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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. 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 film 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 regimens. 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 °C than at 25 °C. However, the contrary was found with regard to AF production. The order of efficacy of EVOH-EO films to control growth of both species and AF production was 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.

1. Introduction

Moulds are responsible for considerable economical losses around the world. Many of them are spoilage agents of agricultural products both 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),

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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. This percentage may rise 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 its 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 films that are effective against aflatoxigenic fungi and aflatoxin production 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 was determined and ii) the effect of these bioactive films to control the production and accumulation of AFB_1, AFB_2, AFG_1, and AFG_2 in the medium under the assayed conditions was 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 (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 ascertain 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). This 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. A helium (He) 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.