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# The influence of ecophysiological factors on growth, *aflR* gene expression and aflatoxin $B_1$ production by a type strain of *Aspergillus flavus*

**Victoria Bernáldez[1], Juan J. Córdoba[1], Naresh Magan[2], Belén Peromingo[1], Alicia Rodríguez[1]***
[1]Food Hygiene and Safety, Meat and Meat Products Research Institute. Faculty of Veterinary Science, University of Extremadura, Avda. de las Ciencias, s/n. 10003-Cáceres, Spain
[2]Applied Mycology Group, Cranfield Soil and AgriFood Institute, Cranfield University, Beds. MK43 0AL, U.K.

Corresponding author: Dr. Alicia Rodríguez
*E-mail address*: aliciarj@unex.es
*URL*: http://higiene.unex.es/

## Abstract
Maize is prone to infection by *Aspergillus flavus*, which can contaminate the product with aflatoxins. The objective of this study was to examine the impact that interactions between water activity ($a_w$) and temperature may have on growth, the expression of a biosynthetic regulatory gene (*aflR*) and aflatoxin $B_1$ ($AFB_1$) production by a strain of *A. flavus* on a maize-based medium. Results showed that there were some differences between lag phases and growth rates of *A. flavus*. The optimum growth rate for *A. flavus* was at 30ºC and 0.99 $a_w$. No growth occurred at 0.90 $a_w$ and 20ºC. Both temperature and $a_w$ had an influence on the relative *aflR* gene expression and $AFB_1$ production by *A. flavus*; however, the results for $AFB_1$ production were not consistent with the effects on gene expression or growth. These findings provide data that are useful to better understand the conditions which represent higher risks from $AFB_1$ production. However, the *aflR* expression was not a good indicator of $AFB_1$ production alone. Thus, further molecular studies of other AF-related genes should be done. These results are discussed in the context of harvesting and storage of maize and the prevailing environmental conditions to minimise $AFB_1$ contamination.

**Keywords:** maize, aflatoxin, *Aspergillus flavus*, *aflR*, environment

## 1. Introduction

Maize (*Zea mays L.*) is one of the most important cereals worldwide, both for human and animal consumption (Pechanova & Pechan, 2015; Valipour, 2014, 2015, 2016; Yannopoulos et al., 2015). Pre-harvest, harvesting and drying, and post-harvest phases need to be efficiently managed to avoid mould deterioration and the potential for $AFB_1$ contamination. It has been shown that during silking airborne spores and damage of the ripening cobs by pests and birds can allow *A. flavus* infection and subsequent toxin contamination (Bhatnagar-Mathur et al., 2015; Mandeel, 2005). After harvest water activity ($a_w$) of the maize cobs can be about 0.90-0.95 $a_w$ which is conducive to infection by toxigenic *Aspergillus* species. Thus effective drying is necessary for medium and long term storage. Delays in drying and poor post-harvest storage can exacerbate toxin contamination which could be increased under unfavourable climate conditions (Magan & Aldred, 2007; Waliyar et al., 2015). $AFB_1$ and total aflatoxin contamination of maize and other commodities has received significant attention because $AFB_1$ is classified as a Class 1A carcinogen and the EU and many countries word wide have strict maximum levels of contamination (European Commission, 2006; 2010; IARC, 2002).

It is important to understand the ecology of *A. flavus* so that there is a better knowledge of methods to delay or prevent growth and $AFB_1$ contamination in maize. The most important factors, that influence colonisation and toxin production, are $a_w$ and temperature. Specifically, the optimum temperature and $a_w$ values for growth and $AFB_1$ production by *A. flavus* range between 16 and 31 °C and 0.82 and 0.99 $a_w$ (Pitt & Hocking, 2009). This range of $a_w$ (moisture content of maize between 16-40%) and temperature values are related to the most common conditions found during maize harvest (Brooking, 1990; Medina et al., 2015).

Previous studies have suggested a relationship between growth and $AFB_1$ production as well as the expression of some structural and regulatory genes in the biosynthetic cluster for aflatoxins (Abdel-Hadi et al., 2010; Al-Saad et al., 2016; Medina et al., 2015; Schmidt-Heydt et al., 2010). Thus, Abdel-Hadi et al. (2010) found a relationship between temporal expression of a structural gene (*aflD*) and $AFB_1$ production in maize grain stored under different $a_w$ levels. From a food safety perspective, the evaluation of the induction of the aflatoxin biosynthesis genes can be a good indicator for determining the risk from specific toxigenic species. The key regulatory genes involved in the aflatoxin biosynthetic gene pathway for aflatoxin biosynthesis are the *aflR* and *aflS* genes. The relative expression of the two regulatory genes (*aflR*, *aflS*) is of particular interest and importance. Schmidt-Heydt et al. (2010) showed that the ratio of expression of these two genes varied with temperature and $a_w$. Thus, high ratios were related to high phenotypic $AFB_1$ production and low ratios to low toxin production. Thus, the increase or decrease in the temporal expression of the *aflR* gene could be a good indicator of control of aflatoxin biosynthesis. The gene transcription always precedes phenotypic production. If the window between gene activation and phenotypic production is long enough, predictions could be made and some preventive or corrective actions taken in the food industry that could prevent $AFB_1$ accumulation in maize. To our knowledge, this is the first study that integrates temporal molecular ecology, ecophysiological data and secondary metabolite data to better understand the dynamics of the process of toxin production by *A. flavus* on a maize-based medium.

The objectives of this study were to (a) examine the effect of $a_w$ (0.99, 0.95 and 0.90 $a_w$) and temperature (20, 25 and 30ºC) conditions usually found during harvesting and post-harvest stages of maize relevant to *A. flavus* on (a) lag phases prior to growth, (b) growth, (c) temporal changes in *aflR* gene expression and (d) phenotypic $AFB_1$ production by such mould species on a maize-based medium.

## 2. Materials and Methods

### 2.1. Fungal strain

An aflatoxin-producing strain, *A. flavus* NRRL 3357, provided by Dr. D. Bhatnagar (USDA Agricultural Research Service, New Orleans, LA, USA) and held in the Culture Collection of the Applied Mycology group at Cranfield University was used in this study. The strain has been previously used for molecular ecological studies (Abdel-Hadi et al., 2010; 2012). This strain was maintained by regular sub-culturing on Malt Extract Agar (MEA; Oxoid Ltd., Bansingstoke, UK) at 25ºC for 4-5 days and then stored at 4ºC until required.

### 2.2. Inoculum preparation

The spores were collected using 10 mL sterile water containing 0.05% Tween 80 (Acros Organics, USA) by using a sterile glass road to rub the surface of the colony to release the spores. The spore suspension was decanted into a sterile 25 ml Universal bottle and counted using a haemocytometer (Fisher Scientific, United Kingdom). The amount of spores was adjusted to $10^7$ conidia $mL^{-1}$ with sterile water. For storage of spores, the spore suspension was kept in glycerol (Fisher Scientific, UK) solutions at −80°C and a new starter culture used for each experiment.

### 2.3. Media preparation, inoculation and incubation

A 3% milled maize agar (MMA) was used in these studies and was prepared with 30 g of maize meal previously ground, 20 g of technical agar and 1000 mL water. The basic medium was modified with 23 and 32.2 g of glycerol per 100 mL of water to obtain 0.95 and 0.90 $a_w$ levels, respectively, as detailed by Dallyn and Fox (1980). The culture media were prepared by autoclaving in an autoclave (J.P. Selecta S.A., Spain) for 20 min at 121 °C (103 kPa). The medium was vigorously shaken prior to pouring into 9-cm diameter Petri plates. The accuracy of the $a_w$ modifications was confirmed using an Aqualab $a_w$-meter device (Decagon Devices Inc., USA).

For lag phases prior to growth, growth rates and $AFB_1$ analyses, treatment agar plates were centrally inoculated with 2 μL of the inoculum. For gene expression studies, a 0.2 mL aliquot was spread plated on the surface of MEA. To obtain an inoculum, this was incubated for 24 h. Then 3 mm agar discs containing germinated conidia were used to inoculate treatment plates containing sterile cellophane overlays in 3 places equidistant from each other.

The inoculated agar plates were enclosed in separate polyethylene bags and placed in polyethylene plastic chambers (25 litres) accompanied by 2x500 mL beakers of glycerol/water solution of the same $a_w$ as the treatment condition to maintain the equilibrium relative humidity (ERH) in the chamber during incubation. The $a_w$ of each maize-based medium was measured at the end of the incubation period. Treatments were incubated in temperature-controlled rooms at 20, 25 and 30ºC for up to 12 days. All experiments were done with three replicates per treatment and repeated once.

## 2.4. Growth assessment

Assessment of growth was made daily during the incubation period in each $a_w$ and temperature treatment condition. Measurements were performed in two directions at right angles to each other. These data were utilised for determination of lag times (days, $\lambda$) and growth rates (diameter, mm/day, $\mu$). Data plots showed, after a lag phase, a linear trend with time. Data was fitted using a linear model obtained by plotting the colony diameter against time. The growth rate (mm/day) was calculated from the slope of the regression line. Lag times (in days) were calculated by equalling the regression line formula to the original inoculum size (diameter, mm).

## 2.5. Gene expression studies

For gene expression studies, sampling was done by 3, 6, 9 and 12 days of the experiment. After each incubation time, the cellophane disks containing the whole colonies were harvested under sterile conditions in a laminar flow hood (Telstar BV-100, United Kingdom). The samples were quickly frozen in liquid nitrogen and stored at -80°C until RNA extraction.

### 2.5.1. RNA extraction

Total RNA was extracted according to the method previously optimised by (Bernáldez, Rodríguez, Rodríguez, Sánchez-Montero & Córdoba, 2017) with the exception of RNA purification was performed using the Spectrum Plant Total RNA Kit protocol (Sigma-Aldrich, USA). RNA was diluted in 50 µL of the elution solution provided by the RNA extraction kit and kept at -80ºC until use for reverse transcription (RT). The RNA concentration and purity ($A_{260}/A_{280}$ ratio) were determined spectrophotometrically using a 1.5 µL aliquot on the Nanodrop 200 UV-VIS Spectrophotometer (Thermo Scientific, USA).

### 2.5.2. Two steps reverse transcription quantitative PCR and relative quantification

#### 2.5.2.1. Primers

The primer pair aflRtaq1/aflRtaq2 previously designed from the *aflR* gene involved in the aflatoxin biosynthesis pathway was used (Table 1; Abdel-Hadi et al., 2010). The primer pair F-TubJD/ R-TubJD was used for the *β-tubulin* gene expression (Table 1).

#### 2.5.2.2. cDNA synthesis

cDNA was synthesised using 5 µL of total RNA (500 ng) according to the PrimeScript ™ RT Reagent kit protocol (Takara Bio Inc, France) which consists in the incubation of the reaction mixture under the following conditions: 37 ºC for 15 min (reverse transcription) and 85 ºC for 5 s (inactivation of reverse transcriptase). Subsequently, cDNA samples were kept at -20ºC until use as template for qPCR amplification.

#### 2.5.2.3. Quantitative PCR

Quantitative PCR (qPCR) analyses were carried out in an Applied Biosystems ViiA TM 7 Real-Time PCR System (Applied Biosystems, USA) using the cDNA previously synthesised. They were prepared in triplicates of 12.5 µL reaction mixtures in

MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystem, USA). Three replicates of a control sample without cDNA template were also included in the runs. Individual SYBR Green qPCR reactions with the different primer pairs were performed in all cases. Reaction mixtures consisted of 6.25 µL SYBR Green (SYBR Premix Ex Taq; Takara, Otsu, Shiga, Japan), 300 nM of each primer and 2.5 µL of cDNA template in a final volume of 12.5 µL. The optimal thermal cycling conditions included an initial step of 10 min at 95ºC followed by 40 cycles at 95ºC for 15s and 60ºC for 1min. The specific binding of SYBR Green to the PCR products derived from the amplification of the *aflR* and *β-tubulin* genes were tested after the qPCR, by analysing the melting curve of the PCR products performed, according to the following protocol: slow ramp between 60 and 95ºC in increments of 0.5ºC for 5 s. Quantification cycle (Cq) determinations which is the intersection between each fluorescence curve and a threshold line were automatically performed by the instrument using default parameters.

#### 2.5.2.4. Relative quantification of the *aflR* gene expression

Data was analysed using the Software ViiA™ Software V1.2.4. Relative quantification studies were conducted according to the method described by Livak and Schmittgen (2001). The expression ratio of the target genes was performed using the housekeeping gene *β-tubulin* as an endogenous control. This method allows comparison between the expression ratio of the target gene (*aflR*) of a tested sample and its relative calibrator ("control sample"). In this work the calibrator corresponded to samples incubated for three days.

### 2.6. Extraction and quantification of aflatoxin $B_1$

Extraction of $AFB_1$ from samples and its detection by using a HPLC-FLD system have been made according to the methods validated and optimised by Medina and Magan (2012) and Al-Saad et al. (2016).

#### 2.6.1. Sample preparation and aflatoxin $B_1$ extraction procedure

Samples for $AFB_1$ extraction were conducted on the last day of the experiment. Plugs of 3 mm diameter were collected from each sample using a cork borer and placing them into previously weighted 2 mL volume tubes (Sigma-Aldrich Chemie GmbH, Germany). Next, tubes with plugs were weighted and immediately frozen at -20ºC and stored until use.

$AFB_1$ was extracted by the addition of 1 mL high performance liquid chromatography (HPLC) grade chloroform to the tubes and shaking for 30 min in a rotary shaker. Then the supernatant was pipetted into fresh 2 mL tubes and evaporated overnight. They were collected as previously described using the AOAC method (2000).

#### 2.6.2. Aflatoxin $B_1$ derivatisation procedure

Derivatisation procedure was carried out by adding 200 µL hexane (Acros Organics, Belgium) and 50 µL of trifluoroacetic acid (TFA; Sigma-Aldrich, Misuri, USA) to the residue. The mixture was then vortex for 30 s and then let for 5 min. Next, a mixture of water: acetonitrile (9:1, v/v) was added. The entire content was vortexed for 30 s

and left 10 min to allow separation of layers. Thereafter the aqueous layer was filtered with a syringe nylon filters (13 mm x 0.22 μm; Jaytee Biosciences Ltd., Herne Bay, UK) and introduced directly into amber silanised 2 mL HPLC vials (Agilent Technologies, Inc., Palo Alto, CA, USA) to carry out the HPLC analysis .The hexane layer was discarded.

### 2.6.3. HPLC quantification of aflatoxin $B_1$

The HPLC system used for $AFB_1$ analyses was an Agilent 1200 series system (Agilent, Berks., UK) consisted of an in-line degasser, autosampler, binary pump and a fluorescence (FLD) detector. The stationary phase was a C18 column (Phenomonex Luna 5 µm, 150 × 4.6 mm) joined to a pre-column (security guard, 4 × 3 mm cartridge, Phenomenex Luna). The mobile phase was methanol/water/acetonitrile (30:60:10, v/v/v) using an isocratic flow rate of 1 ml $min^{-1}$. Run time was 25 min. Analyte detection was made at excitation and emission wavelengths of 360 and 440 nm, respectively (Medina and Magan, 2012). The data were integrated and calculated using a ChemStation software.
The recovery rate of the method was >90% when different amounts of aflatoxins were added (range between 0.1-100 ppb).

## 2.7. Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 22.0 software. Data on lag phase prior to growth, maximum growth rate, the relative *aflR* gene expression and toxin production were tested for normality and the homogeneity of variances using the Shapiro-Wilk and Levene´s tests, respectively. All data sets failed the normality test, therefore variable transformation was performed to improve normality or homogenise the variances but without any success. Therefore, non-parametric data analysis was performed using the Kruskal-Wallis rank sum test. After that, the Mann-Whitney test was applied to compare the mean values obtained. The statistical significance was set at $p \leq 0.05$.

## 3. Results

### 3.1. Effect of $a_w$ and temperature on lag times prior to growth and growth rates

### 3.1.1. Effect of environmental factors on lag times prior to growth

Figure 1 shows the effect of temperature and $a_w$ on the lag phases prior to growth of *A. flavus* on MMA. No growth occurred at 0.90 $a_w$ and 20ºC. The longest lag phase for this strain was at 0.90 $a_w$ and 25ºC (≈ 7 days). In contrast, the shortest lag times were at 25 or 30ºC and 0.99 $a_w$ (<1 day). The statistical analysis of data sets showed that there were significant differences between lag phases at 0.90 $a_w$ at all the temperatures tested ($p \leq 0.05$). No differences were found at 0.95 and 0.99 $a_w$. For temperature, lag phases were different at 20 and 25ºC for all the $a_w$ levels tested ($p \leq 0.05$; Table 2).

### 3.1.2. Effects of environmental factors on growth

The effect of temperature and $a_w$ on growth of *A. flavus* is shown in Figure 2. Optimum growth occurred at 0.99 $a_w$ and 30ºC (≈ 6 mm/day). However, the minimum growth

rate was detected at 0.90 $a_w$ and 25ºC (≈ 0.4 mm/day). At the same $a_w$ but at the lowest temperature used in this study (20ºC) no growth of *A. flavus* was observed after 12 days incubation.
Statistical analyses of data showed that there were significant differences between the growth rate at all temperature and $a_w$ levels tested ($p \leq 0.05$; Table 2).

### 3.2. Effect of $a_w$ and temperature on temporal *aflR* gene expression

The effect of the two-way interacting treatments of the expression of the *aflR* regulatory gene at different sampling times is shown in Figure 3. The data are based on those relative to the control samples (samples taken at 3 days of incubation). There were different relative expression profiles of these genes at the temperature and $a_w$ tested ($p \leq 0.05$; Table 2). Also significant differences were found between transcription levels on the different sampling days ($p \leq 0.05$; Table 2).

The relative *aflR* gene expression was detected at either 3 (5.1 log copies of *aflR* gene, data not shown) or 6 days (Figure 3) of incubation at any of the temperature tested. However, it should be noted that in most of the cases there was a stimulation of the *aflR* gene expression at 9 days when compared to the other sampling times, with the highest gene expression value at 30ºC and 0.90 $a_w$ at this incubation time. At 25 ºC the *aflR* relative gene expression was lower than at the other temperatures for most of the $a_w$ and time conditions; this was more noticeable at 0.95 $a_w$ at all the incubation times evaluated. At the lowest temperature tested, the expression of this regulatory genes appeared to be enhanced regarding the control at 0.90 and 0.95 $a_w$ values by days 9 and 12. However at 0.99 $a_w$ there was a repression of the *aflR* gene expression at the different sampling times in the control. Statistical analysis showed that both $a_w$ and temperature had a significant effect on *aflR* gene expression ($p \leq 0.05$; Table 2).

### 3.3. Effect of $a_w$ and temperature on aflatoxin $B_1$ production

Figure 4 shows that $a_w$ and temperature interactions had a strong influence on the $AFB_1$ production. Maximum $AFB_1$ production was at 0.99 $a_w$ for all the tested temperatures with production highest at 25-30ºC. The *A. flavus* strain produced $AFB_1$ at 0.95 $a_w$ only at the highest temperature evaluated (30ºC). No $AFB_1$ was detected at 0.90 $a_w$ at any of the temperature conditions tested. Statistical analysis showed that both $a_w$ and temperature and their interaction had a significant effect on $AFB_1$ production ($p \leq 0.05$; Table 2).

## 4. Discussion

### *4.1. Maize colonisation by A. flavus in relation to harvest season*

This study assessed the impact of interactions between $a_w$ and temperature commonly found at the harvesting and post-harvest phases of maize relevant to *A. flavus* on growth, biosynthetic gene expression and $AFB_1$ production. *A. flavus* is the most common species that contaminates maize during pre-harvest growth due to its survival on crop debris, and its spread by wind or insects, which can result in kernel infection through the silks (Waliyar et al., 2015; Luo et al. 2009; Reese et al. 2011). Once maize is infected by *A. flavus*, poor post-harvest practices including slow drying and inadequate ventilation can allow colonisation of *A. flavus* and further mycotoxin

contamination (Atanda et al., 2011). Thus an understanding of the $a_w$ and temperature which represents optimum and marginal conditions for growth and aflatoxin production are important. This can help to determine the time frames available before the risk of colonisation by *A. flavus* becomes significant. The lag phase data helps to determine these periods of lower risks in the harvesting and drying phases.

*A. flavus* is able to grow and produce $AFB_1$ under a wide variety of environmental conditions, and the synthesis of this mycotoxin is strongly related to the key critical environmental factors of temperature, $a_w$, pH and the maize nutritional composition and the presence of competitive agents (Keller et al., 2013; Magan & Aldred 2007). In the present study, the influence of temperature and $a_w$ on *A. flavus* growth and $AFB_1$ production was studied by culturing the mould under varied temperature and $a_w$ levels on a maize-based matrix only. The 3% milled maize medium has been considered a good approximation for growth and toxin production patterns in natural matrices (Pardo et al. 2005; Patriarca et al., 2014; Al-Saad et al., 2016). Such food-based media have been frequently used as model systems in other ecophysiological studies (Patriarca et al., 2014; Rodríguez et al. 2014, 2015; Al-Saad et al., 2016). The $a_w$ values tested in this study were the most frequently encountered during the maize harvest. Thus, at the early dough stage, the moisture content is about 40% (=0.99 $a_w$) with no water stress effects; this decreases to 30-35 % moisture content at the mid-dough stage (=0.95 $a_w$) and to 20-25% moisture content (0.90-0.85 $a_w$) at full maturity over a period of about 4-6 weeks (Brooking, 1990; Medina et al., 2015). The typical temperature for maize growing season until harvest is between 20 and 30ºC, which is optimum for growth of *A. flavus*.

### *4.2. Effects of maize harvest-related environmental factors on A. flavus growth*

This study showed that there were some differences between lag phases and growth rates of *A. flavus* at the different conditions tested. The optimum growth rate for *A. flavus* on maize-based medium was found at 30 ºC and 0.99 $a_w$. These results agree with those reported by Giorni, Battilani, Pietri & Magan, (2008) who found optimum growth of *A. flavus* at 0.99 $a_w$ and 25-30 ºC on maize. However, our results contrasted with previous studies on *A. flavus* growth on other food commodities including nuts, cereals and cured meats, or culture media based on such food matrices. These differences are probably based on differences on nutritional composition of the different food matrices since growth is strongly influenced by food constituents and substrate composition (Mohale et al., 2013). Previous studies with *A. flavus* on groundnuts or on almond-based medium where authors reported that *A. flavus* growth was maximum at 34ºC and 0.94 $a_w$ and 28ºC and 0.96 $a_w$, respectively (Sanchis & Magan, 2004; Gallo et al., 2016). Peromingo et al. (2016) showed that the growth was optimum for *A. flavus* at 25ºC and 0.95 $a_w$ on a dry-cured ham-based medium. Abdel-Hadi et al. (2012) showed that the optimum growth for *A. flavus* on a conducive YES medium was at 0.99 $a_w$ and 35ºC. Other authors demonstrated that the optimal growth for *A. flavus* was at 30ºC and 0.90-0.92 $a_w$ on polished and brown rice (Mousa, Ghazali, Jinap, Ghazali & Radu, 2013).

In the present study no *A. flavus* growth occurred at 0.90 $a_w$ and 20ºC on maize-based media. These results contrasted with previous studies that found growth limits were close to 10ºC, regardless of $a_w$ condition (Mousa, Ghazali, Jinap, Ghazali, & Radu, 2011; Peromingo et al., 2016) and at temperatures ≤ 15ºC when $a_w$ level was 0.90 $a_w$.

It seems that differences between composition of culture media used and strains used may explain different marginal conditions for *A. flavus* growth.

### *4.3. Effects of maize harvest-related environmental factors on aflR gene expression*

Previous studies have shown that the expression of the regulatory gene *aflR* is a good indicator of *A. flavus* activity under different $a_w$, temperature and time for such species under climate change conditions and against antagonist microorganisms on maize-based media (Medina et al., 2015; Al-Saad et al., 2016). For this reason this key gene was selected for molecular studies. The expression of the *aflR* gene reached a basal level of expression at 25ºC when compared with other temperatures examined. These data are consistent with previous reports by Schmidt-Heydt et al. (2009) who found a basal level of expression of such genes at 25°C as long as the $a_w$ was higher than 0.90 $a_w$. On the other hand, although the *aflR* gene expression levels were quite similar between the all temperatures and $a_w$ levels tested, a strong induction of this gene occurred when $a_w$ was 0.90 at 20 and 30ºC. The induction of expression peak could be because the growth of the mould occurred under imposed stress conditions. This fact appears to be generic across some toxigenic fungi (Schmidt-Heydt et al., 2008).

In this study a weak relationship between the temporal relative expression of the regulatory gene *aflR* and $AFB_1$ production was found. Surprisingly, in those $a_w$ and temperature where higher gene expression was observed, *A. flavus* did not produce significant amounts of the toxin. Other authors have demonstrated that the mycotoxin biosynthetic gene expression may be strain-dependent (Gallo et al., 2016; Rodríguez et al., 2014) and the expression of others genes such as the *aflS* or *aflD* included in the cluster may exert more influence on the phenotypic mycotoxin production (Schmidt-Heydt et al., 2010). In addition, it is possible that sampling time frame for gene expression (every 3 days) did not allow detecting changes in the *aflR* gene expression in relation to $AFB_1$ production under the experimental conditions tested.

### *4.4. Effects of maize harvest-related environmental factors on $AFB_1$ production by* A. flavus

For $AFB_1$ production, a previous study has suggested that depending on the particular combination of external growth parameters, the biosynthesis of this mycotoxin can either be completely inhibited or fully activated (Abdel-Hadi et al., 2012). In the present study using a type strain mycotoxin production did not correlate well with maximal growth. While growth occurred over a wide range of temperatures and $a_w$ levels, $AFB_1$ was only detected at 0.99 $a_w$ and the three temperatures evaluated and 0.95ºC and 30ºC. $AFB_1$ production was optimum at 25-30ºC and 0.99 $a_w$. These results are similar to some previous reports on optimal conditions for *A. flavus* ranging between 25-30ºC and 0.95-0.99 $a_w$ (Abdel-Hadi, Carter & Magan, 2010; Astoreca et al., 2014; Giorni, Magan, Pietri & Battilani, 2011; Giorni, Magan, Pietri, Bertuzzi, & Battilani, 2007; Mousa et al., 2011; Peromingo et al., 2016).

Concerning marginal conditions for toxin production, from our results no production was found when $a_w$ was ≤ 0.95, and temperature was ≤ 25 ºC after 12 days incubation. These findings parallel those reported by Astoreca et al. (2014) who evaluated the effect of $a_w$ and temperature on $AFB_1$ and cyclopiazonic acid production by *A. flavus*

on a corn extract medium after 7, 14, 21 and 28 days. They observed that at $a_w$ levels ≤ 0.94 $a_w$ and temperature ≤ 25ºC, no $AFB_1$ was produced or very low amounts after 7 and 14 days, except for 0.94 $a_w$ and 25ºC after 14 days of incubation. These small discrepancies may be because of the time frame of the experiments, the strain used (Gallo et al., 2016; Klich, 2007) or small differences between the maize-based media used by Astoreca et al. (2014) and that in the present study.

## 5. Conclusions

This study suggests that *A. flavus* can grow over a relatively wide range of $a_w$ and temperature range. The $a_w$ of the maize when ripe is usually 18-24 % (~ 0.85-0.90). At this $a_w$ maize is less likely to be contaminated with *A. flavus* and then with $AFB_1$ if the grain is not damaged. Therefore, good post-harvest practices avoiding high temperatures (≤ 25 ºC) and rapid drying of grain (grain $a_w$ ≤ 0.95) can avoid a decrease in the grain quality and reduce the health risk due to toxin contamination. Under the environmental conditions occurring at harvesting and post-harvest stages of maize, it seems that the temporal relative expression of the regulatory *aflR* gene is not a good indicator for $AFB_1$ production of *A. flavus*. Further molecular studies of other genes associated with the $AFB_1$ biosynthetic pathway should be done to identify which represent a good indicator of biosynthesis of $AFB_1$.

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Table 1. Nucleotide sequences of primers for RT-qPCR assays on the basis of the *aflR* and *β-tubulin* genes.

| Primers | Gene | Nucleotide sequences (5'- 3') | Product size (bp) | Position | Reference |
|---|---|---|---|---|---|
| AflRtaq1 | *aflR* | TCGTCCTTATCGTTCTCAAGG | 110 | 1646[a] | Abdel-Hadi et al. (2010) |
| AflRtaq2 | | ACTGTTGCTACAGCTGCCACT | | 1735[a] | |
| F-TubJD | *β-tubulin* | TCTTCATGGTTGGCTTCGCT | 98 | 844[b] | This study[a] |
| R-TubJD | | CTTGGGTCGAACATCTGCT | | 926[b] | |

[a]Positions are in accordance with the published sequences of *aflR* gene of *A. flavus* (GenBank accession no. AF441435.2).

[b]Positions are in accordance with the published sequence of *β-tubulin* gene of *A. flavus* NRL3357 (GenBank accession no. XM_002383269.1).

Table 2. U-Mann Whitney statistical analyses performed with data about effect on water activity and temperature on lag phase, growth rate, *aflR* gene expression and aflatoxin $B_1$ production by a strain of *A. flavus* grown on maize-based media.

| **Parameter checked** | **Factor studied** | **Two sets of data used** | **P-value of factor studied[a]** | | |
|---|---|---|---|---|---|
| | | | **0.99 $a_w$** | **0.95 $a_w$** | **0.90 $a_w$** |
| Lag phase | Water activity | **20-25 ºC** | [b]- | - | 0.037 |
| | | **25-30 ºC** | - | - | 0.050 |
| | | **20- 30 ºC** | - | - | 0.037 |
| | | | **20 ºC** | **25 ºC** | **30 ºC** |
| | Temperature | **0.90-0.95 $a_w$** | 0.037 | 0.050 | 0.127 |
| | | **0.90-0.99 $a_w$** | 0.037 | 0.050 | 0.050 |
| | | **0.95-0.99 $a_w$** | 0.050 | 0.050 | 0.050 |
| | | | **0.99 $a_w$** | **0.95 $a_w$** | **0.90 $a_w$** |
| Growth rate | Water activity | **20-25 ºC** | 0.050 | 0.050 | 0.037 |
| | | **25-30 ºC** | 0.050 | 0.050 | 0.050 |
| | | **20- 30 ºC** | 0.050 | 0.050 | 0.050 |
| | | | **20 ºC** | **25 ºC** | **30 ºC** |
| | Temperature | **0.90-0.95 $a_w$** | 0.037 | 0.050 | 0.050 |
| | | **0.90-0.99 $a_w$** | 0.037 | 0.050 | 0.050 |
| | | **0.95-0.99 $a_w$** | 0.050 | 0.050 | 0.050 |
| | | | **0.99 $a_w$** | **0.95 $a_w$** | **0.90 $a_w$** |
| *aflR* gene expression | Water activity | **20-25 ºC** | 0.560 | 1.000 | 1.000 |
| | | **25-30 ºC** | 0.210 | 1.000 | 1.000 |
| | | **20- 30 ºC** | 0.210 | 1.000 | 1.000 |
| | | | **20 ºC** | **25 ºC** | **30 ºC** |
| | Temperature | **0.90-0.95 $a_w$** | 1.000 | 0.770 | 0.770 |
| | | **0.90-0.99 $a_w$** | 1.000 | 0.020 | 0.040 |
| | | **0.95-0.99 $a_w$** | 1.000 | 0.020 | 0.020 |
| | | | **0.99 $a_w$** | **0.95 $a_w$** | **0.90 $a_w$** |
| Aflatoxin $B_1$ | Water activity | **20-25 ºC** | - | 1.000 | - |
| | | **25-30 ºC** | - | 0.037 | - |
| | | **20- 30 ºC** | - | 0.037 | - |
| | | | **20 ºC** | **25 ºC** | **30 ºC** |
| | Temperature | **0.90-0.95 $a_w$** | 1.000 | 1.000 | 0.037 |
| | | **0.90-0.99 $a_w$** | 0.037 | 0.037 | 0.037 |
| | | **0.95-0.99 $a_w$** | 0.037 | 0.037 | 0.050 |

[a] $p \leq 0.05$ means that there are significant differences.
[b]- U-Mann Whitney test was not done since no significant differences were observed when Kruskal-Wallis test was performed

**Figure legends**

Figure 1. Effect of water activity x temperature on the lag phases of *Aspergillus flavus* on maize-based media over a 12 days incubation period.

Figure 2. Effect of temperature and water activity on growth of *Aspergillus flavus* on maize-based media over a 12 days incubation period. *Denotes conditions at which the lag time was longer than the duration of the experiment.

Figure 3. Effect of temperature and water activity on temporal *aflR* gene expression by *Aspergillus flavus* grown on maize-based media over a 12 days incubation period.

Figure 4. Effect of water activity and temperature on aflatoxin $B_1$ production by *Aspergillus flavus* grown on maize-based media at 12$^{nd}$ day of incubation. ND means aflatoxin $B_1$ was not detected.

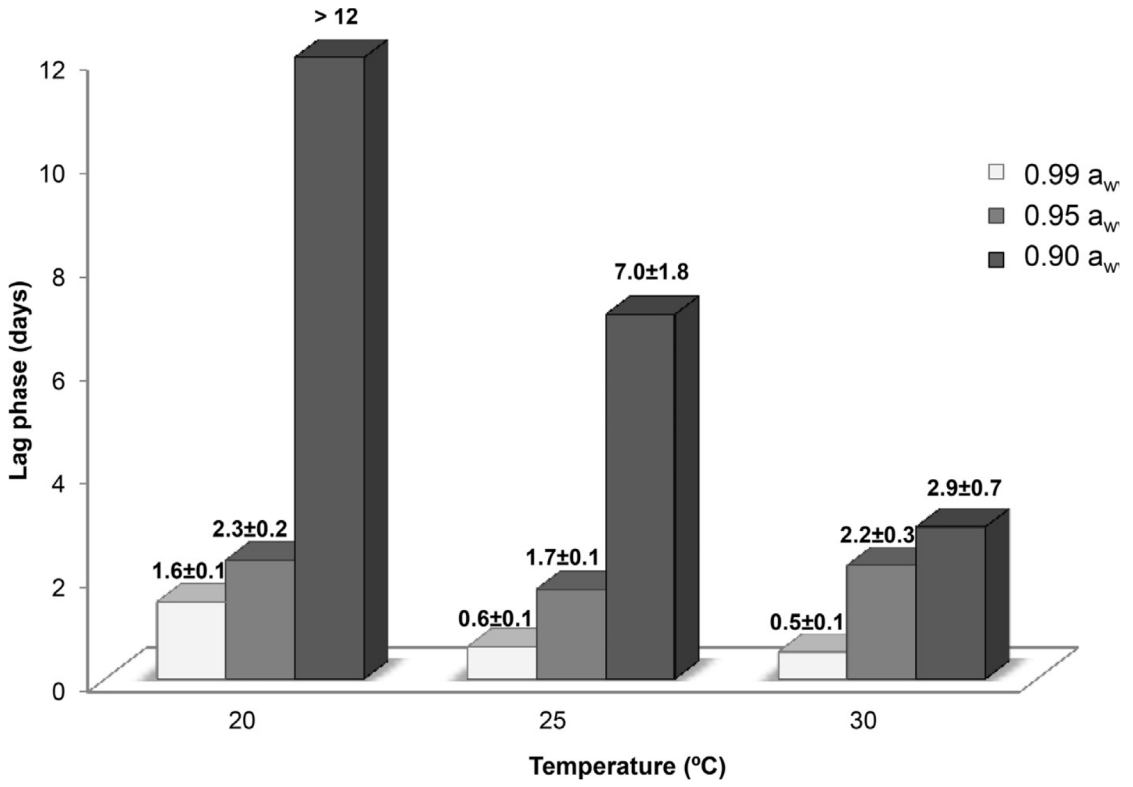


Figure 1.

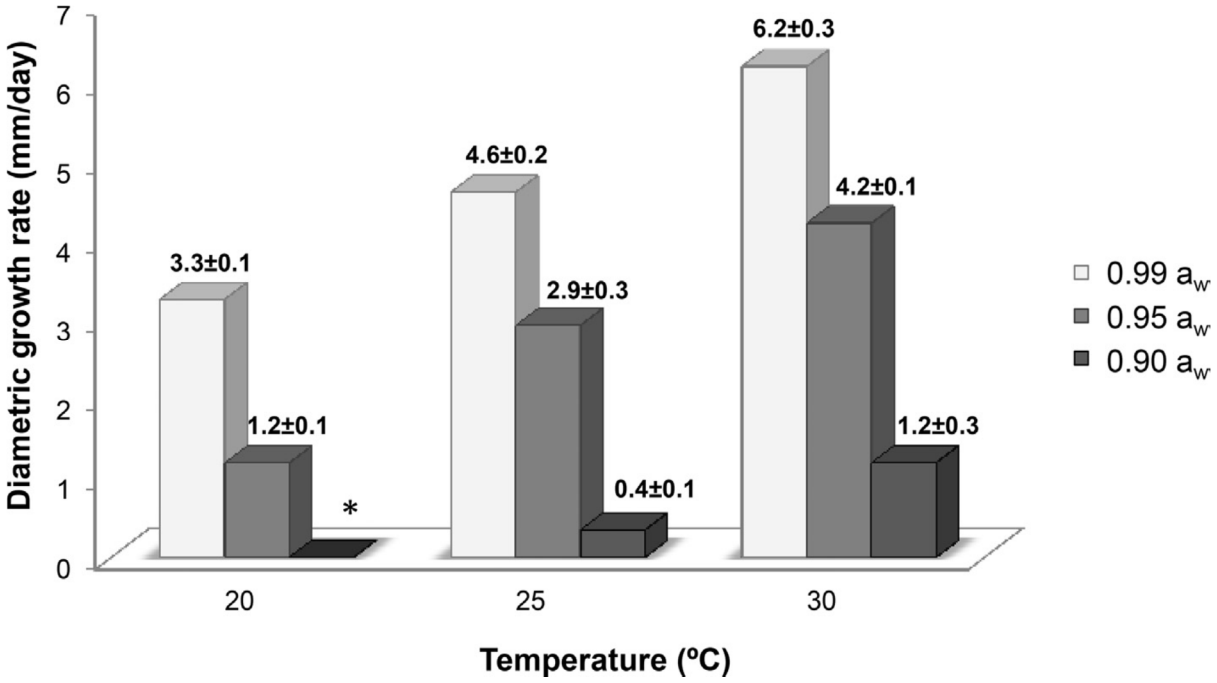
Diametric growth rate (mm/day)
7
6
5
4
3
2
1
0
3.3±0.1
1.2±0.1
*
4.6±0.2
2.9±0.3
0.4±0.1
6.2±0.3
4.2±0.1
1.2±0.3
20
25
30
Temperature (ºC)
0.99 $a_w$
0.95 $a_w$
0.90 $a_w$

Figure 2.

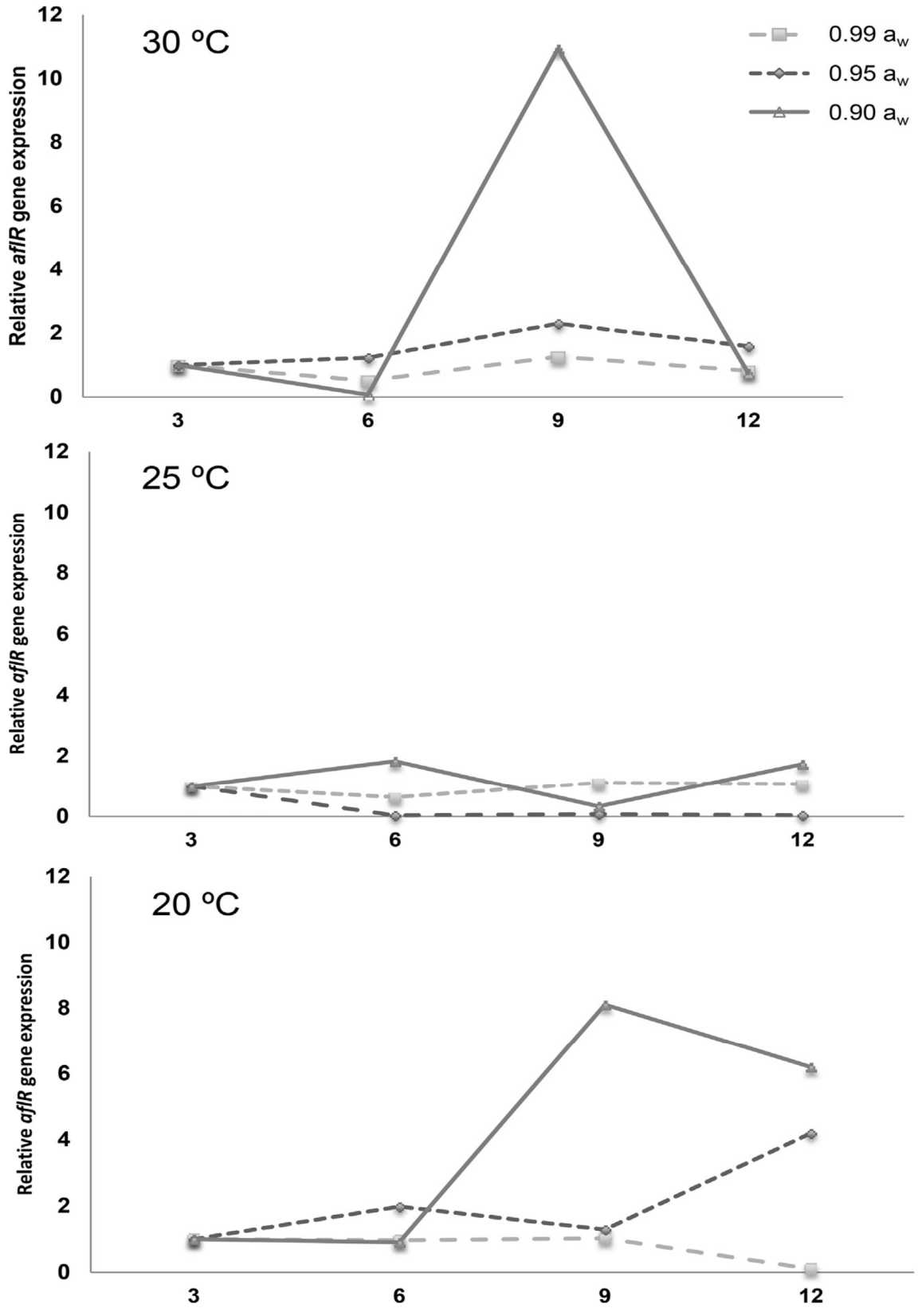
30 °C
25 °C
20 °C
0.99 $a_w$
0.95 $a_w$
0.90 $a_w$
Relative *aflR* gene expression
0
2
4
6
8
10
12
3
6
9
12

Figure 3.

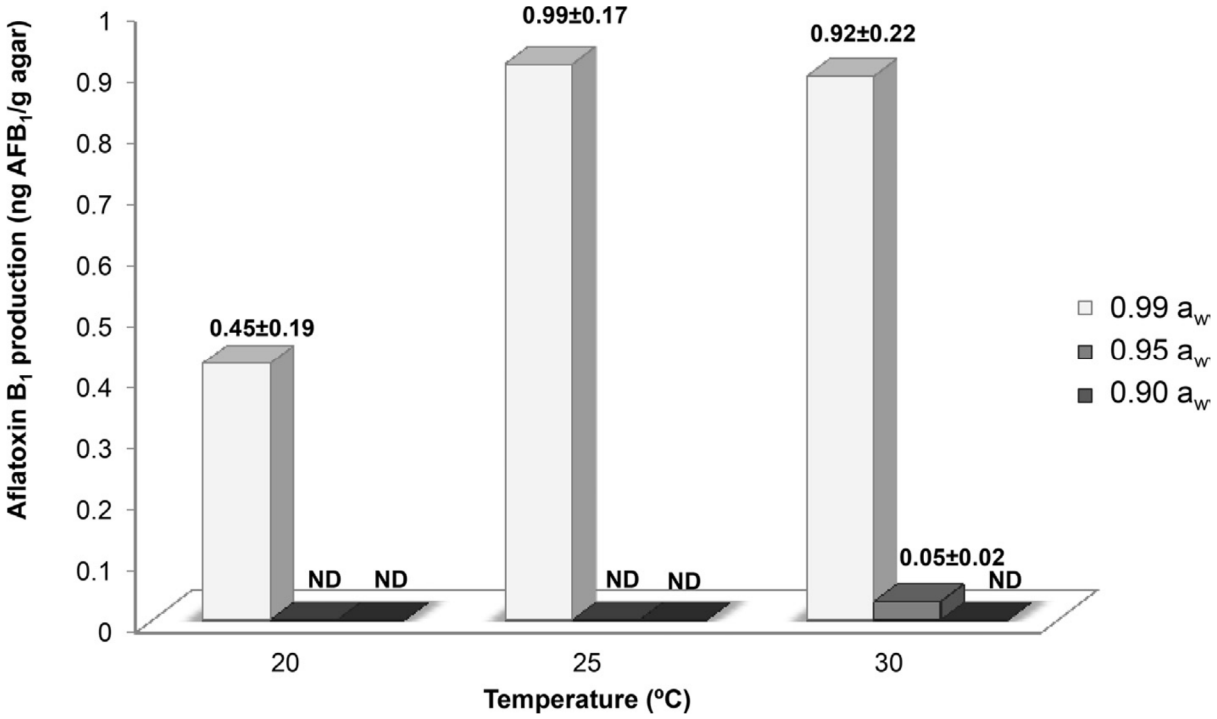
Aflatoxin B1 production (ng AFB1/g agar)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.45±0.19
ND
ND
0.99±0.17
ND
ND
0.92±0.22
0.05±0.02
ND
20
25
30
Temperature (ºC)
0.99 aw
0.95 aw
0.90 aw

Figure 4.

# The influence of ecophysiological factors on growth, aflR gene expression and aflatoxin B1 production by a type strain of Aspergillus flavus

Bernaldez, Victoria

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