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Interactions between water activity and temperature on the *Aspergillus flavus* transcriptome and aflatoxin B_1 production

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ABSTRACT

Effects of *Aspergillus flavus* colonization of maize kernels under different water activities (a_w; 0.99 and 0.91) and temperatures (30, 37 °C) on (a) aflatoxin B_1 (AFB_1) production and (b) the transcriptome using RNAseq were examined. There was no significant difference ($p = 0.05$) in AFB_1 production at 30 and 37 °C and 0.99 a_w. However, there was a significant ($p = 0.05$) increase in AFB_1 at 0.91 a_w at 37 °C when compared with 30 °C/0.99 a_w. Environmental stress effects using gene ontology enrichment analysis of the RNA-seq results for increasing temperature at 0.99 and 0.91 a_w showed differential expression of 2224 and 481 genes, respectively. With decreasing water availability, 4307 were affected at 30 °C and 702 genes at 37 °C. Increasing temperature from 30 to 37 °C at both a_w levels resulted in 12 biological processes being upregulated and 9 significantly downregulated. Decreasing a_w at both temperatures resulted in 22 biological processes significantly upregulated and 25 downregulated. The interacting environmental factors influenced functioning of the secondary metabolite gene clusters for aflatoxins and cyclopiazonic acid (CPA). An elevated number of genes were co-regulated by both a_w and temperature. An interaction effect for 4 of the 25 AFB_1 genes, including regulatory and transcription activators occurred. For CPA, all 5 biosynthetic genes were affected by a_w stress, regardless of temperature. The molecular regulation of *A. flavus* in maize is discussed.

1. Introduction

Crops are commonly colonized by a wide range of microorganisms, including fungi. The species of microorganisms present can be correlated with several abiotic factors. In warm and humid subtropical and tropical conditions maize is prone to infection by *Aspergillus flavus* and *A. parasiticus*, especially via insect damage during silking (Battilani et al., 2011; Magan and Aldred, 2007).

During colonization toxigenic strains of these fungi may secrete toxic secondary metabolites known as mycotoxins, among the most carcinogenic of which is aflatoxin. Aflatoxins are polyketide-derived carcinogenic and mutagenic secondary metabolites which are extremely hepatotoxic, immunosuppressive, and are associated with both acute and chronic toxicity in humans and animals. For these reasons, the IARC has classified aflatoxins, mainly aflatoxin B_1 (AFB_1), as a class 1A carcinogen to humans. Aflatoxins are heat stable and very difficult to destroy during processing. This has resulted in strict legislative limits in many parts of the world for aflatoxins and indeed other mycotoxins in a wide range of food and feedstuffs (European Commission, 2006).

A. flavus is a xerophilic fungus which has developed physiological mechanisms for adaptation to environmental stress factors allowing them to compete and often dominate other fungal communities (Magan, 2007; Nesci et al., 2004; Northolt et al., 1977). Their metabolic plasticity confers on them the ability to produce a battery of extracellular hydrolytic enzymes, secondary metabolites and volatiles that give them a competitive edge (Magan and Aldred, 2007). With regard to abiotic factors, temperature and water availability (water activity, a_w) and their interactions have been demonstrated to be the key factors modulating fungal growth and the production of secondary metabolites (Marín et al., 1998a, b; Schmidt-Heydt et al., 2009, 2010).

Different molecular approaches including reverse transcriptase real-time PCR (RT-qPCR) (Abdel-Hadi et al., 2010, 2012; Jurado et al., 2008; Marín et al., 2010a, b; Rodríguez et al., 2014) and microarrays (Schmidt-Heydt and Geisen, 2007) have been used to elucidate the relationship between these interacting environmental factors and *A. flavus* growth and AFB_1 production. The genes involved in biosynthesis

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of aflatoxins are clustered together on the genome and the expression of the key regulatory (*aflR*; *aflS*) and related structural genes (e.g. *aflD*) have been shown to be influenced by interacting conditions of a_w × temperature. Indeed, Schmidt-Heydt et al. (2010) showed that the ratio of *aflR/aflS* expression was significantly correlated with the amounts of AFB_1 produced. The recent determination of the whole genome sequence and annotation of *A. flavus* NRRL 3357 (Chang et al., 2014; Nierman et al., 2015) has enabled the use of RNA-sequencing (RNA-seq) to interrogate the whole transcriptome of *A. flavus*. This could provide useful functional information on the role of different gene groups in tolerating interacting abiotic stresses. Recently Medina et al. (2015a, b) showed that the expression of sugar transporter genes were significantly changed under a_w × temperature stress. RNA-seq technology has been used in several recent studies of the effect of different abiotic factors such as 5-azacytidine (Lin et al., 2013; Wilkinson et al., 2011), decanal (Chang et al., 2014), a_w (Zhang et al., 2014), temperature (Yu et al., 2011) and resveratrol (Wang et al., 2015a, b) on aflatoxin biosynthesis and mycelial development of *A. flavus*. The majority of these studies have involved addressing only single abiotic factors in defined synthetic broth or semi-solid media. None of these studies have examined the use of RNA-seq for examining the impact of colonization of maize grain under different a_w × temperature conditions by *A. flavus* and the impact on changes in the transcriptome and the effect on phenotypic AFB_1 production.

The objective of the present study was to examine the impact of a_w × temperature (0.99/0.91 a_w and 30/37 °C) on *A. flavus* (NRRL 3357) colonization of stored maize grain to determine effects on (a) the whole genome including the aflatoxin biosynthesis gene cluster using RNA-seq and (b) effects on AFB_1 production.

2. Materials and methods

2.1. Fungal strain

The type *A. flavus* strain (NRRL 3357) obtained from the Southern Regional Research Centre, New Orleans, LA, USA was used in this study. The strain has been previously used for molecular ecology studies (Abdel-Hadi et al., 2010; Abdel-Hadi et al., 2012) with consistent results. Spore stocks were stored at 4 °C or sub-cultured on Malt Extract agar (MEA; CM59, Oxoid LTD., Basingstoke, UK) when required.

2.2. Modification of maize grain water activities

Undamaged French feed maize kernels were used in this study. The a_w of the maize kernels was 0.64 ± 0.02. A water adsorption curve was made by adding known amounts of water to 10 g subsamples, allowing the seeds to equilibrate at 4 °C for 48 h and then measuring the a_w (25 °C, Aqualab 3 TE, Decagon Devices, Pullman, Washington, USA) and moisture content (117 °C, 24 h). The adsorption curve of the amount of water added against a_w was plotted and used to determine the exact amounts required to modify the maize grain to the two water availability treatments: 0.91 and 0.99 a_w.

The a_w of the maize was modified by addition of the required water from the moisture adsorption curve minus 200 µl and equilibrated at 4 °C for 48 h in sealed plastic chambers. The maize grains (15 g) were placed in glass culture vessels containing a microporous lid which allows for moisture and air exchange (Magenta, Sigma Ltd., UK). Subsequently, 200 µl of spore suspension (approx. 10^6 spores/ml) was added to make up the predetermined amounts of water required and thoroughly mixed. The inoculated vessels together with un-inoculated controls were placed in plastic environmental chambers and enclosed with a lid. In each plastic chamber, 2 glass jars (500 ml) containing glycerol-water solutions appropriate to maintaining the equilibrium relative humidity at the target a_w level. The chambers were incubated at 30 and 37 °C for 10 days. The glycerol-water solutions were replaced with fresh solutions every 2 days during the incubation. Three replicates per treatment were used. At the end of the incubation period, samples were snap frozen using liquid N_2 and kept at − 80 °C until subsamples were used for RNA extraction and purification or dried and AFB_1 extraction and clean-up prior to HPLC analysis for quantification.

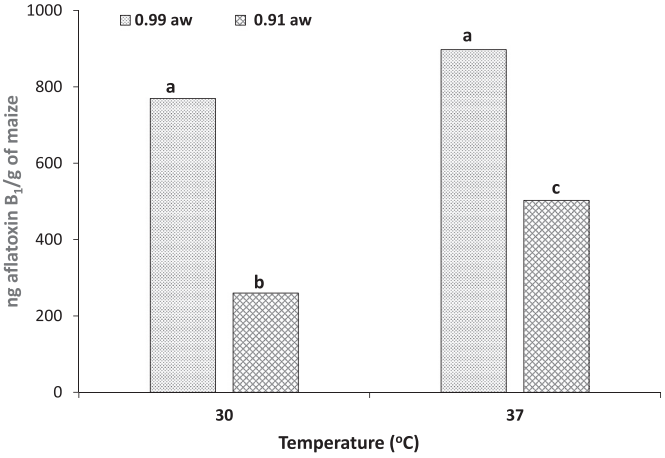

Fig. 1. Effect of interacting conditions of water activity (a_w) × temperature on aflatoxin B_1 production by *A. flavus* NRRL 3357 on maize grain stored for 10 days.

2.3. Aflatoxin analyses

2.3.1. Extraction of aflatoxins from maize

AFB_1 extraction was performed using AflaStar™® - Immunoaffinity Columns (IAC, Romer Labs Inc., MO, USA), following the manufacturers' protocol. Briefly, 5 g of the sample were dried overnight at 80 °C and stored at room temperature. The samples were ground, 4 g weighed into a 50 ml falcon tube and 16 ml of a solution of methanol:water (60/40 v/v) added. The samples were shaken for 1 h at room temperature, and then filtered through qualitative filter paper (QL 110, Fisher Scientific UK Ltd., Loughborough, UK). The extract (1 ml) was diluted in a falcon tube (15 ml) with 9 ml of 1 × PBS buffer (0.05 M/0.15 M NaCl, pH 7.4, Fisher Bioreagents®, Fisher Scientific UK Ltd., Loughborough, UK) and the pH checked with pH strips. The diluted extract was applied to the IAC, and allowed to drip through. After further cleaning, 3 ml of Methanol (HPLC grade) was used to elute the aflatoxins. The eluent was dried in the fume *cupboard for derivatisation*.

2.3.2. Preparation of standards

200 µl aflatoxin (R-Biopharm Rhône Ltd., Darmstadt, Germany) of stock solution comprising of 200 ng AFB1 was prepared. The stock solution was pipetted into 2 ml Eppendorf tubes and left to evaporate to dryness overnight inside a fume cupboard, and thereafter derivatised.

2.3.3. Derivatisation, detection and quantification of aflatoxins by HPLC

Firstly, 200 µl hexane was added to the residue followed by the addition of 50 µl triflouroacetic acid (TFA). The mixture was then vortexed for 30 s and then left for 5 min. Thereafter, a mixture of water:acetonitrile (9:1, v/v) was added and the entire contents of the tube were vortexed for 30 s, after which the mixture was left for 10 min to allow for thorough separation of layers. The hexane layer was discarded and the aqueous layer filtered through nylon syringe filters (13 mm × 0.22 µm; Jaytee Biosciences Ltd., Herne Bay, UK) directly into amber salinized 2 ml HPLC.

A reversed-phase Agilent 1200 series HPLC system with fluorescence detection was used to confirm the identity and quantify AFB_1. This consisted of an in-line degasser, auto sampler, binary pump and a fluorescence detector (excitation and emission wavelength of 360 and 440 nm, respectively). Separation was achieved through the use of a C_{18} column (Agilent Zorbax Eclipse plus C_{18} 4.6 mm × 150 mm, 3.5 µm particle size; Agilent, Berks, UK) preceded by guard cartridge with the