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 AFB_1 and AFB_2 and *A. parasiticus* produces AFB_1, AFB_2, AFG_1, and AFG_2. AFB_1, 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 ED_{50} 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 ºC than at 25 ºC. 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 (ED_{50}) (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 ED_{90} 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). AFB_1 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 AFB_1 and AFB_2 and *A. parasiticus* produces AFB_1, AFB_2, AFG_1, and AFG_2. 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

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

107 Ethylene-vinyl alcohol copolymer (EVOH) is composed of two segment chains:
108 one, olefinic and hydrophobic, comes from ethylene, and the other, with a hydroxyl
109 substituent, presents hydrophilic behaviour. EVOH is a packaging material used to
110 provide high oxygen barrier properties and their hydrophilic nature makes it very
111 sensitive to water. EVOH materials have been used as matrices for the development of
112 active packaging systems, where the polymer protects the active agents during storage
113 and triggers their activity on exposure to humidity (López-de-Dicastillo et al., 2010a,
114 2010b, 2011; Muriel-Galet et al., 2012, 2013). These properties combined with
115 appropriated EOs, could make EVOH a highly suitable material for control of
116 aflatoxigenic fungi (aerobic organisms) and AFs in food and feed, such as maize and
117 by-products.

118 The aims of this work were to develop effective anti-aflatoxigenic fungi and anti-
119 aflatoxin production films for food packaging applications incorporating EOs from
120 oregano (*Origanum vulgare*) (ORE), cinnamon (*Cinnamomum zeylanicum*) (CIN) or
121 their major active constituents, carvacrol (CAR) and cinnamaldehyde (CINHO),
122 respectively, in EVOH copolymer with 29 % ethylene molar content. For this purpose: i)
123 the ability of the designed active films *versus A. flavus* and *A. parasiticus* growth in
124 maize grains under different environmental conditions is determined and ii) the effect of
125 these bioactive films to control the production and accumulation of AFB_1, AFB_2, AFG_1,
126 and AFG_2 in the medium under the assayed conditions is investigated.

127 **2. Materials and Methods**

128 *2.1. Film preparation*

129 Ethylene vinyl alcohol copolymer with 29% ethylene molar content (EVOH-29)
130 was kindly supplied by The Nippon Synthetic Chemical Industry Co., Ltd. (Osaka,
131 Japan). Oregano, 86% carvacrol (ORE) and cinnamon bark, 66.5% cinnamaldehyde
132 (CIN) essential oils (EO) were purchased from Jarpil (Almería, Spain). Carvacrol (CAR)
133 [PubChem CID 10364], the major component of ORE, and cinnamaldehyde (3-phenyl-
134 2-propenal) (CINHO) [PubChem CID 637511], the major component of CIN of Kosher
135 quality were supplied by Sigma-Aldrich (Barcelona, Spain).
136 In this study, films of EVOH containing ORE, CAR, CIN and CINHO, labelled as
137 EVOH-ORE, EVOH-CAR, EVOH-CIN and EVOH-CINHO, and control films (in absence
138 of active substances) were obtained by casting in an oven at 75 ºC for 15 min. For this
139 purpose, 13 g of EVOH-29 were initially dissolved in 100 ml of a 1:1 (v/v) mixture of 1–
140 propanol-distilled water by heating at 75 ºC under reflux. Once the copolymer was
141 completely dissolved, the active component was added to the solution (10 % w/w dry
142 polymer). Then, the mixture was stirred at 40 ºC for 30 min. The mixture was spread on
143 a Teflon-coated glass plate by using a 200-µm spiral bar coater providing films with a
144 thickness of 0.013 ± 0.002 mm.
145 In order to know the final content of the active compounds in the resultant films,
146 three replicates of each film were analysed by thermal desorption-gas chromatography
147 (TD-GC) analysis, using an 890 thermal tube desorber (Dynatherm Analytical
148 Instruments Inc., Kelton, PA, USA). It was connected in series to an HP 5890 Series II
149 Plus gas chromatograph (Agilent Tech., Barcelona, Spain) equipped with a flame
150 ionization detector (FID) and an Agilent HP-1 semi-capillary column of 30 m length,
151 0.53 mm internal diameter and 2.65 µm film thickness (Teknokroma S.C.L., Barcelona,
152 Spain) following the procedure described in previous report (Cerisuelo et al., 2012). In
153 brief, a portion of the tested film (about 20 mg) was placed in the desorption cell and
154 heated at 210 °C for 7 min. He gas stream carried the desorbed gaseous compounds
155 to the GC through a transfer line heated at 230 °C. Chromatographic conditions were
156 as follows: He was the carrier gas, detector temperature was 260 °C, oven temperature

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

164 *2.2. Inoculum preparation*

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

175 *2.3. Preparation of culture media with different EO films and a_w levels*

176 The assays with EO films were carried out in maize grains (*Zea mays*). Culture
177 media were prepared as follow: maize grains (25 g), previously analysed to ensure
178 they had undetectable levels of AFs, were placed in Erlenmeyer flasks and autoclaved
179 for 20 min at 121 ºC. Then, the a_w level was measured using flask controls and once
180 determined it was adjusted in all flasks to 0.96 or 0.99 by addition of sterile distilled
181 water using a moisture adsorption curve for maize previously determined. Flasks with
182 maize grains were closed and refrigerated at 4 ºC 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 ± 0.01 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 x10^6 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 ºC and 37 ºC 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

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

211 *2.5. Aflatoxin determination*

212 *2.5.1. Chemicals, reagents and standard solutions*

213 Chloroform, methanol and acetonitrile were liquid chromatography grade (J.T.
214 Baker, Deventer, The Netherlands). Pure water was from Milli-Q system (Millipore,
215 Billerica, MA, USA). Standards of AFs were from Sigma (Sigma-Aldrich, Alcobendas,
216 Spain). Stock solutions of AFB_1, AFB_2, AFG_1, and AFG_2 were prepared in chloroform to
217 give each a concentration of 500 µg/ml. For maize grain spiking and preparation of
218 calibration standards, appropriate diluted solutions of each AF were prepared in
219 methanol-water 80:20 (v/v). The stock solutions of mycotoxins were stored in freezer
220 (−18 °C) in tightly closed silanized amber glass vials. They were let to equilibrate at
221 room temperature before use. Stock solutions and diluted standards of mycotoxins
222 were gravimetrically controlled over time to avoid change in concentrations.

223 *2.5.2. Extraction*

224 To determinate toxin concentrations in the cultures all maize grains distributed as
225 a homogenous layer on the Petri dish (25 g) were used, regardless of the colony
226 diameter reached after 12 days incubation. Grains were dried at 45 ºC for 48 h, finely
227 milled and homogenized. About two g of ground maize culture was thoroughly mixed
228 with 20 mL of acetone-water (60:40, v/v) in a capped container. The mixture was
229 shaken in orbital shaker for 1 h to extract AFs (Bertucci et al., 2012). Usually, after
230 filtration through filter paper an aliquot of 5 mL of the filtrate was diluted with 45 mL of
231 pure water and cleaned-up through an immunoaffinity column (AflaTest WB, Vicam). In
232 the case of extremely high AF levels, dilution with water was 1:49 (v/v). The column
233 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 N_2 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 AFB_1 and AFB_2, AFG_1 and AFG_2 were prepared by dilution of stock solutions with methanol-water 80:20 (v/v). These solutions contained 0.2 to 4.0 ng AFB_1/ml, 0.1 to 3.0 ng AFG_1/ml and 0.06 to 2.0 ng AFB_2 and AFG_2/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 C_{18} 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 AFB_1 and AFG_1 was achieved using freshly prepared iodine reagent (300 mg I_2/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-µm 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 AFB_1/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 N_2. 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 AFB_1, 0.030 and AFB_2, and 0.040 for AFG_1 and AFG_2. Method LOQ (ng/g) were 0.10 for AFB_1 0.09 for AFB_2, and 0.12 for AFG_1 and AFG_2.

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 ClNHO. 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 ClNHO, 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 ºC than at 25 ºC (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

312 confirmed these results. The response of *A. flavus* (Fig. 1) and *A. parasiticus* (Fig. 2) to
313 the different treatments were used to calculate the ED_{50} and ED_{90} of each EVOH-EO
314 film/plate under all the assayed conditions (Tables 2 and 3, respectively).
315 Taking all the factors (fungal species, a_w, temperature, EO type and dose) into
316 consideration, the statistical analyses by multifactor ANOVA (with interactions, as some
317 of them were significant) showed that single factors EO, dose and temperature
318 significantly influenced the GR (*p*-values < 0.01 for the two former and < 0.03 for the
319 last), while fungal species and a_w factors did not. ANOVA also showed that interactions
320 a_w x temperature, a_w x dose, EVOH-EO x dose, EVOH-EO x fungal species were highly
321 significant (*p*-value < 0.01). The interaction EVOH-EO x temperature was also
322 significant (*p*-value = 0.023).
323 As shown in Table 2 in maize cultures of *A. flavus* the ED_{50} values (mg EO/Petri
324 plate) for EVOH films containing ORE, CAR, CIN, and CINHO were in the ranges
325 1.125-2.575, 1.150-1.625, 2.475-3.500, and 0.125 (fix value), respectively. Concerning
326 maize grains cultures of *A. parasiticus* Table 3 lists the ED_{50} values (mg EO/Petri plate)
327 for EVOH films containing ORE, CAR, CIN, and CINHO which were in the ranges
328 2.000-3.750, 1.175->4.0, 2.275-3.625, and 0.121-0.133, respectively. The efficacy of
329 EVOH-EO films to control growth of *A. flavus* and *A. parasiticus* increased in the order
330 EVOH-CINHO > EVOH-CAR > EVOH-ORE > EVOH-CIN.
331 With regard to the ED_{50} values, multifactor ANOVA (with interactions) of the data
332 including class of EVOH-EO film, a_w, fungal species and temperature revealed that
333 these factors (except for temperature) significantly influenced the ED_{50} values (*p*-value
334 < 0.01). Significant interactions between the factors occurred. The Duncan's test found
335 that with regard to ED_{50} values each of the four EO films tested was different from each
336 other (*p*-value < 0.05).
337 Under the assayed conditions, no studied factor, except the type of EVOH-EO
338 film, showed significant influence on the ED_{90} values. Only for EVOH-CINHO film the
339 ED_{90} values could be estimated and they were 0.22-0.23 mg/plate for both isolates.

340 Duncan's test confirmed two homogeneous non-overlapping groups with regard to the
341 influence of EVOH-EO-film on ED_{90}: the EVOH-CINHO film and the remaining films (*p*-
342 value < 0.05). In the present study, treatments with levels higher than 4.0 mg EO/plate
343 were not tested since such concentrations would be unsuitable and inappropriate in
344 food technology. One of the main drawbacks of the EOs is their flavour, which can alter
345 the organoleptic properties of the food.

346 *3.3. Effect of EVOH-EO films on AF production by A. flavus and A. parasiticus*

347 Cultures of *A. flavus* and *A. parasiticus* under all the assayed conditions were
348 examined to determine AF production. AFB_1 and AFB_2 were analysed in *A. flavus*
349 cultures (Fig. 3) and AFB_1, AFB_2, AFG_1 and AFG_2 in *A. parasiticus* cultures (Fig. 4). In
350 some cases, high dilution ratios (up to 1:400) was performed. In general, multifactor
351 ANOVA with interactions showed that AFB_1 and AFB_2 production was affected by the
352 fungal species, a_w, dose of EO/plate and type of EVOH-EO-film (*p*-value < 0.001 in all
353 cases). Levels of both toxins were lower in cultures of *A. flavus* than in cultures of *A.*
354 *parasiticus*, at 0.96 than at 0.99 a_w, at high EO doses than at low doses and with EVOH-
355 CINHO film than the other film types. Although temperature did not significantly affect
356 AFB_1 and AFB_2 production, levels of both toxins were lower in cultures incubated at 37
357 ºC than at 25 ºC.

358 In cultures of *A. parasiticus*, concentrations of AFG_1 and AFG_2 were also lower at
359 0.96 a_w, high-dose of EOs and 37 ºC. Multifactor ANOVA with interactions showed that
360 factors a_w, EVOH-EO film type, their doses and temperature significantly affected AFG_1
361 and AFG_2 production (*p*-value < 0.05) although a_w and dose were the most significant
362 factors (*p*-value < 0.001). Significant interactions between the factors were revealed.
363 Post-hoc analysis of EO-dose with Duncan's test displays three homogenous groups
364 for AFG_1 and AFG_2 (blanks, 0.25 mg/plate and the remaining doses). With regard to
365 the influence of the EVOH-EO-dose on AF production by *A. flavus* and *A. parasiticus*, it
366 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 AFB_1, AFB_2, AFG_1, and AFG_2 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

394 test has been developed and adopted for evaluating the possible antifungal activity of
395 EOs against food-related fungi. In the present study, the ED_{50} and ED_{90} have been
396 used. These parameters are usually given to describe the response of fungal strains to
397 sub-lethal doses of antifungal agents (Marín et al., 2013; Mateo et al., 2011b, 2013).
398 Additionally, these parameters permit reliable comparisons between similar studies. At
399 present, implementation of lethal doses of EOs against aflatoxigenic species in food, as
400 a single effective measure to prevent fungal development, may become unreliable.
401 Therefore, knowledge of ED_{50} and ED_{90} levels of EOs under standardized conditions
402 can be very useful in food technology (longer periods of lapsing, reduction of others
403 chemical preservatives, application of complementary treatments, etc.).
404 In most studies the EOs are incorporated into the culture medium. In this way,
405 Bluma et al. (2008), found that mycelial growth rate of *Aspergillus* spp. section *Flavi* (*A.*
406 *flavus* and *A. parasiticus*) strains in maize meal extract agar (MMEA) was affected by
407 the addition of 150-500 µg/g of clove, pennyroyal or mountain thyme EOs. At 500 µg/g,
408 all strains were inhibited in percentages higher than 90% by the three EO. Generally, at
409 the lowest a_w tested (0.955 a_w), 150 µg/g of pennyroyal and mountain thyme EO was
410 insufficient to affect fungal growth. Eucalyptus EO was ineffective at all a_w levels tested.
411 Later, these authors (Bluma and Etcheverry, 2008) also found that the dosage for
412 controlling growth rate and AFB_1 production in sterile maize grain was much higher
413 (2000–3000 µg/g) than on MMEA. This finding is similar to those reported by Hope et
414 al. (2002) for the species *F. culmorum*. Low concentrations (50–100 µg/g) of different
415 EOs were effective but much higher concentrations (500 µg/g) were required to control
416 growth of *F. culmorum* on sterile wheat grains. Similar results have been achieved in
417 assays with others antifungal agents (morpholines and azoles) much more effective
418 than EOs but less safe to health (Mateo et al., 2011b, 2013). Thus, the common
419 background indicates that doses of antifungal agents higher than those employed in
420 synthetic or semisynthetic media are required to control fungal growth in food,
421 regardless of environmental conditions, fungal species and active substances. These

422 results demonstrate that, currently, addition of EOs into the food and implementation of
423 this method in food technology seems hardly feasible.
424 Although in the reports cited above, different EO types have been tested by direct
425 contact, considering the weight of culture medium/Petri dish, the masses of EO/plate
426 reported by these authors were far higher than those used in the present study (in the
427 vapour phase). Several researchers have concurred that the best antifungal activity of
428 volatile compounds is achieved by gaseous contact as opposed to aqueous solution or
429 agar contact (Inuoye et al., 2000; Nielsen and Ríos, 2000; Tullio et al., 2006; Tyagi and
430 Malik, 2011a, 2011b; Vilela et al., 2009). Passone et al. (2012, 2013) found that Boldo
431 EO was more effective against *A. flavus* and *A. parasiticus* in the vapour assay than in
432 the contact assay. Moreover, these authors also showed that aflatoxigenic isolates
433 exhibited greater sensitivity to the treatments with pennyroyal and clove EOs applied in
434 vapour phase than into the medium. Currently, with a few exceptions, the advantages
435 of using the EO in vapour phase for food products are that lower doses are required
436 and that its release may be regulated.
437 When applicable, an interesting methodology can be the addition of EO into
438 edible films. The degree of fungal inhibition against *Aspergillus niger* and *Penicillium*
439 *digitatum* by cinnamon, Mexican oregano and lemongrass, incorporated into three
440 edible films (amaranth, chitosan and starch) has been reported (Ávila-Sosa et al.
441 (2012). Amaranth, chitosan and starch edible films were formulated with EO
442 concentrations of 0.00, 0.25, 0.50, 0.75, 1.00, 2.00, or 4.00% (on wet base). Films were
443 placed on the lid's inter side of the Petri dishes (covering the entire surface). The
444 assays were carried out by vapour contact using the inverted lid technique. The level of
445 fungal growth inhibition depended on the type of polymer used to manufacture the film.
446 It appears that each polymer retains EO to different degrees. Chitosan edible films
447 incorporating Mexican oregano or cinnamon EO were more effective in inhibiting *A.*
448 *niger* and *P. digitatum* growth at lower EO concentrations than amaranth edible films. A
449 comparative analysis with our results is difficult because the main component of

450 Mexican oregano EO is thymol and in European oregano (used in the present study)
451 the concentration of carvacrol is much higher (86%) than that of thymol (2%). Thus, the
452 antifungal activity of the ORE EO used here is due to the presence of both carvacrol
453 and thymol along with other minor components. Moreover, cinnamaldehyde is the main
454 component (66.5%) in cinnamon bark oil (used in the present study). However, the oil
455 from leaves and bark of some cinnamon bushes has eugenol as the main component.
456 Differences in film type and EO origins found in the literature also make difficult
457 comparatives studies. In any case, the effective EO doses registered by Ávila-Sosa et
458 al. (2012) are higher than those recorded in the present study. Ávila-Sosa et al. (2012)
459 propose chitosan edible films incorporating Mexican oregano or cinnamon EO to
460 control *A. niger* and *P. digitatum* growth whereas in the present study EVOH films
461 incorporating CINHO have proven to be the best safety proposal in controlling *A. flavus*
462 and *A. parasiticus* growth in maize grains.
463 Active packaging is one of the most innovative food packaging concepts.
464 Polyethylene terephthalate (PET) films containing CIN EO have been tested in vapour
465 phase (without direct contact with the mould) against *A. flavus* (Manso et al., 2013).
466 PET films were placed over the top of the Petri dishes instead of the lid. Culture
467 medium was agar Czapek and incubation temperature 25 ºC. Under these culture
468 conditions the inhibition of *A. flavus* growth exposed to active PET at 2%, 4% and 8%
469 of CIN EO depended largely on the primary inoculum concentration. Using an inoculum
470 of *A. flavus* of 100 µl of a 10^4 CFU/ml suspension, fungal growth was totally inhibited by
471 the 2% PET-CIN EO films, and higher content of essential oil caused total inhibition for
472 all suspensions assayed (10^4, 10^5 and 10^6 CFU/ml).
473 Bioactive EO films can reduce diffusion into the product since the essential oil
474 forms part of the chemical structure of the film and interacts with the polymer and the
475 emulsifying agent, which is generally required to ensure dispersion and formation of a
476 homogeneous coating (Atarés et al., 2016; da Cruz-Cabral et al., 2013). Moreover, the
477 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 ED_{50} values. Overall, the ED_{50} values were higher (less effectiveness) at 37 ºC than at 25 ºC 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;

506 Bluma and Etcheverry, 2008). Although in these studies only AFB_1 production was
507 studied and bioactive EO films were not used, all EOs showed significant impact on
508 AFB_1 accumulation. Only an example related to the study of the anti-aflatoxigenic
509 action of an active polypropylene (PP) film with cinnamon EO in *A. flavus* cultures was
510 found (Manso et al., 2014). Their results show a significant reduction of AFB_1
511 production at PP 2% CIN and total inhibition at PP 4% CIN and PP 6% CIN. In that
512 report, all the surface of Petri dishes was covered with the film. In our study EVOH
513 0.25% CINHO film totally inhibited AF production by *A. flavus* and *A. parasiticus* under
514 all assayed environmental conditions. Stimulation of AF accumulation was registered in
515 some cultures, especially at low or medium EO levels, except for EVOH-CINHO film. It
516 has been reported that mycotoxin production may be stimulated when stressing
517 environmental conditions and low antifungal agent doses are maintained in the medium
518 during the growth of mycotoxin-producing species (da Cruz Cabral et al., 2013; Medina
519 et al., 2007; Mateo et al., 2011b, 2011c, 2013; Prakash et al., 2015). For example,
520 stimulation of AFB_1 accumulation by eucalyptus and mountain thyme EO has been
521 described by Bluma et al. (2008). In the present study, this phenomenon was
522 frequently detected in cultures with EVOH-CAR, EVOH-ORE or EVOH-CIN films.
523 Transcriptome-proteome correlation in biological pathways and secondary metabolism
524 clusters in *A. flavus* in response to temperature have been studied (Bai et al. 2015) and
525 the effect of water activity on development and aflatoxin biosynthesis of *A. flavus* at the
526 transcriptome level has been reported (Zhang et al. 2014). From two different
527 treatments (0.99 a_w and 0.93 a_w), these authors identified differentially expressed
528 genes by transcriptome analysis and they found that numerous metabolic pathways
529 related to biosynthesis of aflatoxins were significantly over-expressed when treated at
530 0.99 a_w. Depending on the particular combination of external growth parameters, the
531 biosynthesis of AFs can be completely inhibited, although normal growth is still
532 possible or the biosynthesis pathway can be fully activated. However, up to date no
533 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 B_1 (AFB_1) and aflatoxin B_2 (AFB_2) 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 B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2
778 (AFG_2) 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

Table 2. ED_{50} and ED_{90} (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 (ºC)	a_w	Essential oils							
		Oregano		Carvacrol		Cinnamon		Cinnamaldehyde	
		ED_{50}	ED_{90}	ED_{50}	ED_{90}	ED_{50}	ED_{90}	ED_{50}	ED_{90}
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. ED_{50} and ED_{90} (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 (ºC)	a_w	Essential oils							
		Oregano		Carvacrol		Cinnamon		Cinnamaldehyde	
		ED_{50}	ED_{90}	ED_{50}	ED_{90}	ED_{50}	ED_{90}	ED_{50}	ED_{90}
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.

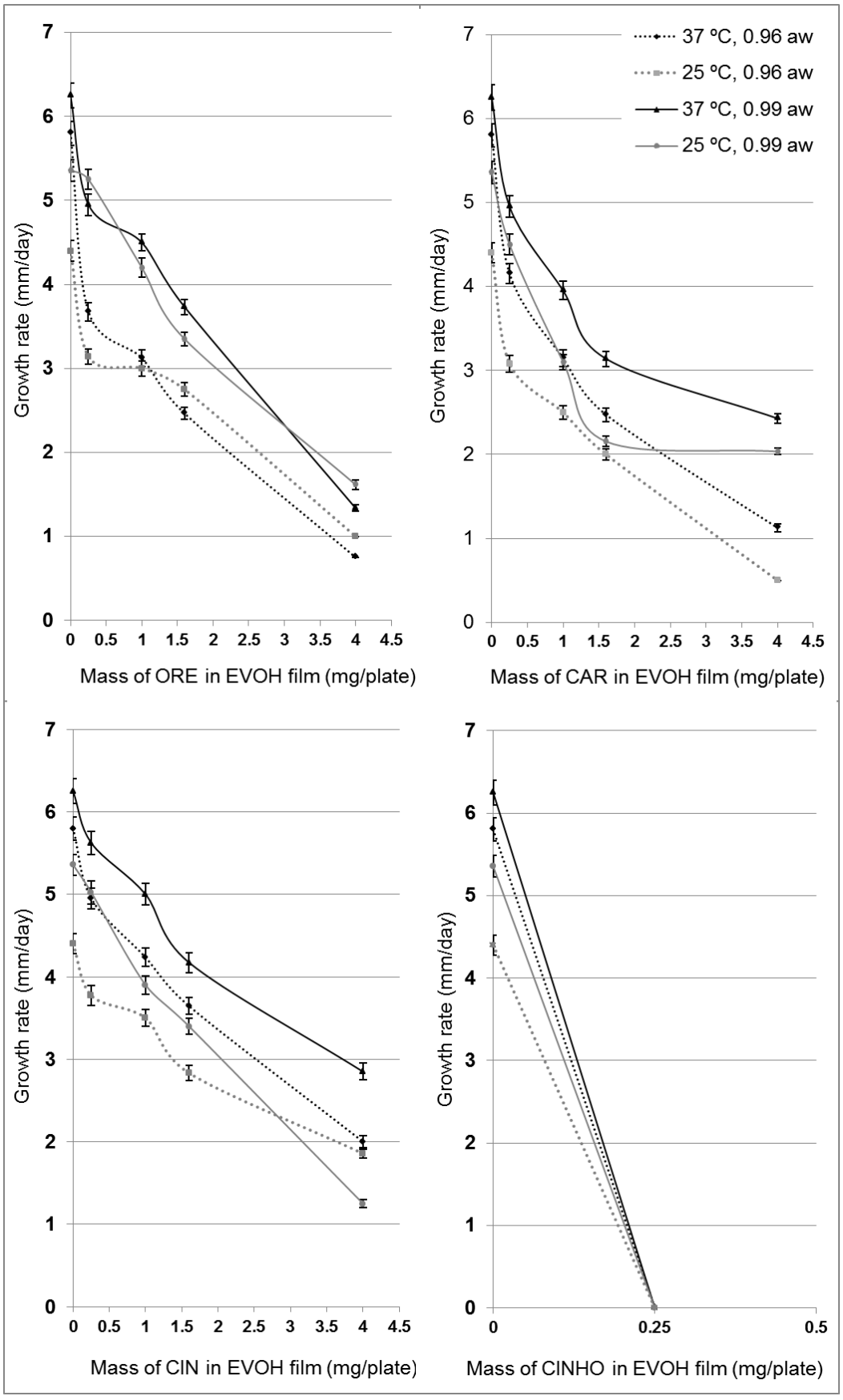

Figure 2

Figure 2

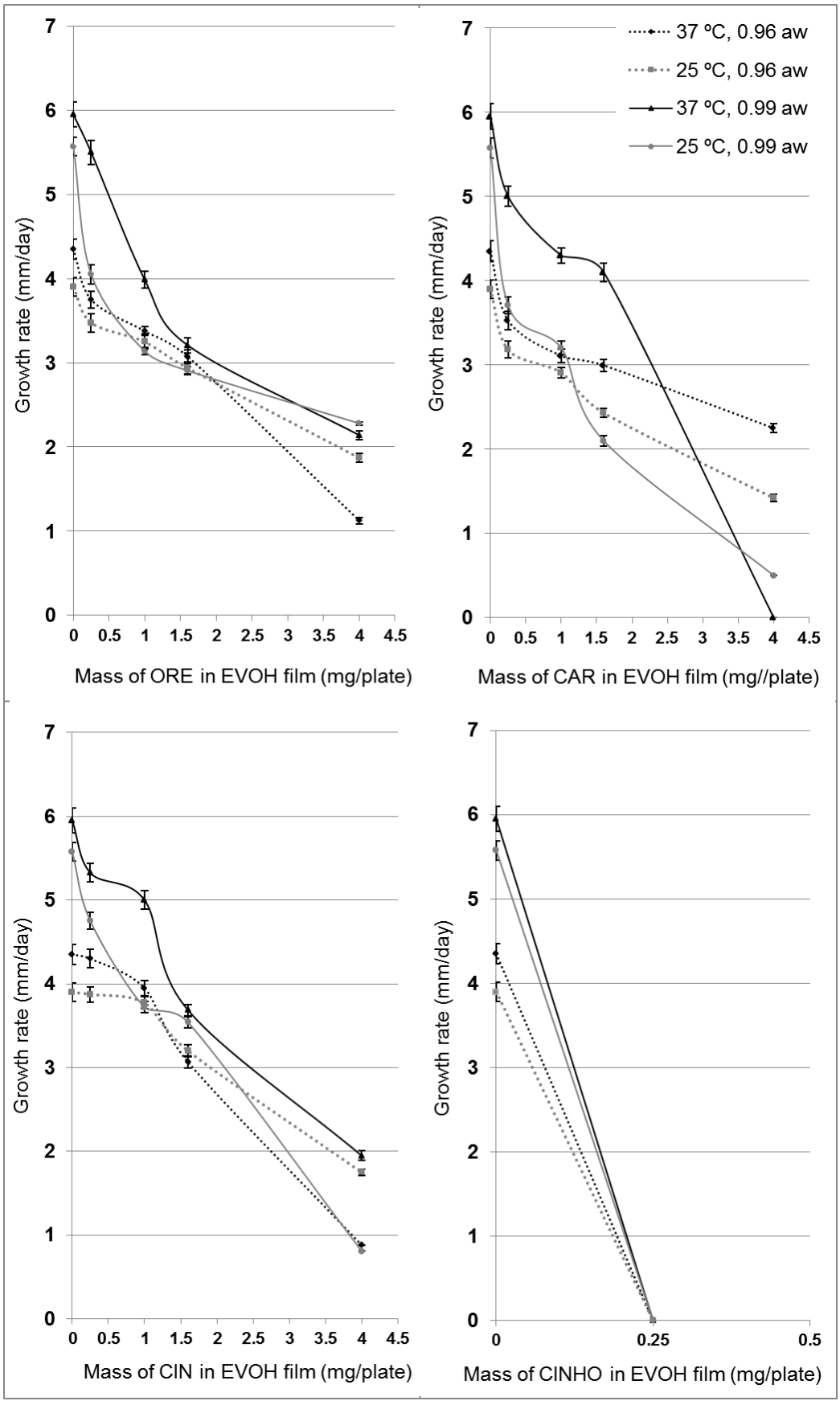

Figure 3

Figure 3.

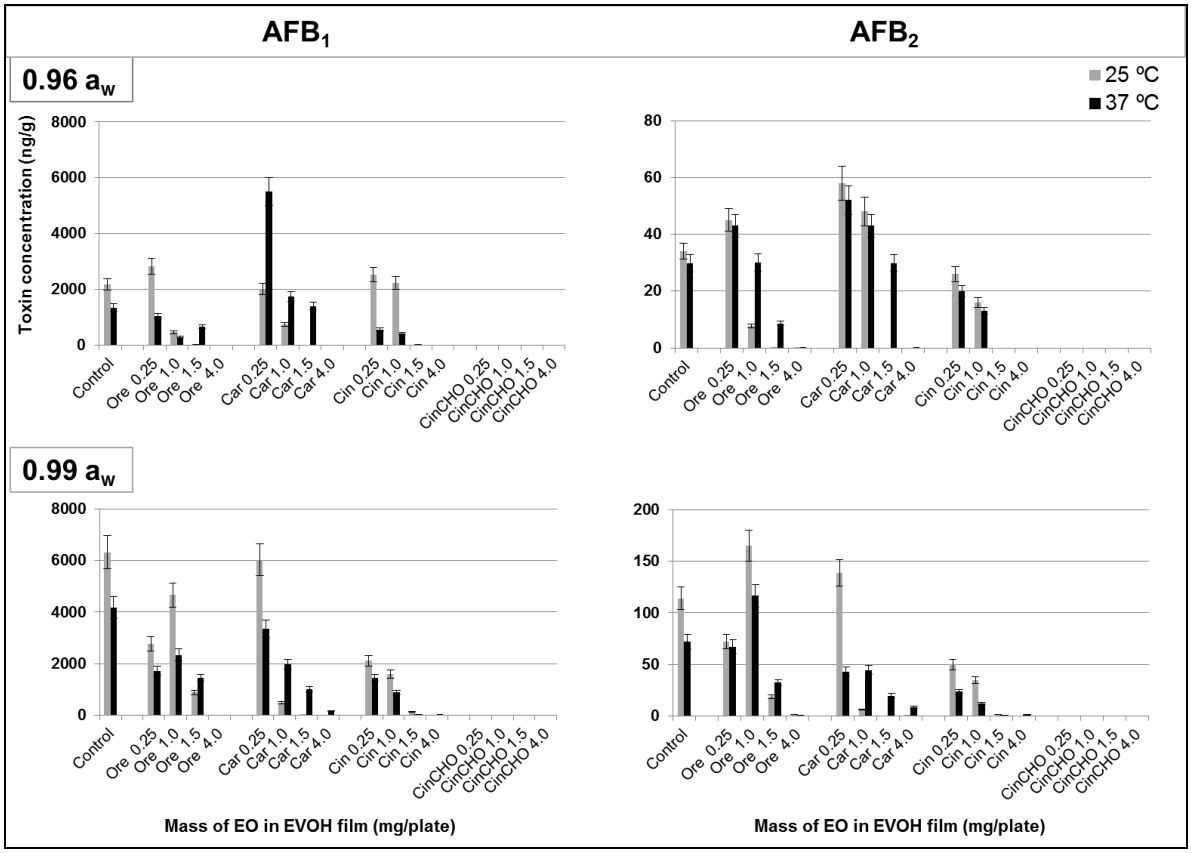

Figure 4

Figure 4.