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Aspergillus steynii and *Aspergillus westerdijkiae* as potential risk of OTA contamination in food products in warm climates

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ABSTRACT

Aspergillus steynii and *Aspergillus westerdijkiae* are the main ochratoxin A (OTA) producing species of *Aspergillus* section *Circumdati*. Due to its recent description, few data are available about the influence of ecophysiological factors on their growth and OTA production profiles. In this work, the effect of temperature (20, 24 and 28 °C) and water activity (a_w) (0.928, 0.964 and 0.995) on growth, sporulation and OTA production by these fungi was examined in CYA and media prepared from paprika, green coffee, anise, grapes, maize and barley. Growth was positively affected by the highest temperature and a_w values indicating that both species might be expected in warm climates or storage conditions. However, optimal growth conditions showed differences depending on the medium. OTA production was markedly affected by substrate and showed qualitative and quantitative differences. Both species, especially *A. steynii*, represent a great potential risk of OTA contamination due to their high production in a variety of conditions and substrates, in particular in barley and paprika-based media. Additionally, neither growth nor sporulation did result good indicators of OTA production by *A. steynii* or *A. westerdijkiae*; therefore, specific and highly-sensitive detection methods become essential tools for control strategies to reduce OTA risk by these species.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by different fungal species. These compounds are considered a high risk to human diet representing the larger group of alert notifications which arrived to the European Rapid Alert System for Food and Feed (RASFF) in 2011 (RASFF, 2012). One of the most important mycotoxins is ochratoxin A (OTA) due to its high toxicity towards both animals and humans and presents nephrotoxic, inmunotoxic and teratogenic properties (Pfohl-Leszkowicz and Manderville, 2007). The International Agency for Research on Cancer (IARC, 1993) classified this toxin as a possible human carcinogen (group 2B). Traditionally, OTA has been considered an important contaminant in different dietary products commonly consumed by humans like cereals and cereal products, coffee, grapes and grape-products (Duarte et al., 2010; Romani et al., 2000; Varga and Kozakiewicz, 2006). However, recent studies regarding OTA incidence have reported its presence in an increasing number of substrates among which nuts and dried fruits, spices, cocoa and chocolate or liquorice are considered the most relevant (Pietri et al., 2010; Serra, 2004; Zaied et al., 2010). The maximum OTA levels in all these products are strongly regulated in the European Union (European Commission, 2006, 2010).

The majority of OTA-producing species are included in the genus *Aspergillus*. Several species of *Aspergillus* section *Circumdati* are capable of producing OTA. Although *Aspergillus ochraceus* used to be considered the most important OTA producer, particularly in warm climates, new species of *Aspergillus* section *Circumdati* have been described and they are also able to produce OTA, in particular *Aspergillus steynii* and *Aspergillus westerdijkiae* (Frisvad et al., 2004). In a recently published work carried out in our group (Gil-Serna et al., 2011), *A. steynii* has been reported as the main OTA-producing species within the section with a 90% of producing strains and toxin levels 1000 times higher than *A. ochraceus*, followed by *A. westerdijkiae* with a 75% of producing isolates and production levels 100 times higher than *A. ochraceus*. Regarding to

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the percentage of producing strains, *A. steynii* is also the most important species (90%), followed by *A. westerdijkiae* (75%) and finally *A. ochraceus* with only a 22% of producing isolates (Gil-Serna et al., 2011). Due to their recent description, the presence of these species has been reported in few food matrices although currently they seem to be more frequent than *A. ochraceus*. *A. westerdijkiae* and *A. steynii* occurrence has been described in different products including coffee (Leong et al., 2007; Noonim et al., 2008), grapes (Díaz et al., 2009), paprika (Santos et al., 2011) or barley (Mateo et al., 2011).

Mycotoxin contamination of foodstuffs is very difficult to predict because it depends on a wide range of ecophysiological factors either intrinsic or extrinsic. D'Mello and MacDonald (1997) and Khalesi and Khatib (2011) suggested that the main factor affecting fungal growth and OTA production is water activity (a_w) followed by temperature, although the effect of substrate should not be disregarded. All these factors are always interacting in the environment; therefore, fungal ability to grow and produce mycotoxins is affected by a complex combination of parameters. Moreover, optimum conditions for fungal growth are usually different from those for mycotoxin production.

The effect of ecophysiological factors on *A. ochraceus* has been extensively studied. Both a_w and temperature have a great influence on this fungus and its optimal intervals to grow and produce OTA were established between 25 and 30 °C and a_w 0.95–0.99 (Pardo et al., 2005, 2006a, 2006b; Ramos et al., 1998). The ability of *A. ochraceus* to produce OTA depends considerably on the substrate on which it is growing. For instance, Pardo et al. (2006b) indicated that its capacity of producing OTA was higher on green coffee beans than on barley.

Up to date, there are few data available regarding the effect of ecophysiological factors on *A. westerdijkiae* and *A. steynii.* Abdel-Hadi and Magan (2009) studied the effect of a_w and temperature on growth, sporulation and OTA production by these species in YES medium. In a recently published work, Wawrzyniak et al. (2013) studied the effect of storage conditions on *A. westerdijkiae* growth in stored barley.

The results obtained from culture media prepared from infusions of selected food products have been considered so far a reasonable and good approximation to the growth and toxin production patterns obtained in natural substrates (Pardo et al., 2005). Furthermore, Ramos et al. (1998) found similar patterns of OTA production in media prepared from infusions of barley and directly on cereal grains.

The aim of this work was to examine the effect of substrate composition, temperature and a_w on fungal growth and OTA production by *A. steynii* and *A. westerdijkiae*. For this purpose, two strains of each species from different origin were cultured in a commonly used medium (CYA) and on matrix-based media prepared from paprika, grapes, coffee, barley, anise and maize.

2. Materials and methods

2.1. Fungal strains

Two strains of *A. westerdijkiae* were used in this study; the type species CECT 2948 and 3.58 isolated from grapes. Likewise, two isolates of *A. steynii* were evaluated in this work Aso2 and 3.53, isolated from grapes and coffee, respectively. These strains were kindly provided by Dr Sanchis (University of Lleida, Spain) and Dr Jimenez (University of Valencia, Spain). The correct identification of all the isolates was confirmed using species-specific PCR assays according to Gil-Serna et al. (2009). They were maintained by regular subculturing on Potato Dextrose Agar (PDA, Pronadisa, Spain) at 25 ± 1 °C for 4–5 days and then stored at 4 °C until required and as spore suspension in 15% glycerol at −80 °C.

2.2. Media

The assays were carried out in CYA medium (Czapek Yeast Agar) and using different matrix-based media. Paprika (*Capsicum annuum*, Spain), green coffee (*Coffea arabica*, Brazil), white grape (*Vitis vinifera*, var. Vinalopo, Spain), anise (*Pimpinella anisum*, Spain), barley (*Hordeum vulgare*, Spain) and maize (*Zea mays*, Spain) were used to prepare the media. In all these food products, *A. westerdijkiae* and/or *A. steynii* have isolated so far or these species have been detected directly on them by our group using specific PCR protocols (Díaz et al., 2009; Leong et al., 2007; Mateo et al., 2011; Noonim et al., 2008; Santos et al., 2011).

The matrix-based media used contained 3% (w/v) of each matrix with 20 g/l of agar (Pronadisa, Spain). They were prepared by boiling 30 g of dry grounded matrix in 1 l of distilled water for 30 min. Subsequently, the mixture was filtered through a double layer of muslin and the volume was adjusted up to 1 l. Water activity was modified with glycerol, a non-ionic solute, up to 0.928, 0.964 and 0.995 (Dallyn and Fox, 1980).

2.3. Inoculation, incubation and measurement of growth

Fungal conidia suspensions were prepared from sporulating cultures (7 day-old) on Czapek-Dox Modified Agar (Pronadisa, Spain) and filtered through Whatman N° 1 paper. Concentrations were measured by microscopy using a Thoma counting chamber and the suspensions were diluted up to a final concentration of 10^7 spores/ml. Two microlitres of these suspensions were placed in the centre of the plates prepared as described above and they were incubated at 20, 24 and 28 °C. Each strain was inoculated in two independent plates in each medium, a_w and temperature condition.

The diameter of the colonies was measured in two directions at right angles to each other after 10 days of incubation and the average of these two values was used as indicator of fungal growth.

2.4. OTA evaluation

OTA was extracted from the plates after 10 days of incubation as described elsewhere (Bragulat et al., 2001). Three agar plugs were removed from different points of the colony and extracted with 1 ml of methanol. OTA was measured in the extracts by High Performance Liquid Chromatography (HPLC) on a reverse phase C18 column (Tracer Extrasil ODS2; 5 μm, 4.6 mm × 250 mm; Teknokroma, Barcelona, Spain) at 45 °C in a Perkin Elmer Series 200 HPLC system coupled with a fluorescence detector (Perkin Elmer, Massachusetts, USA) at excitation and emission wavelengths of 330 and 470 nm respectively. The mobile phase contained monopotassium phosphate 4 mM pH 2.5 and methanol (33:67) and the flow rate was 1 ml/min. OTA was eluted and quantified by comparison with a calibration curve generated from OTA standards (OEKANAL®, Sigma–Aldrich, Steinheim, Germany).

2.5. Sporulation analysis

Three plugs (diameter 5 mm) adjacent of those used for OTA evaluation were removed from each plate and introduced in 2 ml tubes containing 1 ml of saline solution (9 g/l sodium chloride). Spores were separated mechanically from the medium by vortexing and spore concentrations in the extracts were measured by microscopy using a Thoma counting chamber. Spore concentrations