220 °C at 18 °C/min, and 12 min more at 220 °C. At the end of the desorption process the sample was weighed with a 0.1 mg precision balance (Voyager V11140 model, Ohaus, Switzerland). A second desorption process proved that all the volatiles additives were desorbed in the first process. The response of the GC was calibrated by measuring polyethylene and polypropylene samples with known amounts of CAR and CINHO. The additive content is expressed as weight percentage of the compound over dry polymer weight.

2.2. Inoculum preparation

Selected aflatoxigenic strains of *A. flavus* and *A. parasiticus* previously isolated from Spanish maize and characterized following specific PCR protocols described by González-Salgado et al. (2008) and Sardiñas et al. (2010), respectively, were used. These strains are held in the Mycology and Mycotoxins Group Culture Collection (Dep. of Microbiology and Ecology, Valencia University, Spain). The strains were grown on Maize Extract Medium (MEM) (3% w/v of milled maize grains + 2% w/v agar in pure water). The medium was autoclaved at 115 °C for 30 min and poured into Petri dishes. The strains were inoculated on the centre of the plates and incubated at 30 °C for 5 days. Spores of these fresh cultures were used to prepare inocula for further experiments.

2.3. Preparation of culture media with different EO films and a_w levels

The assays with EO films were carried out in maize grains (*Zea mays*). Culture media were prepared as follow: maize grains (25 g), previously analysed to ensure they had undetectable levels of AFs, were placed in Erlenmeyer flasks and autoclaved for 20 min at 121 °C. Then, the a_w level was measured using flask controls and once determined it was adjusted in all flasks to 0.96 or 0.99 by addition of sterile distilled water using a moisture adsorption curve for maize previously determined. Flasks with maize grains were closed and refrigerated at 4 °C for 48 h with periodic shaking to

allow adsorption and equilibration. At the end of this period, a_w -values of moistened maize were checked. The a_w -values of the media were measured with a Novasina RTD 502 equipment (Novasina GmbH, Pfäffikon, Switzerland) using controls of known a_w values supplied by the manufacturer.

The hydrated maize grains were placed into sterile 9-cm Petri dishes with the help of a sterile spatula to form a flat and homogenous layer of grains. The grains were placed exposing the flat face (germen), with minimum space between seeds. Square pieces of each EVOH-film of 2, 4 and 5 cm of side and a circular piece of 4.5 cm of radius containing ORE, CIN or CINHO were attached on the base of the Petri dish lid with double sided adhesive cellophane. In the case of EVOH-CAR film, square pieces of 1.6, 3.2, 4 and 6.4 cm of side were used. The grammage of the EVOH-EO films tested was $1.77 \pm 0.01 \text{ mg/cm}^2$. In this way, the masses of all EO in the assayed film pieces were 0.25, 1.0, 1.5 and 4.0 mg/Petri dish.

2.4. Incubation conditions. Radial growth analysis

All media were inoculated centrally with 15 μl of a fresh spore suspension (1.0×10^6 spores/ml) of *A. flavus* or *A. parasiticus* (on the flat surface of a grain) and immediately capped and sealed with Parafilm M®. Plates were incubated for 12 days at 25 °C and 37 °C in the darkness. Circular green colonies were produced on the flat surface of the inoculated maize grains, which were extended radially to the contiguous seeds. The average of two perpendicular colony diameters was daily registered with a magnifying glass and calculated. Growth rates (GR) were calculated as the slopes of the lines obtained by linear regression of mean radius vs time. The EO doses/plate necessary for 50 and 90% growth inhibition (ED_{50} and ED_{90}) were determined when possible from the plots of GR vs EO dose. All experiments were performed in triplicate and repeated once. The three replicates of inoculated Petri plates of the same treatment (a_w , type and size of EO-film) were enclosed in sealed plastic containers

together with beakers of a glycerol–water solution matching the same a_w as the treatment to maintain a constant equilibrium relative humidity (ERH) inside the boxes.

2.5. Aflatoxin determination

2.5.1. Chemicals, reagents and standard solutions

Chloroform, methanol and acetonitrile were liquid chromatography grade (J.T. Baker, Deventer, The Netherlands). Pure water was from Milli-Q system (Millipore, Billerica, MA, USA). Standards of AFs were from Sigma (Sigma-Aldrich, Alcobendas, Spain). Stock solutions of AFB₁, AFB₂, AFG₁, and AFG₂ were prepared in chloroform to give each a concentration of 500 µg/ml. For maize grain spiking and preparation of calibration standards, appropriate diluted solutions of each AF were prepared in methanol-water 80:20 (v/v). The stock solutions of mycotoxins were stored in freezer (−18 °C) in tightly closed silanized amber glass vials. They were let to equilibrate at room temperature before use. Stock solutions and diluted standards of mycotoxins were gravimetrically controlled over time to avoid change in concentrations.

2.5.2. Extraction

To determinate toxin concentrations in the cultures all maize grains distributed as a homogenous layer on the Petri dish (25 g) were used, regardless of the colony diameter reached after 12 days incubation. Grains were dried at 45 °C for 48 h, finely milled and homogenized. About two g of ground maize culture was thoroughly mixed with 20 mL of acetone-water (60:40, v/v) in a capped container. The mixture was shaken in orbital shaker for 1 h to extract AFs (Bertucci et al., 2012). Usually, after filtration through filter paper an aliquot of 5 mL of the filtrate was diluted with 45 mL of pure water and cleaned-up through an immunoaffinity column (AflaTest WB, Vicam). In the case of extremely high AF levels, dilution with water was 1:49 (v/v). The column was washed with 5 mL pure water. Then, AFs were eluted with 3 mL of methanol and

collected in a vial, the solvent was evaporated in a gentle stream of N₂ and the residue was solved in 250 µl of methanol-water: (80:20, v/v), and centrifuged at 14000 rpm. Fifty µl of the supernatant was injected in the liquid chromatograph. Appropriate dilution of the supernatant with the same solvent was performed before injection when AF concentration was higher than the maximum level for linearity in the calibration curve.

2.5.3. Standard calibration curve of aflatoxins

Calibration solutions of AFB₁ and AFB₂, AFG₁ and AFG₂ were prepared by dilution of stock solutions with methanol-water 80:20 (v/v). These solutions contained 0.2 to 4.0 ng AFB₁/ml, 0.1 to 3.0 ng AFG₁/ml and 0.06 to 2.0 ng AFB₂ and AFG₂/ml. Then, 50 µl of each solution was injected into the liquid chromatograph and the recorded areas were used to obtain the calibration curves for each aflatoxin. More diluted solutions of standards were prepared and injected to estimate the limits of detection (LOD) and quantification (LOQ) of the method.

2.5.4. Chromatographic analysis

AF in culture media and standard solutions were examined by LC using a Waters 600E system controller, a Waters 717 automatic injector, a Waters 474 scanning fluorescence detector and a post-column reaction module (Waters Co., Milford, MA). The system was operated under Waters Millennium 32 software. Separation was performed on a reversed-phase C₁₈ column (Phenomenex Gemini 150 x 4.6 mm, 5 µm particle size). Column temperature was 35 °C. The mobile phase consisted of a mixture of water (A), acetonitrile (B), and methanol (C) that was gradient programmed as follows: 0.00 min: 70%A, 10%B, 20%C (1 min); 1.01 min: 60%A, 10%B, 30%C; 16.01-19.50 min: 30%A, 25%B, 45%C; 19.51-28.00 min: 70%A, 10%B, 20%C. The flow-rate was 1.0 ml/min. Post-column derivatization of AFB₁ and AFG₁ was achieved using freshly prepared iodine reagent (300 mg I₂/l solved in water-methanol 10:90, v/v), which was delivered at a flow-rate of 0.5 ml/min. The mobile phase and the post-column

reagent were filtered through a 0.45- μ m filter and degassed before use. The temperature of the post-column reactor was 70 °C. Detection was performed via fluorescence detector and excitation and emission wavelengths were set at 362 and 450 nm, respectively.

2.5.5. Method validation

Blank maize grains finely ground were spiked (n=5) with 0.15, 0.5, 1.0, 5.0, 50 and 100 ng AFB₁/g. For the remaining aflatoxins, the levels added were 0.15, 0.3, 0.6, 3.0, 10 and 50 ng/g. The highest levels were appropriately diluted before injection to maintain the concentration within the linear part of the calibration curve. Spiked millet maize kernels were allowed to equilibrate in the dark for 2 h prior to extraction and the solvent was evaporated with a slight stream of N₂. The results of the recovery experiments appear in Table 1. The calibration limits of detection (LOD) and quantification (LOQ) were estimated, respectively, as 3.3 and 10 times the ratios between the standard errors of the estimates (obtained by linear regression) and the calibration line slopes. Method LOD (ng/g) were 0.033 for AFB₁, 0.030 and AFB₂, and 0.040 for AFG₁ and AFG₂. Method LOQ (ng/g) were 0.10 for AFB₁ 0.09 for AFB₂, and 0.12 for AFG₁ and AFG₂.

2.6. Statistics

Data were analysed by multifactor analysis of variance (ANOVA) and *post hoc* Duncan's multiple range test using Statgraphics Centurion XV.II statistical package (StatPoint, Inc., VA, USA). For calculation purposes, detectable mycotoxin levels below the LOQ were estimated as 50% of those limits and undetectable levels were assumed to be zero.

3. Results

3.1. Active films

Four different films based on EVOH-29 were successfully obtained by incorporating ORE and CIN EOs as well as their main components CAR and CINHO. All the obtained films were colourless and transparent, continuous, and presented the typical smell to ORE or CIN. Due to the high volatility of these kinds of compounds, the content of the active substances could significantly decrease during the film making step doing determination of their final content necessary. Similar contents for CINHO, CIN and ORE were found in the films, being close to 3.5 ± 0.75 % w/w of dry polymer. However, a higher amount (5.5 ± 1.0 % w/w of dry polymer) was obtained for CAR in EVOH-CAR films.

3.2. Effect of EVOH-EO films on growth of *A. flavus* and *A. parasiticus*

Circular colonies of *A. flavus* and *A. parasiticus* were observed on maize grain cultures under all conditions when growth was detected. Growth delay was between 2-3 days depending on culture conditions. The GR of the isolates under the different conditions assayed are shown in Figs. 1 and 2.

In control cultures without any film or with films without EO, the radial development of fungal colonies under the same conditions was similar. Therefore, the values represented in Figs. 1 and 2 for control cultures are the average of these six replicates (3 replicates each). In control cultures and treatments (cultures with EVOH-EO-films), GR were usually higher at 37 °C than at 25 °C (regardless of a_w) and at 0.99 than at 0.96 a_w (regardless of temperature).

In cultures treated with films containing EVOH-EO, GR generally decreased with increasing EO dose/plate regardless of the remaining factors a_w , temperature, fungal species and type of film. However, different response profiles were observed depending on the EVOH-EO film type. Multifactor ANOVA showed that there were significant differences concerning film type (p -value < 0.01) and their doses. The order of film effectivity was: EVOH-CINHO > (EVOH-CAR or EVOH-ORE) > EVOH-CIN. The order of dose effectivity was that of their concentration. The post-hoc Duncan's test

confirmed these results. The response of *A. flavus* (Fig. 1) and *A. parasiticus* (Fig. 2) to the different treatments were used to calculate the ED₅₀ and ED₉₀ of each EVOH-EO film/plate under all the assayed conditions (Tables 2 and 3, respectively).

Taking all the factors (fungal species, a_w , temperature, EO type and dose) into consideration, the statistical analyses by multifactor ANOVA (with interactions, as some of them were significant) showed that single factors EO, dose and temperature significantly influenced the GR (p -values < 0.01 for the two former and < 0.03 for the last), while fungal species and a_w factors did not. ANOVA also showed that interactions a_w x temperature, a_w x dose, EVOH-EO x dose, EVOH-EO x fungal species were highly significant (p -value < 0.01). The interaction EVOH-EO x temperature was also significant (p -value = 0.023).

As shown in Table 2 in maize cultures of *A. flavus* the ED₅₀ values (mg EO/Petri plate) for EVOH films containing ORE, CAR, CIN, and CINHO were in the ranges 1.125-2.575, 1.150-1.625, 2.475-3.500, and 0.125 (fix value), respectively. Concerning maize grains cultures of *A. parasiticus* Table 3 lists the ED₅₀ values (mg EO/Petri plate) for EVOH films containing ORE, CAR, CIN, and CINHO which were in the ranges 2.000-3.750, 1.175->4.0, 2.275-3.625, and 0.121-0.133, respectively. The efficacy of EVOH-EO films to control growth of *A. flavus* and *A. parasiticus* increased in the order EVOH-CINHO > EVOH-CAR > EVOH-ORE > EVOH-CIN.

With regard to the ED₅₀ values, multifactor ANOVA (with interactions) of the data including class of EVOH-EO film, a_w , fungal species and temperature revealed that these factors (except for temperature) significantly influenced the ED₅₀ values (p -value < 0.01). Significant interactions between the factors occurred. The Duncan's test found that with regard to ED₅₀ values each of the four EO films tested was different from each other (p -value < 0.05).

Under the assayed conditions, no studied factor, except the type of EVOH-EO film, showed significant influence on the ED₉₀ values. Only for EVOH-CINHO film the ED₉₀ values could be estimated and they were 0.22-0.23 mg/plate for both isolates.

Duncan's test confirmed two homogeneous non-overlapping groups with regard to the influence of EVOH-EO-film on ED₉₀: the EVOH-CINHO film and the remaining films (p -value < 0.05). In the present study, treatments with levels higher than 4.0 mg EO/plate were not tested since such concentrations would be unsuitable and inappropriate in food technology. One of the main drawbacks of the EOs is their flavour, which can alter the organoleptic properties of the food.

3.3. Effect of EVOH-EO films on AF production by *A. flavus* and *A. parasiticus*

Cultures of *A. flavus* and *A. parasiticus* under all the assayed conditions were examined to determine AF production. AFB₁ and AFB₂ were analysed in *A. flavus* cultures (Fig. 3) and AFB₁, AFB₂, AFG₁ and AFG₂ in *A. parasiticus* cultures (Fig. 4). In some cases, high dilution ratios (up to 1:400) was performed. In general, multifactor ANOVA with interactions showed that AFB₁ and AFB₂ production was affected by the fungal species, a_w , dose of EO/plate and type of EVOH-EO-film (p -value < 0.001 in all cases). Levels of both toxins were lower in cultures of *A. flavus* than in cultures of *A. parasiticus*, at 0.96 than at 0.99 a_w , at high EO doses than at low doses and with EVOH-CINHO film than the other film types. Although temperature did not significantly affect AFB₁ and AFB₂ production, levels of both toxins were lower in cultures incubated at 37 °C than at 25 °C.

In cultures of *A. parasiticus*, concentrations of AFG₁ and AFG₂ were also lower at 0.96 a_w , high-dose of EOs and 37 °C. Multifactor ANOVA with interactions showed that factors a_w , EVOH-EO film type, their doses and temperature significantly affected AFG₁ and AFG₂ production (p -value < 0.05) although a_w and dose were the most significant factors (p -value < 0.001). Significant interactions between the factors were revealed. Post-hoc analysis of EO-dose with Duncan's test displays three homogenous groups for AFG₁ and AFG₂ (blanks, 0.25 mg/plate and the remaining doses). With regard to the influence of the EVOH-EO-dose on AF production by *A. flavus* and *A. parasiticus*, it must be stressed that low-doses of EO (0.25-1.00 mg/plate) in vapour phase (except

for the EVOH-CINHO film), frequently stimulated AF production. The levels of toxins in these cultures surpassed the levels of toxins in control cultures under the same conditions (Figs. 3 and 4).

4. Discussion

In the present study, two simultaneous analyses were carried out. In the first one, the activity of bioactive EVOH films containing oregano, carvacrol, cinnamon or cinnamaldehyde EO against *A. flavus* and *A. parasiticus* in maize grains has been determined. In the second one, the effect of these active films, containing sub-inhibitory EO doses, on the biosynthesis of AFB₁, AFB₂, AFG₁, and AFG₂ by these fungi has been shown. The study also shows the impact of the interactions between environmental conditions and these active films on fungal growth and AF production. The isolates of *A. flavus* and *A. parasiticus* used in these assays were previously isolated from maize grown in Spain. Maize is the cereal with the highest levels of AFs worldwide (EFSA, 2012). Therefore, maize was chosen as a substrate in this study. The two a_w and temperature levels used have been selected on the basis of the exceptional adaptation and competitiveness of these species to a wide range of relative humidity and environmental temperature (EFSA, 2012). All this highlights the importance and usefulness of the present study in food safety, quality and technology.

As far as we know, no previous studies have examined the impact that interacting environmental conditions and bioactive EVOH-ORE films, EVOH-CIN films, EVOH-CAR films or EVOH-CINHO films have on aflatoxigenic fungal growth and AF production. Lack of such reports hinders a critical and comparative discussion of the results.

Until now, a wide variety of reports have shown that fungal growth may be inhibited by plant EOs and some reviews about this topic have been published (da Cruz Cabral et al., 2013; Nguyen Van Long et al., 2016). However, in these reports, the methods used for monitoring fungal growth have been very dissimilar. No standardized

test has been developed and adopted for evaluating the possible antifungal activity of EOs against food-related fungi. In the present study, the ED₅₀ and ED₉₀ have been used. These parameters are usually given to describe the response of fungal strains to sub-lethal doses of antifungal agents (Marín et al., 2013; Mateo et al., 2011b, 2013). Additionally, these parameters permit reliable comparisons between similar studies. At present, implementation of lethal doses of EOs against aflatoxigenic species in food, as a single effective measure to prevent fungal development, may become unreliable. Therefore, knowledge of ED₅₀ and ED₉₀ levels of EOs under standardized conditions can be very useful in food technology (longer periods of lapsing, reduction of others chemical preservatives, application of complementary treatments, etc.).

In most studies the EOs are incorporated into the culture medium. In this way, Bluma et al. (2008), found that mycelial growth rate of *Aspergillus* spp. section *Flavi* (*A. flavus* and *A. parasiticus*) strains in maize meal extract agar (MMEA) was affected by the addition of 150-500 µg/g of clove, pennyroyal or mountain thyme EOs. At 500 µg/g, all strains were inhibited in percentages higher than 90% by the three EO. Generally, at the lowest a_w tested (0.955 a_w), 150 µg/g of pennyroyal and mountain thyme EO was insufficient to affect fungal growth. Eucalyptus EO was ineffective at all a_w levels tested. Later, these authors (Bluma and Etcheverry, 2008) also found that the dosage for controlling growth rate and AFB₁ production in sterile maize grain was much higher (2000–3000 µg/g) than on MMEA. This finding is similar to those reported by Hope et al. (2002) for the species *F. culmorum*. Low concentrations (50–100 µg/g) of different EOs were effective but much higher concentrations (500 µg/g) were required to control growth of *F. culmorum* on sterile wheat grains. Similar results have been achieved in assays with others antifungal agents (morpholines and azoles) much more effective than EOs but less safe to health (Mateo et al., 2011b, 2013). Thus, the common background indicates that doses of antifungal agents higher than those employed in synthetic or semisynthetic media are required to control fungal growth in food, regardless of environmental conditions, fungal species and active substances. These

results demonstrate that, currently, addition of EOs into the food and implementation of this method in food technology seems hardly feasible.

Although in the reports cited above, different EO types have been tested by direct contact, considering the weight of culture medium/Petri dish, the masses of EO/plate reported by these authors were far higher than those used in the present study (in the vapour phase). Several researchers have concurred that the best antifungal activity of volatile compounds is achieved by gaseous contact as opposed to aqueous solution or agar contact (Inuoye et al., 2000; Nielsen and Ríos, 2000; Tullio et al., 2006; Tyagi and Malik, 2011a, 2011b; Vilela et al., 2009). Passone et al. (2012, 2013) found that Boldo EO was more effective against *A. flavus* and *A. parasiticus* in the vapour assay than in the contact assay. Moreover, these authors also showed that aflatoxigenic isolates exhibited greater sensitivity to the treatments with pennyroyal and clove EOs applied in vapour phase than into the medium. Currently, with a few exceptions, the advantages of using the EO in vapour phase for food products are that lower doses are required and that its release may be regulated.

When applicable, an interesting methodology can be the addition of EO into edible films. The degree of fungal inhibition against *Aspergillus niger* and *Penicillium digitatum* by cinnamon, Mexican oregano and lemongrass, incorporated into three edible films (amaranth, chitosan and starch) has been reported (Ávila-Sosa et al. (2012). Amaranth, chitosan and starch edible films were formulated with EO concentrations of 0.00, 0.25, 0.50, 0.75, 1.00, 2.00, or 4.00% (on wet base). Films were placed on the lid's inner side of the Petri dishes (covering the entire surface). The assays were carried out by vapour contact using the inverted lid technique. The level of fungal growth inhibition depended on the type of polymer used to manufacture the film. It appears that each polymer retains EO to different degrees. Chitosan edible films incorporating Mexican oregano or cinnamon EO were more effective in inhibiting *A. niger* and *P. digitatum* growth at lower EO concentrations than amaranth edible films. A comparative analysis with our results is difficult because the main component of

Mexican oregano EO is thymol and in European oregano (used in the present study) the concentration of carvacrol is much higher (86%) than that of thymol (2%). Thus, the antifungal activity of the ORE EO used here is due to the presence of both carvacrol and thymol along with other minor components. Moreover, cinnamaldehyde is the main component (66.5%) in cinnamon bark oil (used in the present study). However, the oil from leaves and bark of some cinnamon bushes has eugenol as the main component. Differences in film type and EO origins found in the literature also make difficult comparatives studies. In any case, the effective EO doses registered by Ávila-Sosa et al. (2012) are higher than those recorded in the present study. Ávila-Sosa et al. (2012) propose chitosan edible films incorporating Mexican oregano or cinnamon EO to control *A. niger* and *P. digitatum* growth whereas in the present study EVOH films incorporating CINHO have proven to be the best safety proposal in controlling *A. flavus* and *A. parasiticus* growth in maize grains.

Active packaging is one of the most innovative food packaging concepts. Polyethylene terephthalate (PET) films containing CIN EO have been tested in vapour phase (without direct contact with the mould) against *A. flavus* (Manso et al., 2013). PET films were placed over the top of the Petri dishes instead of the lid. Culture medium was agar Czapek and incubation temperature 25 °C. Under these culture conditions the inhibition of *A. flavus* growth exposed to active PET at 2%, 4% and 8% of CIN EO depended largely on the primary inoculum concentration. Using an inoculum of *A. flavus* of 100 µl of a 10⁴ CFU/ml suspension, fungal growth was totally inhibited by the 2% PET-CIN EO films, and higher content of essential oil caused total inhibition for all suspensions assayed (10⁴, 10⁵ and 10⁶ CFU/ml).

Bioactive EO films can reduce diffusion into the product since the essential oil forms part of the chemical structure of the film and interacts with the polymer and the emulsifying agent, which is generally required to ensure dispersion and formation of a homogeneous coating (Atarés et al., 2016; da Cruz-Cabral et al., 2013). Moreover, the EOs are gradually released from the polymer matrix on the product surface over time,

maintaining a proper concentration of antimicrobial components during the incubation period and allowing the use of smaller amounts compared with direct application of EOs into the medium or in direct contact with a food surface. The results obtained in the present study, especially in the cultures with EVOH-CINHO films, show that extraordinarily low levels of EO (0.121-0.229 mg/plate) were able to inhibit growth of *A. flavus* and *A. parasiticus* (50%-90%) under the different assayed conditions. The rest of assayed bioactive films presented relevant antifungal activity although were less effective than EVOH-CINHO films. Total growth inhibition of *A. flavus* and *A. parasiticus* was registered with EVOH-CINHO at 0.25 mg/plate.

From the statistical analysis of the data it can be inferred that the class of EO-film, their doses, a_w , fungal species and temperature significantly influence ($p < 0.05$) the fungal GR and the ED₅₀ values. Overall, the ED₅₀ values were higher (less effectiveness) at 37 °C than at 25 °C and at 0.99 a_w than at 0.96 a_w . This finding agrees with the results obtained by others authors (although no films were used). Thus, Passone et al. (2012, 2013) found that the inhibition effect of three EO (boldo, pennyroyal and clove) increased when a_w decreased. Treatment with 1000 µl/l of pennyroyal EO resulted in inhibition growth rates varying in the ranges 16.1-75.8% and 3.7-74.7% at 0.98 and 0.95 a_w , respectively, while significant inhibitions ($p < 0.05$) by 39.4-72.8% were observed at 0.93 a_w . Bluma et al. (2008) also reported that the efficacy of anise, boldo, mountain thyme, clove and pennyroyal EOs against *Aspergillus* spp. section *Flavi* under different a_w conditions (0.982, 0.955, and 0.90) increased when EO concentration increased and a_w levels decreased.

In our study, AF production was generally inhibited by EVOH films containing ORE, CAR, CIN or CINHO EO (in vapour phase) at the highest doses assayed, regardless of temperature and a_w . For EVOH films containing ORE, CAR and CIN at medium/low level doses, this effect was closely dependent on a_w and the EO dose per plate. This is in agreement with the results obtained by others authors with different EOs (boldo, pennyroyal, clove, anise, and mountain thyme) (Passone et al., 2012;

Bluma and Etcheverry, 2008). Although in these studies only AFB₁ production was studied and bioactive EO films were not used, all EOs showed significant impact on AFB₁ accumulation. Only an example related to the study of the anti-aflatoxigenic action of an active polypropylene (PP) film with cinnamon EO in *A. flavus* cultures was found (Manoso et al., 2014). Their results show a significant reduction of AFB₁ production at PP 2% CIN and total inhibition at PP 4% CIN and PP 6% CIN. In that report, all the surface of Petri dishes was covered with the film. In our study EVOH 0.25% CINHO film totally inhibited AF production by *A. flavus* and *A. parasiticus* under all assayed environmental conditions. Stimulation of AF accumulation was registered in some cultures, especially at low or medium EO levels, except for EVOH-CINHO film. It has been reported that mycotoxin production may be stimulated when stressing environmental conditions and low antifungal agent doses are maintained in the medium during the growth of mycotoxin-producing species (da Cruz Cabral et al., 2013; Medina et al., 2007; Mateo et al., 2011b, 2011c, 2013; Prakash et al., 2015). For example, stimulation of AFB₁ accumulation by eucalyptus and mountain thyme EO has been described by Bluma et al. (2008). In the present study, this phenomenon was frequently detected in cultures with EVOH-CAR, EVOH-ORE or EVOH-CIN films.

Transcriptome-proteome correlation in biological pathways and secondary metabolism clusters in *A. flavus* in response to temperature have been studied (Bai et al. 2015) and the effect of water activity on development and aflatoxin biosynthesis of *A. flavus* at the transcriptome level has been reported (Zhang et al. 2014). From two different treatments (0.99 a_w and 0.93 a_w), these authors identified differentially expressed genes by transcriptome analysis and they found that numerous metabolic pathways related to biosynthesis of aflatoxins were significantly over-expressed when treated at 0.99 a_w. Depending on the particular combination of external growth parameters, the biosynthesis of AFs can be completely inhibited, although normal growth is still possible or the biosynthesis pathway can be fully activated. However, up to date no study has reported the effect of antifungal agents, such as essential oils, to control

growth of *A. flavus* or *A. parasiticus* and AF production under different environmental conditions and their possible interactions.

In summary, EVOH is a family of copolymers valid for direct food contact. They are thermoplastic and present excellent barrier and thermal properties including heat sealability. These films can be used alone or as a coating onto a film substrate. Several papers has been published with these films and structures and tested as a final package for vegetables, nuts, fresh meat, surimi, stock, soaps, or dressing and sauces. In the present study, the EVOH-CINHO film proved the most effective in controlling fungal growth of aerobic toxigenic fungi *A. flavus* and *A. parasiticus*, and AF production, because CINHO acts at very low concentration, as mentioned above, regardless of relative humidity and environmental temperature, and the EO is slowly released in vapour phase. The advantages of using the EO in vapour phase for food products are that lower doses are required and that its release may be regulated. This minimizes the alteration of the organoleptic properties of foods. Without a doubt, the results of the present study show that EVHO-CINHO films could be an excellent tool to control growth of the most important aflatoxigenic species that have been found in maize, and other agricultural crops, and to control AF production in maize, maize products, and other agricultural commodities susceptible to contamination, such as nuts and dried fruits.

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764 Figure captions

765 Fig 1. Growth rates (GR, mm/day) of *A. flavus* on maize grains in the presence of
766 EVOH-EO-films containing oregano (ORE), carvacrol (CAR), cinnamon (CIN) or
767 cinnamaldehyde (CINHO) at different doses, a_w and temperature regimes. Error bars
768 represent standard deviations.

769 Fig. 2. Growth rates (GR, mm/day) of *A. parasiticus* on maize grains in the presence of
770 EVOH-EO-films containing oregano (ORE), carvacrol (CAR), cinnamon (CIN) or
771 cinnamaldehyde (CINHO) at different doses, a_w and temperature regimes. Error bars
772 represent standard deviations.

773 Fig. 3. Aflatoxin B₁ (AFB₁) and aflatoxin B₂ (AFB₂) production by *A. flavus* in maize
774 grains in the presence of EVOH-EO-films containing oregano (ORE), carvacrol (CAR),
775 cinnamon (CIN) or cinnamaldehyde (CINHO) at different doses, a_w and temperature
776 regimes. Incubation time was 12 days. Error bars represent standard deviations.

777 Fig. 4. Aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁) and aflatoxin G₂
778 (AFG₂) production by *A. parasiticus* in maize grains in the presence of EVOH-EO-films
779 containing oregano (ORE), carvacrol (CAR), cinnamon (CIN) or cinnamaldehyde
780 (CINHO) at different doses, a_w and temperature regimes. Incubation time was 12 days.
781 Error bars represent standard deviations.

Table 1

Table 1. Recovery data for blank maize grains spiked with standards of aflatoxins

Aflatoxin	Spiking level (ng/g)	Mean recovery (%) (n = 5)	Recovery range (%)	RSD (%)
AFB ₁	0.15	86.4	75 - 90	7.1
	0.50	89.6	80 - 92	6.1
	1.00	94.4	91 - 97	2.6
	5.0	96.2	94 - 100	2.4
	50.0	90.4	84 - 94	5.1
	100.0	97.0	94 - 99	2.2
AFB ₂	0.15	76.2	71 - 86	7.6
	0.30	78.2	76 - 94	5.6
	0.60	88.0	82 - 98	8.1
	3.0	88.2	83 - 96	7.1
	10.0	89.4	85 - 96	6.3
	50.0	90.2	86 - 96	5.4
AFG ₁	0.15	83.0	75 - 91	7.6
	0.30	84.8	78 - 92	7.1
	0.60	89.2	84 - 96	6.1
	3.00	89.8	85 - 98	6.9
	10.0	88.8	84 - 96	6.9
	50.0	73.8	69 - 80	5.5
AFG ₂	0.15	73.6	68 - 85	8.9
	0.30	75.0	69 - 89	11.6
	0.60	81.0	72 - 91	10.7
	3.00	79.2	75 - 85	5.7
	10.0	82.4	73 - 91	8.4
	50.0	85.8	81 - 93	5.4

Table 2. ED₅₀ and ED₉₀ (mg of EO/Petri plate) of EVOH films containing oregano, carvacrol, cinnamon or cinnamaldehyde in vapor phase against *A. flavus* in maize grains under different environmental conditions.

Temperature (°C)		Essential oils							
		Oregano		Carvacrol		Cinnamon		Cinnamaldehyde	
		ED ₅₀	ED ₉₀	ED ₅₀	ED ₉₀	ED ₅₀	ED ₉₀	ED ₅₀	ED ₉₀
a _w									
37	0.99	2.150	>4.0	1.625	>4.0	3.500	>4.0	0.125	0.225
	0.96	1.125	>4.0	1.150	>4.0	2.650	>4.0	0.125	0.225
25	0.99	2.575	>4.0	1.150	>4.0	2.475	>4.0	0.125	0.229
	0.96	2.300	>4.0	1.300	>4.0	3.063	>4.0	0.125	0.222

Table 3

Table 3. ED₅₀ and ED₉₀ (mg of EO/Petri plate) of EVOH films containing oregano, carvacrol, cinnamon or cinnamaldehyde in vapor phase against *A. parasiticus* in maize grains under different environmental conditions.

Temperature (°C)		Essential oils							
		Oregano		Carvacrol		Cinnamon		Cinnamaldehyde	
		ED ₅₀	ED ₉₀	ED ₅₀	ED ₉₀	ED ₅₀	ED ₉₀	ED ₅₀	ED ₉₀
a _w									
37	0.99	2.000	>4.0	2.250	3.675	2.475	>4.0	0.133	0.229
	0.96	2.688	>4.0	>4.0	>4.0	2.550	>4.0	0.125	0.225
25	0.99	2.125	>4.0	1.175	3.975	2.275	>4.0	0.125	0.225
	0.96	3.750	>4.0	2.625	>4.0	3.625	>4.0	0.121	0.226

Figure 1

Figure 1.

Figure 2

Figure 2

Figure 3

Figure 3.

Figure 4

Figure 4.