International Journal of Food Microbiology 217 (2016) 162–169

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Effect of temperature and water activity on gene expression and aflatoxin biosynthesis in *Aspergillus flavus* on almond medium

Antonia Gallo *[,1], Michele Solfrizzo, Filomena Epifani, Giuseppe Panzarini, Giancarlo Perrone

Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Amendola 122/O, 70126 Bari, Italy

ARTICLE INFO

Article history:
Received 4 August 2015
Received in revised form 15 October 2015
Accepted 25 October 2015
Available online 26 October 2015

Keywords:
Aspergillus flavus
Aflatoxin B_1
Almond
Temperature
Water activity
Biosynthetic genes

ABSTRACT

Almonds are among the commodities at risk of aflatoxin contamination by *Aspergillus flavus*. Temperature and water activity are the two key determinants in pre and post-harvest environments influencing both the rate of fungal spoilage and aflatoxin production. Varying the combination of these parameters can completely inhibit or fully activate the biosynthesis of aflatoxin, so it is fundamental to know which combinations can control or be conducive to aflatoxin contamination. Little information is available about the influence of these parameters on aflatoxin production on almonds. The objective of this study was to determine the influence of different combinations of temperature (20 °C, 28 °C, and 37 °C) and water activity (0.90, 0.93, 0.96, 0.99 a_w) on growth, aflatoxin B_1 (AFB_1) production and expression of the two regulatory genes, *aflR* and *aflS*, and two structural genes, *aflD* and *aflO*, of the aflatoxin biosynthetic cluster in *A. flavus* grown on an almond medium solidified with agar. Maximum accumulation of fungal biomass and AFB_1 production was obtained at 28 °C and 0.96 a_w; no fungal growth and AFB_1 production were observed at 20 °C at the driest tested conditions (0.90 and 0.93 a_w). At 20° and 37 °C AFB_1 production was 70–90% lower or completely suppressed, depending on a_w. Reverse transcriptase quantitative PCR showed that the two regulatory genes (*aflR* and *aflS*) were highly expressed at maximum (28 °C) and minimum (20 °C and 37 °C) AFB_1 production. Conversely the two structural genes (*aflD* and *aflO*) were highly expressed only at maximum AFB_1 production (28 °C and 0.96–0.99 a_w). It seems that temperature acts as a key factor influencing aflatoxin production which is strictly correlated to the induction of expression of structural biosynthesis genes (*aflD* and *aflO*), but not to that of aflatoxin regulatory genes (*aflR* and *aflS*), whose functional products are most likely subordinated to other regulatory processes acting at post-translational level.

The results of this study are useful to select conditions that could be used in the almond processing chain to suppress aflatoxin production in this important product.

1. Introduction

Aflatoxins (AF) are the most toxic and carcinogenic of the mycotoxins that contaminate agricultural products, posing a worldwide food safety issue (Bennett and Klich, 2003; Payne and Brown, 1998). In particular, there is a great concern about the risk derived from consumption of food and feed contaminated by aflatoxin B_1 (AFB_1), the most potent natural hepatocarcinogen that has been characterized to date (Groopman and Kensler, 2005; Wogan, 1992). Aflatoxins are produced by several species in *Aspergillus* Sect. *Flavi*, but *Aspergillus flavus* and *Aspergillus parasiticus* are the main species commonly implicated as global contaminants of a wide variety of commodities, even though some reports recently showed the importance of species such as *Aspergillus nomius*, *Aspergillus minisclerotigenes* and *Aspergillus arachidicola* as aflatoxin producers (Ehrlich et al., 2007; Pildain et al., 2008). Tree nuts are commodities with moderate to high risk of AF contamination as they are produced under environmental conditions favoring growth of aflatoxigenic fungi and toxin production (Arrus et al., 2005; Molyneux et al., 2007; Stroka et al., 2000). As with other tree nuts, including pistachios, Brazil nuts, walnuts and hazelnuts, almonds must comply with European Union legislative limits for aflatoxin contamination (8 μg/kg of AFB_1 and 10 μg/kg of total aflatoxins in nuts for direct human consumption). Thus, they are subject to the potential costs associated with the health hazards and economical losses of aflatoxin contamination.

In 2012 and 2013, the Rapid Alert System for Food and Feed (RASFF) reported a total of 525 alerts and 405 notifications for mycotoxins; 90% were for AFs, mostly nuts, nut products, and seeds. Among these, 12 notifications were for almonds, mainly from the USA and a few from Australia (Rapid Alert System for Food and Feed (RASSF), 2013).

* Corresponding author at: Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Prov.le Lecce-Monteroni, Lecce 73100, Italy.
E-mail addresses: antonia.gallo@ispa.cnr.it (A. Gallo), michele.solfrizzo@ispa.cnr.it (M. Solfrizzo), filomena.epifani@ispa.cnr.it (F. Epifani), giuseppe.panzarini@ispa.cnr.it (G. Panzarini), giancarlo.perrone@ispa.cnr.it (G. Perrone).
[1] Present address: Antonia Gallo, Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Prov.le Lecce-Monteroni, Lecce 73100, Italy.

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.10.026
0168-1605/

The almond tree (*Prunus dulcis*) is cultivated mainly in Mediterranean countries such as Portugal, Spain, Italy and France which have climatic conditions that are comparable to those of the almond growing areas of California (USA), Australia, South Africa, Chile and Argentina. Aflatoxin contamination of nuts occurs both pre-harvest, due to fungal infection in the field, and post-harvest if storage conditions are suitable for fungal development and aflatoxin production (Turner et al., 2005). However, fungal growth and mycotoxin production only occur under favorable conditions which vary for each species and crop. In particular, AF production is always strongly associated with high-carbohydrate and high-fat food because *Aspergillus* Sect. *Flavi* have a large diversity of enzymes able to degrade these substrates, including proteases, lipases, amylases and pectinases.

In almonds, the sources of contamination are usually from the soil, previously infected almonds and various pests. Mold spores can be carried by the pests and grow on damaged nutmeats. In addition to crop composition, temperature and water activity are the two key determinants in pre and post-harvest environments influencing both the rate of fungal spoilage and mycotoxin production (Magan and Aldred, 2007). There are numerous studies about the best conditions for fungal growth and toxin production; some contrasting results obtained are due to the diversity of strains and availability of nutrients and structure of media used (Gqaleni et al., 1996; Mayne et al., 1967; Schmidt-Heydt et al., 2010; Vaamonde et al., 2006). Generally, the ideal temperature for aflatoxin production is 28–30 °C, and production decreases at temperature below 25 °C and approaching 37 °C. Better fungal growth and toxin production are detected at higher water activity values. Growth and spore germination rates slow at water activity below 0.85 and are completely inhibited at values between 0.70 and 0.75 (Bhatnagar et al., 2006; Schmidt-Heydt et al., 2009). As reported above, the composition of substrate can also affect aflatoxin production by *A. flavus*. By varying the combination of the parameters involved in AF biosynthesis, toxin production can be completely inhibited or fully activated. It is therefore fundamental to know which combinations can control or be conducive to aflatoxin production in crops/substrates (Abdel-Hadi et al., 2012; Molina and Giannuzzi, 2002).

Recent studies have investigated the aflatoxin biosynthesis genes in response to the variation of temperature and water activity, in terms of activation rate and regulation. Expression profiling has been performed on aflatoxigenic species using microarray analyses (Abdel-Hadi et al., 2011; O'Brian et al., 2007; Schmidt-Heydt et al., 2009, 2010) or RNA seq technologies (Yu et al., 2011; Zhang et al., 2014). These studies have produced conflicting results on the role of *aflS* and *aflR* transcription in determining aflatoxin biosynthesis under variations of environmental conditions, probably due to the diversity of analysis methods used. In this regard, the effects of temperature and water activity on the production of aflatoxins by *A. flavus* have been widely studied but very little information is available about the influence of these parameters on aflatoxin production on almonds. The objective of this study was to determine the influence of different combinations of temperature (20 °C, 28 °C, and 37 °C) and water activity (0.90, 0.93,0.96, 0.99 a_w) on growth, AFB_1 production and expression of some genes of the aflatoxin biosynthetic cluster in *A. flavus* grown on almond medium solidified with agar. In particular, we focused on the expression of the two regulatory genes, *aflR* and *aflS*, and two structural genes, *aflD* and *aflO*, involved in early and late stages of the aflatoxin biosynthesis pathway, respectively, by using the reverse transcriptase quantitative PCR approach.

2. Material and methods

2.1. Fungal strain and culture conditions

The producing strain *A. flavus* ITEM 7828 (Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, CNR, Bari, Italy) was used for this work. An almond enriched medium was prepared by blending sterile water and 4% (wt/vol) ground almonds and then adding 2% (wt/vol) agar. The a_w in the medium was modified with the appropriate amount of glycerol: 14.26 mL in 100 mL of medium for 0.96 a_w; 21.46 mL in 100 mL for 0.93 a_w; 28.29 mL in 100 mL for 0.90 a_w; glycerol was not added to obtain 0.99 a_w. The medium was autoclaved and Petri plates (diameter 90 mm) were prepared with layers of cellophane membranes placed on top to keep mycelium separate from medium (Schmidt-Heydt et al., 2010; Zhang et al., 2014). Single point inoculations were made with 10^5 spores of *A. flavus* in 100 μL of water. Cultures were incubated at 20 °C, 28 °C, and 37 °C and different a_w values (0.99, 0.96, 0.93, 0.90) for a maximum of 15 days; the experiments were performed in triplicate for chemical and molecular analyses. The collection of mycelium grown on cellophane membranes started at different time points according to the first appearance of fungal colonies at the different conditions of water activity and temperature (3 days after inoculation at 20 °C, 2 days at 28 °C, and 1 day at 37 °C). Then the mycelium was harvested every 24 h until the 7th day of incubation, and after 10 and 15 days of growth at 20 °C and at 28 °C, whereas at 37 °C the last sampling was made after 10 days of incubation. For growth assessment, the fungal biomass was scraped from plates and weighed. For each plate, half of mycelium was frozen in liquid nitrogen and then stored at −80 °C prior to RNA extraction and the other half was stored at −20 °C prior to extraction for the determination of aflatoxin content.

2.2. Nucleic acid extraction and cDNA synthesis

DNA was isolated using the Fungal DNA miniprep kit (E.Z.N.A., Omega Bio-Tek Inc., Doraville, GA) according to the manufacturer's protocol. Total RNA was extracted from frozen mycelium pulverized in liquid nitrogen using the RNeasy kit and the RNase-Free DNase Set (Qiagen, Valencia, CA) to eliminate possible trace amounts of contaminating DNA, according to the manufacturer's protocol. For each time point, RNA was isolated from two plates for each of the three replicates. RNA aliquots were preserved at −80 °C. First strand cDNA was synthesized using about 1.5 μg of total RNA, oligo $(dT)_{18}$ primer, random hexamers and the SuperScript III Reverse Transcriptase (Invitrogen, San Diego, CA) according to the manufacturer's protocol.

2.3. Reverse transcriptase quantitative PCR (qRT-PCR)

The transcription profiles of four genes of aflatoxin biosynthesis cluster (*aflR*, *aflS*, *aflD*, *aflO*) and of *β-tubulin* gene, as reference gene, were analyzed by using reverse transcriptase quantitative PCR (qRT-PCR). The sequence of primers designed in this study is listed in Table 1. Amplifications were performed in a total reaction volume of 10 μL, in a MicroAmp Fast Optical 96-well reaction plate (Applied Biosystem, Warrington, UK). For each reaction 4.5 μL of 2.5× Real Master Mix SYBR-ROX (5 PRIME GmbH, Hamburg, DE) and different concentrations of primers pairs for each gene were added (Table 1). Real time reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystem, Warrington, UK) with the following

Table 1
Primers designed in this study.

Gene	Concentration	Primer code	Primer sequence (5′–3′)
aflR	100 nM	RT_aflR_for	CGGCACAGCTTGTTCTGAGT
		RT_aflR_rev	GCATCGTCTCCACCTTCTTG
aflS	150 nM	RT_aflS_for	CTGGCAAAACTTGGGAATGG
		RT_aflS_rev	CACGAGGAAACGGAGTGATG
aflD	50 nM	RT_aflD_for	GCGCAAGTTCCACTTTGAGA
		RT_aflD_rev	CCTTGGTCGCCCATATCAGT
aflO	50 nM	RT_aflO_for	GTGCGGTGGTGCAACTATTC
		RT_aflO_rev	TCTCTCGGCCAGGAAGTCA
β-tubulin	50 nM	RT_AFtub_for	GGTCGTTACCTCACCTGCTCT
		RT_AFtub_rev	GGATGTTGCGCATCTGGT