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Gene expression analysis to predict aflatoxins B_1 and G_1 contamination in some plant origin foods

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ABSTRACT

Maize, peanuts and wheat are exposed to colonisation by *Aspergillus flavus* and *Aspergillus parasiticus,* which are mould species able to produce aflatoxins B_1 (AFB_1) and G_1 (AFG_1). New tools based on molecular data can be used to obtain critical information to prevent AFs accumulation in these crops. The objective of this study was to analyse the time course of the relative expression of the *aflR*, *aflS* and *aflP* genes by reverse transcription real-time PCR (RT-qPCR) in relation to AFs production by *A. flavus* and *A. parasiticus* on maize, peanuts and wheat under controlled conditions during nine days of incubation. Different gene expression profiles for the three target genes were observed in maize, peanut and wheat. In general, AFB_1 amounts produced by both species were increased over incubation time, although *A. parasiticus* produced higher AFB_1 quantities than *A. flavus* in peanuts and wheat. Regarding AFG_1 production, quantities were higher at the end of the incubation time but lower than AFB_1 amounts. High relationships were found between gene expression by day 4 and AFs production by days 7 and 9. Thus, gene expression data allow predicting early AFs contamination of maize and wheat. In the case of peanuts further studies are needed.

1. Introduction

Maize (*Zea mays L.*), peanut seeds (*Arachis hypogaea*) and wheat (*Triticum* spp.) are staple foods worldwide and have become ones of the most important products in the economy of many countries of the world (Pechanova & Pechan, 2015). They are prone to infection by filamentous fungi at both pre- and post-harvest stages, leading to mycotoxin contamination. According to the Food and Agricultural Organization (FAO), 25% of the abovementioned crops are contaminated with mycotoxins around the world (Waliyar et al., 2015). Mycotoxins are a chemically diverse group of secondary fungal metabolites which have some negative effects on consumer health. Among them, aflatoxins (AFs) have been reported in several agricultural crops, mainly maize, peanuts, and even in wheat and oat (Al-Sagga, Alian, Ismail, & Mohamed, 2015; Kara, Ozbey, & Kabak, 2015; Kuzdraliński, Kot, Szczerba, Nowak, & Muszyńska, 2017; Kuzdraliński, Solarska, & Mazurkiewicz, 2013; Kuzdraliński et al., 2017; Schatzmayr & Streit, 2013). These mycotoxins can occur in host crops infected by some species of *Aspergillus*, mainly *A. flavus* and *A. parasiticus* that are well adapted to warm and dry weather conditions (Al-Sagga et al., 2015; Payne & Brown, 1998). *A. flavus* toxigenic strains produce aflatoxins B_1 and B_2; while *A. parasiticus* produces B_1, B_2, G_1 and G_2. AFB_1 is classified by IARC as class 1A human and animal carcinogen and AFB_2 within group 2B, as probable human carcinogens. AFs are immunosuppressive, mutagenic, teratogenic and hepatocarcinogenic compounds (IARC, 2012).

Due to their toxicity and prevalence in plant origin foods, many of them processing crops, the European Union and many countries worldwide have strict maximum levels of contamination (The Commission of the European Communities, 2006, 2010). These limits have generated a high number of alerts in the Rapid Alert System for Food and Feed (RASFF) in the European Union (http://ec.europa.eu/food/safety/rasff_en) due to the detection of contaminated crop samples exceeding the level for AFs. This implies large economic losses to farmers and producers and a significant risk to the consumer health since these products and their derivatives are staple foods with high consumption.

The high stability of AFs in foods makes difficult their removal during food processing. Thus, crops can be colonised by *A. flavus* and *A. parasiticus* at either pre- or post-harvest stages. However, AFs contamination, mainly by the presence of AFB_1 and AFG_1, occurs during post-harvest conditions whether they are not quickly dried and/or

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maintained at proper storage conditions according to good agricultural practices (Prelle, Spadaro, Garibaldi, & Gullino, 2014). In this sense, poor hygienic conditions during storage, packaging and transport seem to encourage post-harvest mould growth and further AFs production (Santos, Marin, Sanchis, & Ramos, 2008). From a food safety point of view development of new tools, able to predict AFB_1 and AFG_1 contamination of maize, peanuts and wheat before they occur, could be a step forward in order to take preventive or corrective actions in the field and food industry for controlling AFs accumulation in agricultural crops. Since gene expression always precedes phenotypic production, the use of a molecular approach based on reverse transcription real-time PCR (RT-qPCR), could be useful to obtain critical information on the activation or inactivation of specific genes involved in AFB_1 and AFG_1 biosynthesis.

Among the genes involved in the AFs biosynthesis, the two regulatory genes (*aflR* and *aflS*) and the structural *aflP* gene have demonstrated to be necessary for production of these mycotoxins. The *aflR* gene, encodes a sequence-specific DNA-binding binuclear zinc cluster ($Zn(II)_2CyS_6$) protein, required for transcriptional activation of most of the structural genes, while the *aflS* gene encodes a transcription enhancer and interacts with the *aflR* gene, but its role in the AFs biosynthesis still remains unclear (Georgianna & Payne, 2009). The *aflP* gene, one of the structural genes activated by the *aflR* gene product, encodes an O-methyltransferase A or an O-methyltransferase II, that converts sterigmatocystin to O-methylsterigmatocystin or dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin (Yu et al., 2004). So far, only the relationship between the functioning of such genes and AFs production by *A. flavus* and *A. parasiticus* contaminating different food-based or synthetic culture media has been studied (Gallo, Solfrizzo, Epifani, Panzarini, & Perrone, 2016; Lozano-Ojalvo, Rodríguez, Bernáldez, Córdoba, & Rodríguez, 2013; Medina, Rodríguez, Sultan, & Magan, 2015; Schmidt-Heydt, Abdel-Hadi, Magan, & Geisen, 2009). However, time-course studies that relate changes in the relative expression of genes associated with AFs to phenotypic mycotoxin biosynthesis on maize, peanuts and wheat themselves have not been conducted yet. This is so important due to the expression of AFs-related genes is influenced by food composition (Luchese & Harrigan, 1993). Then, utilisation of the food matrix itself assures obtaining reliable data to be further used for controlling AFB_1 and AFG_1 production in the Hazard Analysis Critical Control Point (HACCP) programs of food industry.

The objectives of this study were: (a) to analyse the time course of the relative expression of the *aflR*, *aflS* and *aflP* genes and AFB_1 and AFG_1 production by *A. flavus* and *A. parasiticus* on maize, peanuts and wheat, and (b) to examine the relationship between the changes in relative expression of the *aflP*, *aflR* and *aflS* genes throughout incubation time and the phenotypic AFB_1 and AFG_1 production.

2. Materials and methods

2.1. Mould strains

One strain of *A. flavus* (CECT 2687) and one strain of *A. parasiticus* (CECT 2688) obtained from the Spanish Type Culture Collection (CECT) were used in this work. Both strains showed the ability to produce AFB_1 and aflatoxin G_1 (AFG_1) (only in the case of *A. parasiticus*) when incubated under permissive conditions at 25 °C for 5 days on Malt Extract Agar (MEA; Scharlab S.L., Spain).

The strains were maintained by regular subculturing on MEA at 25 °C for 7 days and then stored at 4 °C until required. The spore suspension was maintained in 100 mL L^{-1} glycerol (Scharlau Chemie S.A., Spain) at −80 °C.

2.2. Inocula preparation

The mould strains were initially cultured on MEA and incubated at 25 °C for 7 days. The spores were collected by flooding 3 plates of MEA with 5 mL of sterile nanopure water containing glycerol (100 mL L^{-1}), and rubbing the surface with a glass rod for removing conidia. The spore suspensions were filtered through Whatman paper No 1, diluted in 1 g L^{-1} sterile Peptone Water as necessary, and counted using a Thoma counting chamber Blaubrand® (Brand, Wertheim, Germany). The suspensions were adjusted to 10^6 conidia mL^{-1} and used as an inoculum. The spore suspensions were stored at −80 °C in glycerol solution (100 mL L^{-1}) and new starter cultures were used for each experiment.

2.3. Sample preparation and inoculation

Samples of about 2 g of non-sterile commercial maize kernels, peanuts seeds and wheat grains were aseptically transferred into 55 mm Ø Petri dishes and then exposed to 254 nm UV light for 10 min to eliminate any surface contamination (Samsudin, Rodríguez, Medina, & Magan, 2017). The a_w of the cereals and nuts was checked using a Novasina Lab Master water activity meter (Novasina AG, Lachen, Switzerland). Subsequently, all the treatments were equilibrated at 0.97 a_w. Maize, peanut and wheat in Petri dishes were placed separately in pre-sterilised oblong plastic chambers with a saturated K_2SO_4 solution (0.97 a_w) put at the bottom to maintain the equilibrium relative humidity (RH) during incubation. Equilibration was achieved by incubating for 24 h at 4 °C. The experimental chambers were subsequently allowed to equilibrate at the target temperature of 25 °C for 4 h. Maize kernels, wheat grains and peanut seeds were used as cereal and nut model systems.

Each food matrix was centrally inoculated with 200 μL of the inoculum which was spread on its surface with a sterile glass rod, and then the inoculated food was incubated at 25 °C for up to 9 days.

2.4. Sampling

Destructive sampling was performed at different incubation times depending on the sample processing. For gene expression analysis, samples were taken at 2, 4, 6, 7, 8 and 9 days of incubation, while for determining AFB_1 and AFG_1 production, samples were collected at 2, 4, 7 and 9 days of incubation. All experiments were made in triplicate and repeated once.

2.5. Gene expression studies

2.5.1. Sample preparation

After each incubation time, samples weighing around 1 g were collected under sterile conditions, quickly frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.5.2. RNA extraction

RNA was isolated according to a method previously optimised (Bernáldez, Rodríguez, Rodríguez, Sánchez-Montero, & Córdoba, 2017). The RNA obtained was eluted in 50 μL of the elution buffer (provided by *Spectrum Plant Total* RNA extraction kit, Sigma Aldrich Química S.A., Spain) and kept at −80 °C until use. The RNA concentration and purity (A_{260}/A_{280} ratio) was determined spectrophotometrically using a 1.5 μL aliquot on Nanodrop™ 200 UV-VIS Spectrophotometer (Thermo Scientific, Spain). Samples were diluted to 0.1 μg μL^{-1} and treated with DNAse I kit (Thermo Fisher Scientific, USA) to remove genomic DNA as described by manufacturer.

2.5.3. RT-qPCR reactions and relative quantification

2.5.3.1. Primers. Nucleotide sequences of primers used in this study are included in Table 1. The primer pairs F/R-omt, AflRTaq1/2 and AflSqPCR1/2 designed from the *aflP*, *aflR*, and *aflS* genes, involved in the AFs biosynthetic pathway (*target genes*) (Lozano-Ojalvo et al., 2013; Peromingo, Rodríguez, Delgado, Andrade, & Rodríguez, 2017; Schmidt-