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Risk management of ochratoxigenic fungi and ochratoxin A in maize grains by bioactive EVOH films containing individual components of some essential oils

Andrea Tarazona[a], José V. Gómez[a], Rafael Gavara[b], Rufino Mateo-Castro[c], José V. Gimeno-Adelantado[c], Misericordia Jiménez[a], Eva M. Mateo[d,*]

[a] Department of Microbiology and Ecology, University of Valencia, Dr. Moliner 50, 46100 Burjassot, Valencia, Spain
[b] Packaging Lab, Institute of Agrochemistry and Food Technology, CSIC, Avenida Agustín Escardino 7, 46980 Paterna, Valencia, Spain
[c] Department of Analytical Chemistry. University of Valencia, Dr. Moliner 50, 46100 Burjassot, Valencia, Spain
[d] Microbiology Service, Hospital Clínico Universitario, Institute for Research INCLIVA, Av. Menéndez y Pelayo 4, 46010, Valencia, Spain

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ABSTRACT

Aspergillus steynii and *Aspergillus tubingensis* are possibly the main ochratoxin A (OTA) producing species in *Aspergillus* section *Circumdati* and section *Nigri*, respectively. OTA is a potent nephrotoxic, teratogenic, embryotoxic, genotoxic, neurotoxic, carcinogenic and immunosuppressive compound being cereals the first source of OTA in the diet. In this study bioactive ethylene-vinyl alcohol copolymer (EVOH) films containing cinnamaldehyde (CINHO), linalool (LIN), isoeugenol (IEG) or citral (CIT) which are major components of some plant essential oils (EOs) were produced and tested against *A. steynii* and *A. tubingensis* growth and OTA production in partly milled maize grains. Due to the favourable safety profile, these bioactive compounds are considered in the category "GRAS". The study was carried out under different water activity (0.96 and 0.99 a_w), and temperature (24 and 32 °C) conditions. ANOVA showed that class of film, fungal species, a_w and temperature and their interactions significantly affected growth rates (GR), ED_{50} and ED_{90} and the doses for total fungal growth inhibition and OTA production. The most effective EVOH films against both species were those containing CINHO. ED_{50}, ED_{90} and doses for total growth and OTA inhibition were 165–405, 297–614, 333–666 μg of EVOH-CINHO/plate (25 g of maize grains), respectively, depending on environmental conditions. The least efficient were EVOH-LIN films. ED_{50}, ED_{90} and doses for total growth and OTA inhibition were 2800– > 3330, > 3330 and > 3330 μg of EVOH-LIN/plate (25 g of maize grains), respectively. The effectiveness of the bioactive films increased with increasing doses. Overall, *A. tubingensis* was less sensitive to treatments than *A. steynii*. Depending on the species, a_w and temperature affected GR and OTA production in a different way. In *A. steynii* cultures, optimal growth occurred at 0.96 a_w and 32 °C while optimal OTA production happened at 0.99 a_w and 32 °C. In *A. tubingensis* cultures optimal growth happened at 0.99 a_w and 32 °C, although the best conditions for OTA production were 0.99 a_w and 24 °C. Thus, these species can be very competitive in warm climates and storage conditions. The EVOH-CINHO films followed by EVOH-IEG and EVOH-CIT films, designed in this study and applied in vapour phase, can be potent antifungal agents against *A. steynii* and *A. tubingensis* and strong inhibitors of OTA biosynthesis in maize grains at very low doses. This is the first study on the impact that interacting environmental conditions and bioactive films containing individual components of EOs have on the growth of these ochratoxigenic fungi and on OTA production in maize grains.

1. Introduction

Maize (*Zea mays* L.) is the world's largest cultivated cereal crop. The major contributors to maize grains spoilage and mycotoxin contamination are species of the genera *Aspergillus*, *Fusarium* and *Penicillium* (Pitt and Hocking, 2009), although their distribution is closely linked to the climate of the region. Several mycotoxins have been detected in maize and by-products in different countries, among them ochratoxin A (OTA) occupies a relevant position (Duarte et al., 2010a; Pereira et al., 2014). OTA is also frequently found in other cereals such as wheat, barley or rice (Kara et al., 2015; Lai et al., 2015; Mateo et al., 2011a), beer and wine (Mateo et al., 2007) and in many

* Corresponding author.
E-mail address: Emateo@incliva.es (E.M. Mateo).

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basic foods of the diet such as grapes (Covarelli et al., 2012), bread (Duarte et al., 2010b), coffee (Benites et al., 2017), cocoa (Copetti et al., 2013), dried fruits (Bircan, 2009) and spices (Prelle et al., 2014), among others. Due to its slow elimination from the body and its permanence in blood (Medina et al., 2010), OTA intake can lead to prolong internal exposure. OTA acts as a potent nephrotoxin (kidney is the primary target organ) (JECFA, 2008). It also exhibits teratogenic, embryotoxic, genotoxic, neurotoxic and immunosuppressive effects (affecting both humoral and cell-mediated immunity) (Gan et al., 2017) and it has been classified as a possible human carcinogen (group 2B) by the International Agency for Research in Cancer (IARC, 1993).

In warm climates the majority of OTA-producing species in maize, in pre- and post-harvest, are *Aspergillus* spp. included in the sections *Circumdati* and *Nigri*. Although *A. ochraceus* has been considered the most important OTA producer in cereals in these regions for a long time, new *Aspergillus* spp. in section *Circumdati* have been described by DNA based methods and they are also able to produce OTA, in particular *A. steynii* and *A. westerdijkiae* (Frisvad et al., 2004). *A. steynii* has been reported as the main OTA producing species within the section with a 90% of producing strains (Gil-Serna et al., 2011). The second ochratoxigenic *Aspergillus* spp. in cereals are members of *Aspergillus* section *Nigri*. *A. niger* has been considered the most important OTA-producing species (in this section) in maize in different countries (Magnoli et al., 2007; Xing et al., 2017). However, since our group described for the first time the ability of *A. tubingensis* to produce OTA (Medina et al., 2005), ochratoxigenic isolates of this species have been frequently detected in grapes (Chiotta et al., 2013; Medina et al., 2005; Perrone et al., 2006; Selouane et al., 2009). More recently, it has been reported that *A. tubingensis* is the predominant ochratoxigenic species of the *Aspergillus* section *Nigri* in maize from USA and Italy (Susca et al., 2014). Although similar studies by molecular methods are required to know the real incidence and distribution of *A. steynii* and *A. tubingensis* in cereals and other crops in the world, currently, *A. steynii* and *A. tubingensis* are already recognized as target ochratoxigenic species that must be controlled.

Fungal growth and OTA biosynthesis, depend mainly on the species and isolate, substrate, environmental conditions (temperature and water activity) (Gil-Serna et al., 2015a, 2015b; Khalesi and Khatib, 2011), treatments with antifungal agents and possible interactions between all these factors. Several chemical fungicides have been applied to prevent and control the growth of toxigenic fungi and production of mycotoxins (Bendaha et al., 2011; Mateo et al., 2011b, 2011c, 2013, 2017a). Nevertheless, this strategy, though effective, is not without risk in practice (da Luz et al., 2017). The application of chemical fungicides increases the risk of toxic residues in foods and often leads to fungal resistance. In order to reduce the utilization of these compounds, alternative treatments are being studied (Ribeiro-Santos et al., 2017; Zhu et al., 2016).

Essential oils (EOs) from plants and their individual constituents are natural substances categorized as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration (FDA, 2016). They have been the focus of extensive research not only for being natural products but also because they have demonstrated benefits in human health and food preservation such as anti-tumour, analgesic, anti-diabetic, anti-inflammatory, insecticidal, antioxidant, and antimicrobial properties (Periasamy et al., 2016; Yen et al., 2015). EOs are bioactive compounds able to control mold growth and numerous reports and some reviews about this topic have been published (da Cruz Cabral et al., 2013; Prakash et al., 2015). Most of the studies have been carried out by contact methods such as agar disk diffusion assay (different modifications of the method commonly known as Kirby Bauer test), agar well diffusion assay, broth dilution method or the poisoned food technique (Das et al., 2010). This methodological variety together with the high volatility of the EOs makes comparative analysis difficult. In addition, the studies have been focused on spoilage fungi and too little attention has been paid to ochratoxigenic fungi and OTA production (Lappa et al., 2017; Passone et al., 2012).

EOs have the potential advantage of being bioactive in their vapour phase, a characteristic that makes them attractive as possible fumigants of stored products or as active ingredients in food packaging in order to be released during transportation and/or storage of food. This reduces the possibility of undesirable flavour development compared to their direct addition into food and improves their effectiveness (Ribeiro-Santos et al., 2017). Consequently, in line with the rapid technological advances, active packaging containing EOs to control fungi have emerged (Nguyen Van Long et al., 2016). In these studies, the antifungal effect of active packaging is analysed but in very little cases mycotoxin biosynthesis is considered (Manso et al., 2014; Mateo et al., 2017b). No study on *A. steynii* and *A. tubingensis* growth and OTA production in relation to EOs or active packaging systems containing EOs has been reported so far.

The antifungal activity of the EOs is mainly related to their chemical structure, concentration and interactions with the matrix, substrate and the environmental conditions. Ethylene-vinyl alcohol copolymer (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). EVOH is composed of two segment chains: one, olefinic and hydrophobic, comes from ethylene, and the other, with a hydroxyl substituent, presents hydrophilic behaviour. It is a packaging material used to provide high oxygen barrier properties and their hydrophilic nature makes it very sensitive to water. These properties combined with appropriate EOs, or their individual constituents, could make bioactive EVOH films an effective and sustainable strategy in the management of risks from ochratoxigenic fungi (aerobic organisms) and OTA in food and feed.

The aims of this study were to develop effective bioactive films for controlling *A. steynii and A. tubingensis* growth and OTA production in maize grains and, by extension, in other possible food and feed. For this purpose: i) bioactive EVOH films (29% ethylene molar content) incorporating cinnamaldehyde, linalool, isoeugenol or citral were prepared, ii) the ability of the designed active films to reduce/inhibit the growth of *A. steynii* and *A. tubingensis* in partly milled maize grains under different environmental conditions was determined and iii) the effect of these active films to inhibit OTA accumulation in the seeds under all the assayed conditions was tested.

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). Cinnamaldehyde (3-phenyl-2-propenal) (CINHO) [PubChem CID 637511], linalool (LIN) [PubChem CID 6549], isoeugenol (IEG) [PubChem CID 853433] and citral (CIT) [PubChem CID 638011] were supplied by Sigma-Aldrich (Barcelona, Spain).

In this study, films of EVOH containing CINHO, LIN, IEG and CIT, labelled as EVOH-CINHO, EVOH-LIN, EVOH-IEG and EVOH-CIT, and control films (in absence of active substances) were obtained by casting in an oven at 75 °C for 15 min. The films were made following the methodology described in Mateo et al. (2017b). 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 (1, 2, 5 and 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