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A systems approach to model the relationship between aflatoxin gene cluster expression, environmental factors, growth and toxin production by *Aspergillus flavus*

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Abstract

A microarray analysis was used to examine the effect of combinations of water activity (a_w; 0.995-0.90) and temperature (20-42^oC) on the activation of aflatoxin biosynthetic genes (30 genes) in *A. flavus* grown on a conducive YES medium. The relative expression of 10 key genes (*aflF, aflD, aflE, aflM, aflO, aflP, aflQ, aflX, aflR, aflS*) in the biosynthetic pathway were examined in relation to different environmental factors and phenotypic aflatoxin B_1 (AFB_1) production. This data, plus data on relative growth rates, and AFB_1 production under different a_w x temperature conditions, were used to develop a mixed-growth-associated product formation model. The gene expression data was normalised and then used as a linear combination of the data for all 10 genes and combined with the physical model. This was used to relate gene expression to a_w and temperature conditions to predict AFB_1 production. The relationship between the observed AFB_1 production provided a good

linear regression fit to the predicted production based in the model. The model was then validated by examining data sets outside the model fitting conditions used (37, 40^{o}C and different a_w levels). The relationship between structural genes (*aflD, aflM*) in the biosynthetic pathway and the regulatory genes (*aflS, aflJ*) was examined in relation to a_w and temperature by developing ternary diagrams of relative expression. These findings are important in developing a more integrated systems approach by combining gene expression, ecophysiological influences, and growth data to predict mycotoxin production. This could help in developing a more targeted approach to develop prevention strategies to control such carcinogenic natural metabolites which are prevalent in many staple food products. The model could also be used to predict the impact of climate change on toxin production.

1. **1. INTRODUCTION**

Aflatoxins are produced by *Aspergillus* section *Flavi* group species and are thought to be one of the most cancerous natural substances known. Economically and biologically the most important fungal species able to produce aflatoxins are *A. flavus* and *A. parasiticus* (Bhatnagar *et al.* 2002). The aflatoxin biosynthesis gene cluster of *A. parasiticus* has been completely elucidated (Yu *et al.*, 2002; Yu *et al.* 2004a). Indeed a whole genome microarray of *A. flavus* has been used to study the regulation of aflatoxin biosynthesis genes (O´Brian *et al.*, 2007). Generally, the aflatoxin biosynthesis genes of *A. flavus* and *A. parasiticus* are highly homologous and the order of the genes within the cluster have been shown to be the same (Yu *et al.*, 1995). *A. flavus* strains produce only aflatoxin B_1 and B_2 while *A. parasiticus* produce aflatoxins B_1, B_2, G_1, and G_2 (Vaamonde *et al.* 2003; Giorni *et al.* 2007).

The biosynthesis of mycotoxins is strongly dependent on growth conditions such as substrate composition (Luchese & Harrigan 1993) or physical factors including pH, water activity, temperature or modified atmospheres (Ellis *et al.* 1993; Molina & Gianuzzi 2002; Sanchis & Magan 2004; Ribeiro *et al.* 2006; Giorni *et al.* 2008). Water activity (a_w) is a measure of the amount of freely available water in a substrate for microbial growth and is related to pure water, which has a a_w of 1.00 or 100% equilibrium relative humidity. This a_w is related to the total moisture content of a specific substrate by a moisture sorption curve (Magan 2007). Depending on the particular combination of external growth parameters the biosynthesis of aflatoxin can

either be completely inhibited, or the pathway fully activated. Knowledge about these relationships enables an assessment of which parameter combinations can control aflatoxin biosynthesis and which are conducive to phenotypic aflatoxin contamination. For example, a mathematical model which delineated the relationship between pH, propionic acid concentration and temperature on aflatoxin biosynthesis by *A. parasiticus* was described by Molina & Giannuzzi (2002). However, with the exception of this data at the phenotypic level, very little information is available on the influence of abiotic factors on the regulation of the aflatoxin biosynthesis genes. Sweeney *et al*. (2000) developed a reverse transcription polymerase chain reaction to analyse the expression of the *aflR* and *ord1* genes of the aflatoxin pathway in relation to various nutritional media. Price *et al.* (2005) used a whole genome microarray approach to analyse the influence of substrate composition and pH on the activation of aflatoxin biosynthesis genes. O'Brian *et al*. (2007) using the whole genomic microarray, demonstrated that conducive and non-conducive temperatures affected functioning of the genes, with transcript levels of *aflR* and *aflR* protein present at lower concentrations at 37 than at 35^{o}C for a strain of *A. flavus*.

Using a mycotoxin microarray with sub-arrays for specific mycotoxins, developed by Schmidt-Heydt & Geisen (2007), the impact of key environmental factors (a_w, temperature) were shown to significantly affect the aflatoxin gene clusters. The ratio of the key regulatory genes (*aflR* and *aflS* [=*AflJ*]) was important in encoding the enzymes in the biosynthetic pathway of both *A. flavus* and *A. parasiticus* (Schmidt-Heydt *et al.* 2009; 2010). Other studies have also demonstrated the impact that such environmental factors may have, especially marginal stress conditions, on gene cluster expression and phenotypic toxin production for a number of species (Schmidt-Heydt *et al.* 2008). Recently, Schmidt-Heydt *et al.* (2011) showed that for *Fusarium culmorum* and *Fusarium graminearum* it is possible to integrate such microarray data on relative *TRI* gene expression under different environmental conditions, growth and deoxynivalenol (DON) production and develop models which can be used to predict DON concentrations for the first time. No such integrated systems approach has been attempted for *A. flavus*.

In *A. flavus* the clustered pathway genes have been detailed, and in some cases new gene names have been recently given (Yu *et al.* 2004a, b). Some of the key genes in the aflatoxin biosynthesis include *aflF* (old name: *norB*), *aflD* (*nor-1*) and *aflE* (*norA*) which encode a dehydrogenase and two reductases which convert norsolorinic

acid to averantin; *aflM* (=*ver-1*) is a dehydrogenase which converts versicolorin A to demethylsterigmatocystin; *aflaO* (=*omtB*) is a *O*-methyltransferase I or *O*-methyltransferase B which is involved in the conversion of demethylsterigmatocystin to sterigmatocystin and dihydro-demethylsterigmostocystin to dihydrosterigmatocystin; *aflP* (=*omtA*) is an O-methyltransferase A or II which converts sterigmatocystin to O-methylsterigmatocystin as well as demethylsterigmatocystin to dihydro-O-methylsterigmatocystin; other genes such as *aflQ* (=*ordA*) and *aflX* (=*ordB*) have been shown to be involved in the final part of the biosynthetic pathway, as oxidoreductase-P450 monooxygenase and monoxygenase oxidase. The two key regulatory genes which are important in transcription activation are *aflR* which is involved in both aflatoxin and sterigmatocystin production and *aflS* (=*aflJ*) which is involved in the regulation of aflatoxin.

We have utilised the mycotoxin microarray sub-array for the aflatoxin genes as a tool for examining the changes that interacting environmental factors may have on the relative expression of *A. flavus* gene clusters, as well as effects on growth and phenotypic aflatoxin production. The objectives of this study were to (a) examine the effect of a_w x temperature conditions on growth, aflatoxin B_1 (AFB_1) and relative expression of 10 key genes (*aflD, aflE, aflF, aflM, aflN, aflP, aflQ, aflX;* and the regulatory genes *aflR* and *aflS*) in the biosynthetic pathway using the mycotoxin gene microarray; (b) to quantify the amounts of AFB_1 produced under these interacting conditions; (c) to mathematically model the relationship between expression of these genes, environmental factors, growth and AFB_1 production; (d) to validate the model with data sets outside the range of the model and (e) to examine the possible ternary interactions and relationships between some genes in the early part (*aflD*, *aflM*) of the biosynthetic pathway and key regulatory genes (*aflR*, *aflS*) in relation to a_w, temperature and AFB_1 production.

2. MATERIALS AND METHODS

2.1 Fungal strain used

The *A. flavus* strain (NRRL 3357) was previously used in molecular ecology studies (Schmidt-Heydt *et al.* 2009). This was kindly provided by Dr. D. Bhatnagar, USDA, New Orleans, USA. It was stored at 4^{o}C and sub-cultured on a 2% maize meal agar when required. It has a known aflatoxin B_1 (AFB_1) production capacity (Schmidt-Heydt *et al.* 2009).

2.2 Growth studies

These were carried out with a conducive YES medium (20 g Yeast extract, 150 g sucrose, 1 g $MgS0_4 \bullet 7H_20$, 1 L). The agar medium was modified with glycerol to adjust the water availability to 0.99, 0.95, 0.90 and 0.85 a_w. The modification was done by first modifying the water with glycerol (46.1, 230, 506, 782 g l^{-1} respectively) and then substituting this mixture as if adding water. The advantage of using glycerol is its stability over the experimental temperature range for modifying a_w. The accuracy of the modifications was confirmed using an Aqualab 3TE instrument (Decagon, Pullman, WA, USA) and found to be within ±0.005 of the target a_w.

Spores from a 7 days old culture grown at 25^{o}C were dislodged with a sterile loop and placed in 10 ml sterile water + 0.05% Tween 20, a surfactant, in a 25 ml Universal bottle. The spores were counted and a 10^6 spores ml^{-1} concentration prepared. The 9 cm Petri plates containing media treatments were all over-laid with sterile 8.5 cm cellophane discs (P400, Cannings Ltd, Bristol, UK) and then centrally inoculated with a 5 µl spore suspension. Replicates (5 per treatment) were incubated at 20, 25, 30, 35 for model design and temperatures outside the model boundaries: 37, 40 and 42^{o}C were use to validate the model. Growth was measured daily by taking two diametric measurements at right angles to each other for a period of 9 days. Previous kinetics studies suggested that this was an optimum time under some conditions for gene expression using RT-PCR (Schmidt-Heydt *et al.* 2008). At the end of this period the whole colony biomass was scraped from the cellophane surface into Eppendorf tubes and frozen at -80^{o}C.

2.3 Isolation of RNA from samples

To perform microarray experiments RNA was isolated using the RNAeasy Plant Mini kit (Qiagen, Hilden, Germany). An amount of 1 g of the mycelium was ground with a mortar and pestle in liquid nitrogen. Two hundred and fifty mg of the resulting powder was used for isolation of total RNA. The powder was suspended in 750 µl lysis buffer, mixed with 7.5 µl β-mercaptoethanol and 100 glass beads with a diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) in a 2 ml RNase free micro reaction tube. The extracts were mixed thoroughly and incubated for 15 min at 55 °C and 42 kHz in an S10H ultrasonic bath (Elma, Singen, Germany).

All further procedures were essentially the same as suggested by the manufacturer of the kit.

2.4 Microarray experiments

cDNA synthesis and labelling were performed using the Micromax Direct Labeling kit (Perkin Elmer Life And Analytical Sciences, Inc. Boston, USA). For this purpose an amount of 50 µg of the DNase I treated total RNA was used according to the specifications of the kit. After cDNA synthesis and labelling, the cDNA was purified with the QiaQuick Min Elute kit (Qiagen, Hilden, Germany). The labelled and purified cDNA was brought to dryness in a vacuum concentrator (Speed Vac, Savant Instruments, Farmingdale, USA), re-suspended in 60 µl hybridization buffer (Scienion, Berlin, Germany), heated for 2 min. at 95 °C, placed on ice to prevent strand rearrangement and hybridized for 18 h at 42°C to the microarray by using an automatic hybridization station (Perkin Elmer, Boston, USA). After hybridization the array was scanned with a confocal laser system (Scanarray lite, Perkin Elmer) at a resolution of 5 µm. The analysis of the results was performed using the Scanarray software (Perkin Elmer, Boston, USA). The results were normalized using the Lowess algorithm (locally weighted scatter plot smoothing) together with subtraction of the background signal. As a control, the constitutively expressed β-tubulin gene was used.

2.5 Aflatoxin analyses

2.5.1 Derivatisation procedure. Agar plugs (4-5 plugs, approx. 0.5 g) were cut out of the agar medium across the 9 cm Petri plates. These were placed into 2 ml Eppendorf tubes and weighed. Aflatoxins were extracted by adding 1 ml chloroform and shaking for 1 hour. The biomass was discarded after centrifugation and the chloroform was evaporated to dryness. The residue was derivatised (AOAC, 2000) using the following method:

1. 200 µl HPLC hexane were added to the residue in a 2 ml Eppendorf tube.
2. 50 µl TFA (Triflouroacetic acid) was added and vortexed for 30 seconds; Eppendorf tubes were left for 5 minutes
3. 950 µl water: acetonitrile (9:1) was added
4. Eppendorf tubes were vortexed for 30 seconds.
5. They were left for 10 minutes for separation of the layers. The upper layer was discarded. The extracts were filtered through Nylon 13 mm, 0.2 µm filter

(SMI- Lab Hut LTD, UK) directly into amber HPLC vials (Jaytee Biosciences LTD, UK).

2.5.2 Sample analysis. Sample extracts were analyzed by HPLC using a Waters 600 E system controller, 470 fluorescence detector (Millipore Waters, Corporation Massachusetts USA) (λ_{exc} 360 nm; λ_{em} 440 nm) and a C_{18} column (Phenomenex Luna ODS2 150 x 4.6 mm, 5 µm) all under control Waters Millennium[32] software. The analysis was performed at a flow rate of 1 ml min^{-1} of the mobile phase (30 % Methanol: 60 % Water: 10 % Acetonitrile) and the run time was 25 minutes. A 200 µl stock solution of an aflatoxin mixed standard in methanol (Supelco, Bellefonte, PA, USA), containing 200 ng B_1, 60 ng B_2, 200 ng G_1 and 60 ng G_1, was dried under nitrogen gas and derivatised as for samples. Four concentrations (AFB_1; 50-200 ng ml^{-1}) were prepared for HPLC injection to make a standard curve (r^2=0.9999). The limit of detection for AFB_1 using HPLC was 0.8 ng g^{-1} medium.

2.6 Data analysis and model development

This study has used a mixed-growth-associated product formation model (Shuler and Kargi, 2007), which takes account of both specific growth rate and metabolite accumulation. This includes the fact that product formation is a combination of growth rate and the specific rate of product formation which is given by Eq. 1. This has been previously used for production of compounds such as xanthan gum and a range of secondary metabolites of pharmaceutical interest.

2.7 Generation of ternary contour surfaces of interactions between gene expression and environmental factors, and on aflatoxin B_1 production

In order to plot the ternary contour surfaces of the genetic expression a standardised signal from the microarray was calculated as follows for a_w and for temperature to relate the relative abundance of mRNA of the structural genes (e.g. *aflD, aflM*) to the regulatory genes (*aflR, aflS*):

$$(\quad) = \frac{}{(\quad + \quad + \quad)}, \frac{}{(\quad + \quad + \quad)}, \frac{}{(\quad + \quad + \quad)}$$

$$(\qquad) = \frac{}{(\quad + \quad + \quad)}, \frac{}{(\quad + \quad + \quad)}, \frac{}{(\quad + \quad + \quad)}$$

Where the standardised value = actual value – minimum value/maximum value – minimum value.

For AFB_1 production the model is:

$$(\qquad (\quad)) = \frac{}{(\quad + \quad + \quad)}, \frac{}{(\quad + \quad + \quad)}, \frac{}{(\quad + \quad + \quad)}$$

3. **RESULTS**

3.1 Effect of environmental factors on growth and aflatoxin B_1 production by a strain of A. flavus

Figure 1a shows the effect of interacting conditions of a_w and temperature on growth of the *A. flavus* strain used in this study. This shows that optimum was at 0.99 a_w and 30-35^{o}C, with good growth at 0.95 a_w and 25-40^{o}C. At the driest conditions examined, 0.85 a_w, growth only occurred at 30-37^{o}C.

Figure 1b shows the effect of these parameters on AFB_1 production. This shows a very different pattern from that for growth. Very little, if any AFB_1 was produced at 40^{o}C, except at 0.95 a_w. It is interesting to note that at sub-optimal growth conditions, AFB_1 production occurred at 20-37^{o}C with often higher production at 0.99-0.98 a_w. These data sets were used in conjunction with the relative gene expression of the 10 genes encoding for enzymes involved in the biosynthesis of AFB_1 for modelling their relationship.

3.2. Modelling the relationship between environmental factors, gene expression and aflatoxin B_1 production.

We have used a mixed-growth-associated product formation model, with specific rate of product formation given by the following equation (Shuler and Kargi, 2007)

$$q_P = \alpha\mu + \beta \qquad \text{(Eq. 1)}$$

Where q_P is the total amount of aflatoxin B_1 produced; α and β are constants of aflatoxin B_1 production associated with primary and secondary metabolism and μ is the specific growth rate.

The specific rate of product formation is proportional to the total biomass and the rate of product formation.

$$q_P = \frac{1}{X}\frac{dP}{dt} \qquad \text{(Eq. 2)}$$

The rate of product formation for a growth associated product is related to the initial biomass (X_o) and the specific growth rate (μ) and the time (t)

Thus combining Eq. 1 and Eq. 2.

$$\frac{dP}{dt} = (\alpha\mu + \beta)X \qquad \text{(Eq. 3)}$$

$$\frac{dP}{dt} = X_0 e^{\mu t} \qquad \text{(Eq. 4)}$$

The rate of microbial growth is characterized by the specific growth rate, defined as

$$\mu = \frac{1}{X}\frac{dX}{dt} \qquad \text{(Eq. 5)}$$

After integration form from t=0 to t (days) and $X(0)=X_0$ to $X(t)=X$

$$X = X_0 e^{\mu t} \qquad X_0 = Xe^{-\mu t} \qquad \text{(Eq. 6)}$$

Substituting Eq. 6 and 3

$$\frac{dP}{dt} = (\alpha\mu + \beta)X_0 e^{\mu t} \qquad \text{(Eq. 7)}$$

A temperature-dependent rate coefficient for growth represented by Arrhenius's empirical equation is given by:

$$\mu = e^{-\frac{Ea}{RT}} \qquad \text{(Eq. 8)}$$

Where E_a is the activation energy and R is the universal constant of the gases (8.31 10^{-3} J/mol-K) and T is the absolute temperature (K). If we assume that the rate of production is affected directly by fungal growth rate and activation energy.

$$\frac{dP}{dt} = (\alpha\mu + \beta)X_0 e^{\mu \cdot t} \cdot e^{-\frac{E_a}{RT}} \qquad \text{(Eq. 9)}$$

Based on previous experiments (data not shown) it was observed that the activation energy could be adjusted as a quadratic function

$$E_a = b_1 a_w \qquad \text{(Eq. 10)}$$

Thus

$$\frac{dP}{dt} = (\alpha\mu + \beta)X_0 e^{-\frac{b_1 a_w}{RT}} e^{\mu \cdot t} \qquad \text{(Eq. 11)}$$

$$\int_0^P dP = (\alpha\mu + \beta)X_0 e^{-\frac{b_1 a_w}{RT}} \int_0^t e^{\mu \cdot t} dt \qquad \text{(Eq. 12)}$$

After integration

$$P = \left(\alpha + \frac{\beta}{\mu}\right) X_0 e^{-\frac{b_1 a_w}{RT}} \left(e^t - 1\right) \qquad \text{(Eq. 13)}$$

For assessing the relationship between physiological and thermodynamic conditions and AFB_1 production and the expression of the gene clusters involved in toxin

production the physical model was combined with the gene expression data as a linear combination. The generic cluster can be described as a linear function:

$$[g] = a_1 aflD + a_2 aflR + a_3 aflS + a_4 aflE + a_5 aflM + a_6 aflO + a_7 aflP + a_8 aflQ \; a_9 aflX + a_{10} aflF \quad \text{(Eq. 14)}$$

where a_1 to a_{10} are the parameter estimates from the linear combination of the expression of genes so that [g] represents the sum of the effect of the individual genes expressed under specific conditions. The final model which considers a_w, temperature, growth rate and gene expression on the regulation of AFB_1 is given by:

$$P = [g] \times \left(\alpha + \frac{\beta}{\mu} \right) X_0 e^{-\frac{b_1 a_w}{RT}} \left(e^{\mu \cdot t} - 1 \right) \quad \text{(Eq. 15)}$$

where P is the AFB_1 production ( ) and b1, α, and β are parameter estimates from the model and μ was calculated based on a period of 9 days growth and the assumption that growth occurs in cylindrical fungal hyphal extension with a constant radius simplified as follows:

$$= \ln \; — \; = \ln \; \frac{\times}{\times} \; = \ln \; \frac{\times —}{\times —} \; = \ln \; \frac{\times \; \times}{\times \; \times} \; = \quad — \quad \text{(Eq. 16)}$$

Where X_f and X_i are final and initial biomass, m_f and m_i are initial and final fungal mass, v_f and v_i are the initial and final fungal road volume, r_A is the *Aspergillus flavus* hyphal radius and L_f and L_i are the radial growth and ρ is the fungal density.

Table 1 shows the actual mean data (n=3) for AFB_1 production and that predicted by the model in relation to different combinations of temperature and a_w, the gene expression data, and the relative mean growth rate.

Table 2 shows the ANOVA for the fit of the model and the regressed coefficients and the corrected totals. This suggests that the model showed a good fit to the data and was statistically significant. Table 3 shows the overall estimates and the dependence for the main areas that are related to the model. All the parameters are statistically significant at $p<0.01$. The model fit for the observed vs. the predicted effects on AFB_1 production (μg g^{-1}) gave a good correlation between the parameters ($r^2=0.9495$; residual square difference $R_{msd}=0.0440$). The model was used to construct contour

maps of the relationship between environmental factors, growth and AFB_1 production (Figure 2 a, b). This shows that optimum growth was at about 27°C and 0.98 a_w. The marginal conditions for growth were in the region <0.90 a_w and temperatures <20°C and >35°C. For AFB_1 production optimum conditions were at 0.98-0.99 a_w and 25-33°C.

3.3. Validation of the model

The model was subsequently tested to examine whether it could be used at temperatures of 37 and 40°C at different a_w levels. These conditions were not originally included in the model because of the limited data at these conditions. Table 4 shows the effect on growth rate, and on the observed and predicted AFB_1 production under these conditions. At 37°C and 0.90, 0.95 and 0.99 a_w the model predicted slightly higher AFB_1 production than was actually observed. The divergence between the predicted and observed at 0.95 to 0.99 a_w increased from 30% to 56% at this temperature. At 40°C and 0.90 a_w there was very slow growth but no AFB_1 produced. This was similar to the predicted value.

3.3. Ternary relationships between gene expression, environmental factors and aflatoxin B_1 production.

By using a standardised signal from the microarray data set for some of the key structural and regulatory genes it was possible to examine the relative relationship between the activity of either *aflD* or *aflM* and the two regulatory genes *aflR* and *aflS* at the same time in ternary diagrams in relation to a_w, temperature and AFB_1 production. Figure 3 shows the effect of a_w and temperature on the relative gene expression (standardised) of the genes *aflD*, *aflS* and *aflR*. These were calculated using the relative fractions (*f*) of the three individual genes as shown below:

$$= 1.37 \frac{}{(\qquad)} + 0.840 \frac{}{(\qquad)} + 0.982 \frac{}{(\qquad)} \quad \text{(Eq. 17)}$$

and for temperature:

(℃)

$$= 55.69 \frac{}{(\quad + \quad + \quad)}$$

$$+ 25.49 \frac{}{(\quad + \quad + \quad)} + 19.13 \frac{}{(\quad + \quad + \quad)}$$

(Eq. 18)

As the a_w increased, the expression of *aflD* was reduced. The regulatory genes *aflR* and *aflS* were less sensitive to water availability. From the model these genes have a similar sensitivity to a_w. For temperature, the genes *aflD* and *aflR* expression were inversely related to this parameter. As the temperature was increased the expression of *aflD* and *aflR* was reduced and that of *aflS* increased. The model shows higher co-efficients of *aflD* and *aflR* suggesting that a slight change in temperature causes a large change in expression of these genes.

Similar ternary diagrams were constructed for the interaction between *aflM* and the regulatory genes *aflR* and *aflS* (Figure 4). The relative fraction (*f*) of the three genes was calculated in relation to a_w and temperature:

For a_w this was:

$$= 0.97 \frac{}{(\quad)} + 0.876 \frac{}{(\quad)} + 0.991 \frac{}{(\quad)}$$

(Eq. 19)

and for temperature:

$$T(℃) = 47.95 \frac{}{(\quad)} + 30.52 \frac{}{(\quad)} + 17.53 \frac{}{(\quad)}$$

(Eq. 20)

The effect of interactions between *aflM*, *aflS* and *aflR* showed that with more available water (e.g. 0.98 a_w) there was a higher expression of the genes *aflM* and *aflS* (Figure 5a). In contrast, under drier conditions, there was a higher *aflR* signal. There was an inverse proportional effect of temperature on the expression of *aflM* and *aflR*.

Thus the higher the temperature, the lower *aflR* and higher *aflM* gene signal (Figure 5b). The expression of *aflS* was similar across a wide spectrum of temperatures.

Figure 5 shows the relative expression of (a) *aflD, aflR* and *aflS* and (b) *aflM, aflR* and *aflS* in relation to AFB_1 production. The relationships were calculated based on:

$$\text{aflatoxin B production } (\mu g\ g\ \ ) = 3.66\ \frac{}{(\quad + \quad + \quad)} - 0.802\ \frac{}{(\quad + \quad + \quad)} + 1.91\ \frac{}{(\quad + \quad + \quad)} \qquad \text{(Eq. 21)}$$

and for *aflM* and the regulatory genes:

$$\text{aflatoxin B production } (\mu g\ g\ \ ) = 3.21\ \frac{}{(\quad + \quad + \quad)} - 0.63\ \frac{}{(\quad + \quad + \quad)} + 1.85\ \frac{}{(\quad + \quad + \quad)} \qquad \text{(Eq. 22)}$$

4 DISCUSSION

This study has examined the relationship between growth of *A. flavus* and the impact that environmental factors can have on key structural and regulatory genes and the impact that this will have on AFB_1 production. This has shown that temperature and water availability have a profound effect on both gene expression of key biosynthetic genes as well as significantly affecting the phenotypic production of the toxic secondary metabolite actually quantified analytically.

The data has shown that while growth can occur over a wider range of temperatures x a_w levels, AFB_1 production is over a narrower range of conditions. Thus optimum conditions for growth of this strain of *A. flavus* were 30-35^{o}C and 0.99

a_w, with marginal conditions at 15 and 40°C at 0.99 a_w. For AFB_1 production optimum conditions were 25-30°C at 0.99 a_w and this changed to 30-35°C at 0.95 a_w. While few previous studies have considered interactions between environmental conditions, Sanchis and Magan (2004) did integrate data based on growth and AFB_1 production on different nutritional matrices and this showed that optimum a_w and temperatures on groundnuts were: 0.94 a_w and 34°C for growth and 0.99 a_w and 32°C for AFB_1 production. More recently, an Italian isolate of *A. flavus* from maize was shown to have a wide temperature tolerance range for growth (15-45°C) but much narrower for AFB_1 (20-35°C) production (Giorni *et al.* 2011).

In the present study expression data were available for the whole aflatoxin gene cluster. However, we decided to use 8 key biosynthetic genes and the two regulatory genes which were relevant to the biosynthesis of AFB_1 (Yu *et al.* 2002; Georgianna and Payne 2009). This showed that both temperature and a_w influenced their relative expression. Schmidt-Heydt *et al.* (2009, 2010) showed that the ratio of the regulatory genes *aflR* and *aflS* may be important, as a low ratio under certain a_w x temperature levels resulted in low AFB_1 production while higher ratios resulted in significantly higher toxin production in both *A. flavus* and *A. parasiticus*. Because of this we examined the relative activity of two genes (*aflD, aflM*) present in the early part of the biosynthetic pathway with the regulatory genes in relation to changes in a_w and temperature. This showed that there was some relationship which was influenced by both temperature and water stress.

O'Brian *et al.* (2007) analysed the influence of elevated temperature on aflatoxin gene expression. They found by microarray analysis a differential expression of certain genes at 28 °C, which was conducive, compared to 37 °C which was repressive, for aflatoxin biosynthesis in their strain of *A. flavus*. However, in agreement with the data presented here, transcript levels of both *aflR* and *aflS* did not change significantly between these temperatures.

It is necessary to also consider this study in the context of what might happen when biotic variables are included. Thus, the interaction with ripening maize kernels, especially where plant physiological and nutritional effects may be important, also needs to be considered (Georgianna & Payne, 2009). Some relevance from the present study can be inferred from the changes in a_w of maize kernels in ripening maize cobs. At the early dough stage the moisture content (m.c.) is about 40% (=0.99 a_w) with no water stress effects, this decreases to 30-35% m.c. (= 0.95 a_w) at the mid-dough stage

and to 20-25% (0.90-0.85 a_w) at full maturity over a period of about 4-6 weeks (Brooking, 1990). This will influence infection and colonisation by *A. flavus*. Recently, Giorni *et al.* (2011) showed that the nutritional media made from maize kernels at different ripening stages had little effect on growth of *A. flavus* and AFB_1 production. This suggests that a_w x temperature stresses may play an important role in influencing both gene expression and aflatoxin contamination in the fungus/plant interface during this period. This may further influence the interaction with other mycobiota which colonise the ripening maize cobs during these critical phases of plant development.

Oxidative stress may also be an important factor as it has been shown that this can stimulate aflatoxin production (Reverberi *et al.* 2008). For example, antioxidants such as gallic acid found in walnuts were found to inhibit several aflatoxin biosynthetic pathway genes including *aflD* and *aflM* (Mahoney and Molyneux 2004). Kim *et al.* (2008) showed that caffeic acid down regulated most of the aflatoxin biosynthetic genes. These studies suggest that several oxidative stress genes such as catalases, super oxidase dismutases and MAPKinase genes may be required to overcome such stress. These effects may be further influenced by environmental parameters which may add another layer of complexity to attempts to model the system.

Recently Abdel-Hadi *et al.* (2010) suggested that relative expression of *aflD* was an important indicator of colonisation under different environmental regimes. They were able to develop a contour map in relation to a_w and to time of colonisation of groundnuts. While the expression of *aflD* was not found to be directly correlated with populations of *A. flavus* (CFUs) isolated from stored groundnuts its activity showed some pattern in relation to the relative amount of water stress imposed.

Very few attempts have been made to try and integrate molecular expression data under different environmental stresses with phenotypic secondary metabolite data to develop predictive models. Schmidt-Heydt *et al.* (2011) used a polynomial model to examine the relationship between ecophysiological factors, growth and deoxynivalenol production by strains of *Fusarium culmorum* and *F. graminearum* for the first time. This suggested that it was possible to use quantitative PCR data for specific genes (e.g. *TRI5, TRI6*) under different environmental conditions to predict DON production using contour maps. However, validation was not possible using this model. The present study has used a mixed growth model to try and relate the relative

expression of 10 biosynthetic genes under different interacting environmental factors to growth and AFB_1 production. This made it possible to use this approach to develop a predictive model which gave a good relationship between the observed and predicted AFB_1 production. Contour maps could be developed to show the relationship between a_w x temperature on AFB_1 production. It also enabled the inclusion of the gene expression data for 10 genes as a linear function to relate this to growth and toxin production under a range of interacting stress conditions. Validation of the mixed growth model was possible under conditions which were not included in the model development. Thus the effect of slight changes in temperature to 35^{o}C and 40^{o}C at different water stress levels could be examined. The model gave a better fit under some conditions, but deviated markedly under very wet conditions, which are conducive to growth. The approach could be a powerful tool in examining the impact that climate change factors, including elevated temperature conditions, water stress and elevated CO_2 have on growth, gene expression and potential for toxin production (Magan *et al.* 2011).

The development of ternary diagrams to examine the relationship between structural and regulatory genes in relation to environmental conditions and toxin production has not been studied in detail previously. This has shown that the relative expression of *aflD* or *aflM* to that of *aflR* and *aflS* is important, and is related to and influenced by both a_w and temperature. The fact that *aflS* expression remains relatively consistent across a range of interacting environmental conditions suggests that this is a key regulatory gene in the biosynthetic pathway for aflatoxin biosynthesis. The relative expression of sugar transporter genes (*nadA, hxtA, glcA, sugR*) may also be important, especially under water stress conditions where utilisation of alternative carbon sources can be important. It has been shown that this cluster of genes is important in *A. parasiticus* and may be related to the aflatoxin biosynthetic gene cluster (Yu *et al.* 2000). This approach may enable the links between different genes to be examined in order to better understand how they impact on the phenotypic production of aflatoxins.

We believe that the effective integration of molecular, ecophysiological and secondary metabolite data sets could be critical in predicting the relative risk of mycotoxin contamination under different biotic and abiotic stress scenarios which could have an impact on both food quality and security (Magan *et al.* 2011).

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Table 1. Experimental data and model estimation for aflatoxin B_1 at different temperatures, water activity and observed cluster gene expression for the 10 genes and the effect on growth rate.

Temp. (°C)	a_w	*aflD*	*aflR*	*aflS*	*aflE*	*aflM*	*aflO*	*aflP*	*adlQ*	*aflX*	*aflF*	μ±S.E. (cm day^{-1})	Aflatoxin B_1 (μg g^{-1}) Observed	Predicted
20	0.99	1887	1897	20645	6940	4982	3341	1571	1918	1937	1404	0.406±0.002	1.703	1.701
25	0.9	1472	4063	2207	3654	1810	1696	1399	1705	2903	1301	0.127±0.000	0.354	0.336
25	0.95	217	241	1785	278	288	237	269	325	243	354	0.580±0.003	0.857	0.859
25	0.99	505	890	1335	628	553	1155	548	566	659	589	0.572±0.002	3.289	3.289
30	0.9	1362	1187	1751	1986	1569	1554	1656	1757	3895	1160	0.187±0.003	0.541	0.509
30	0.95	315	186	1323	363	516	390	391	340	346	303	0.577±0.000	1.641	1.637
30	0.99	266	286	793	369	429	1	509	193	354	232	0.679±0.004	2.788	2.786
35	0.9	1344	3389	1134	1741	1418	1436	1241	1070	1505	1255	0.205±0.001	0.000	0.006
35	0.95	203	259	141	212	337	248	253	159	180	216	0.588±0.004	1.678	1.677
35	0.99	400	313	312	295	238	282	269	230	326	233	0.687±0.000	1.881	1.881

Table 2. Analysis of variance of the developed model

	SS	**DF**	**MS**	**F-value**	***p*-value**
Regression	92.26	14	6.59	63.90	0.0000
Residual	1.44	14	0.10		
Total	93.71	28			
Corrected Total	30.07	27			
Regression vs. Corrected Total	92.26	14	6.59	5.91	0.0000

SS= Sum of squares, DF= Degrees of freedom, MS= Mean squares

Table 3. The estimates and the dependence on the main areas that are related to the model. All the parameters are statistically significant at $p<0.01$.

	Parameter	**Estimate**	**p-level**
Gene cluster for aflatoxin biosynthesis	$\mathbf{a_1}$ *(aflD)*	0.4874464	0.00
	$\mathbf{a_2}$ *(aflR)*	-0.5495745	0.00
	$\mathbf{a_3}$ *(aflS)*	-0.0168165	0.00
	$\mathbf{a_4}$ *(aflE)*	-0.0785165	0.00
	$\mathbf{a_5}$ *(aflM)*	0.2303169	0.00
	$\mathbf{a_6}$ *(aflO)*	0.2979484	0.00
	$\mathbf{a_7}$ *(aflP)*	0.155606	0.00
	$\mathbf{a_8}$ *(aflQ)*	-2.527789	0.00
	$\mathbf{a_9}$ *(aflX)*	1.90013	0.00
	$\mathbf{a_{10}}$ *(aflF)*	0.4844927	0.00
Mixed-growth-associated product formation	$\boldsymbol{\alpha}$	0.1963598	0.00
	$\boldsymbol{\beta}$	-0.0048802	0.00
Initial inoculum	$\mathbf{X_0}$	0.0027675	0.00
Arrhenius's coefficient	$\mathbf{b_1}$	-401.0787	0.00

Table 4. Model validation outside the regions in which the model was developed

Factors		**Condition**		
Temperature (^{o}C)	37	37	37	40
Water activity (a_w)	0.90	0.95	0.99	0.9
Growth rate (cm day^{-1})	0.29	0.59	0.42	0.14
Observed aflatoxin production (μg^{-1})	3.96±0.20	2.68±0.14	2.42±0.16	0.00
Predicted aflatoxin production (μg^{-1})	4.90±0.00	3.75±0.18	3.78±0.14	0.00

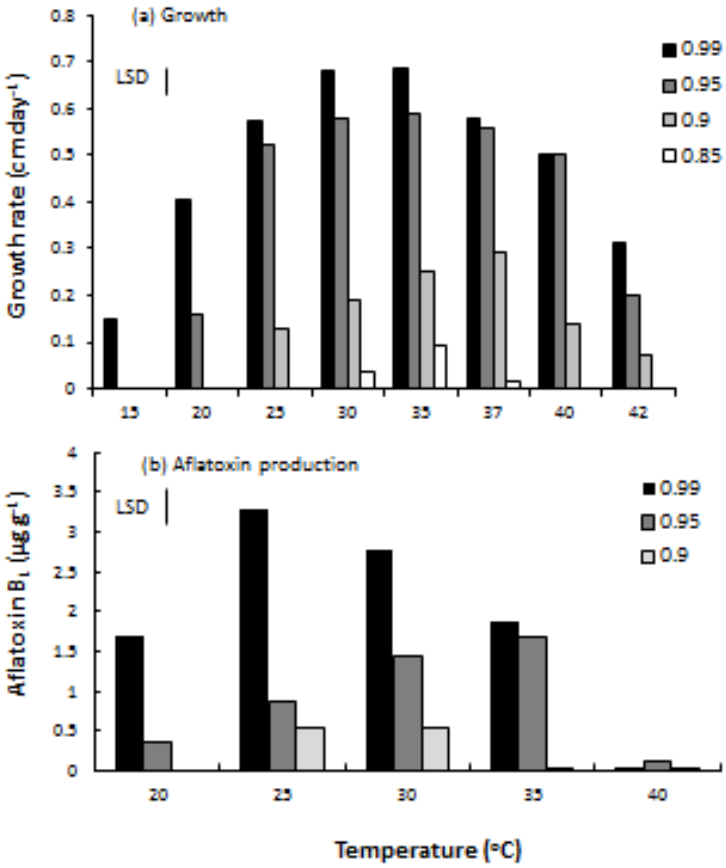

Figure 1. Abdel-Hadi et al.

Figure 1. Effect of water activity x temperature effects on (a) mean growth rate and (b) aflatoxin B_1 production on a conducive YES medium grown for 10 days. LSD, Least Significant Difference (P=0.05).

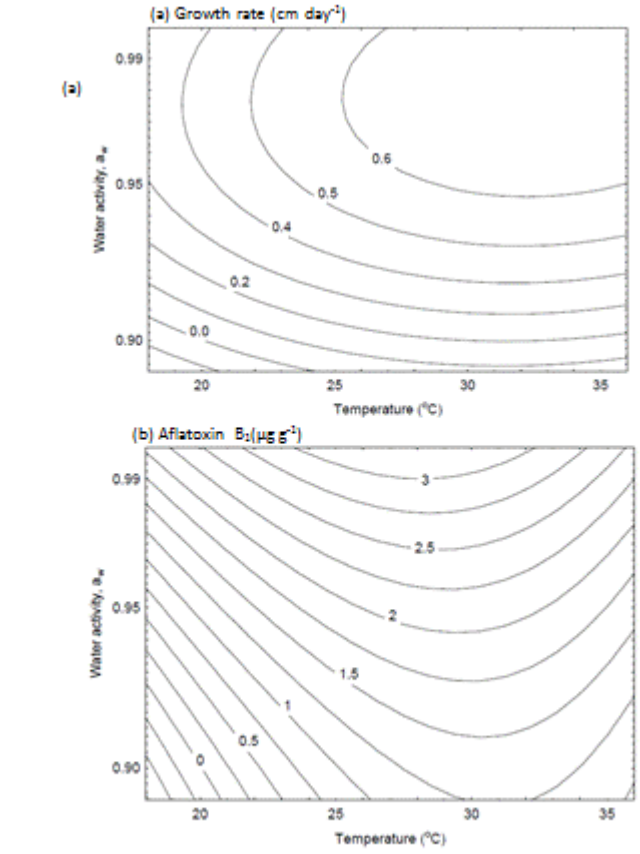

Figure 2. Abdel-Hadi et al.

Figure 2. (a) Contour plot for temperature *vs.* water activity on growth rate (mm day^{-1}). Numbers on the isopleths lines join conditions of the same growth rate; (b) Contour plot for effect of temperature *vs.* water activity conditions on aflatoxin B_1 production (µg g^{-1}). The isopleths lines join conditions at which similar quantities of aflatoxin B_1 are produced.

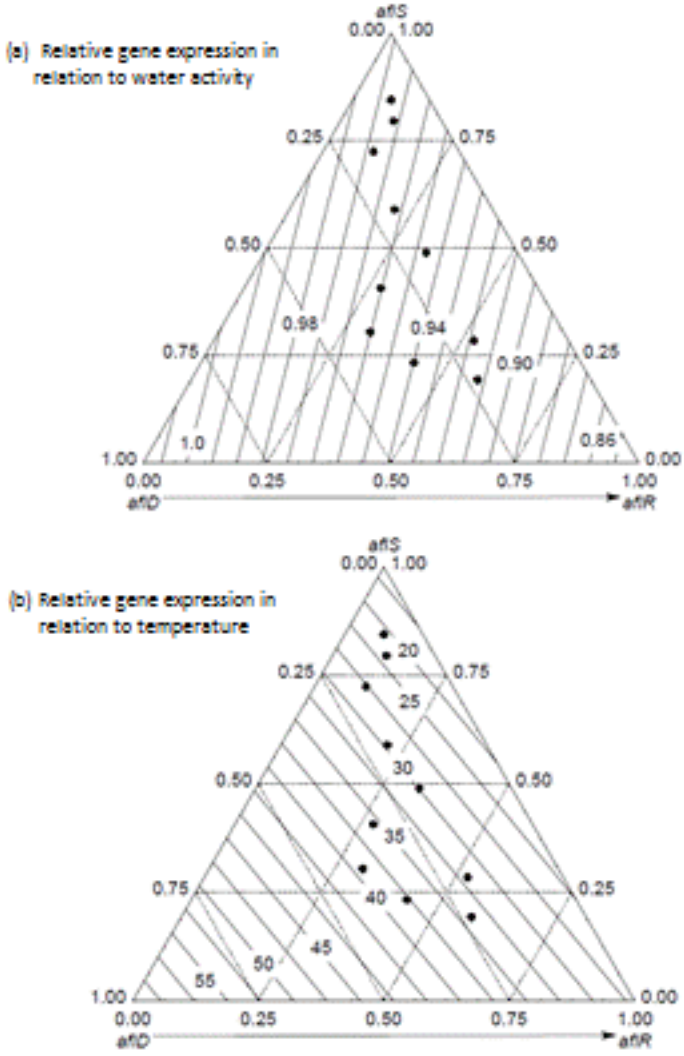

Figure 3. Abdel-Hadi et al.

Figure 3. Ternary diagrams of the relative relationship between expression of *aflD*, *aflR* and *aflS* in response to (a) water activity in the range 0.86 to 1.00 and (b) temperature in the range 15 to 55^{o}C. Legends indicate relative expression. The closed circles (●) indicate the experimental data in relation to gene expression.

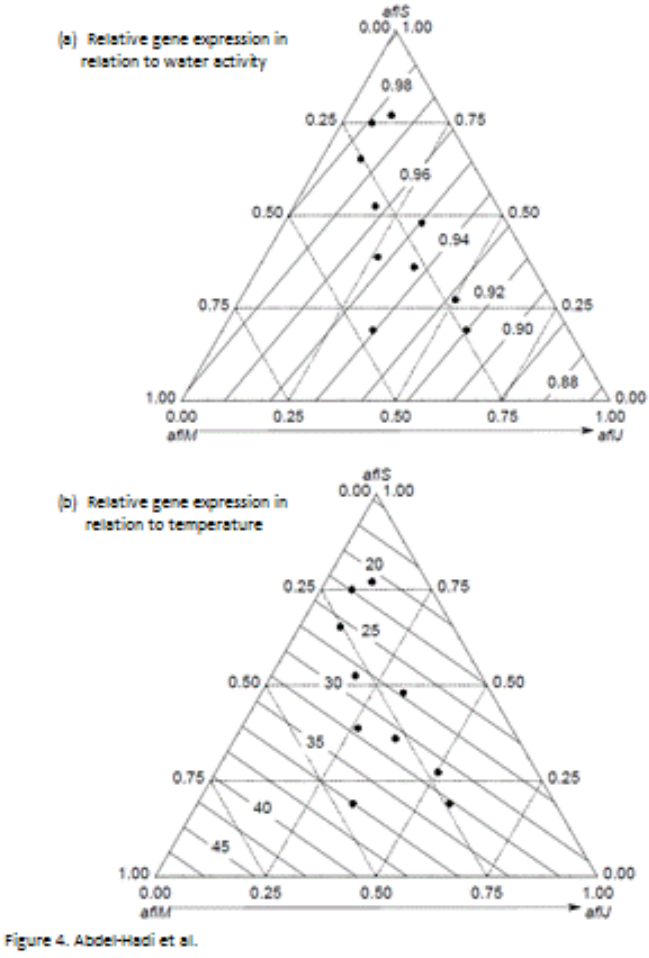

Figure 4. Abdel-Hadi et al.

Figure 4. Ternary diagram of the relative relationship of expression of *aflM*, *aflR*, and *aflS* in response to (a) water activity in the range 0.90 and 0.98 and (b) temperature in the range 20 to 45^{o}C. Legends indicate relative expression of each gene. Closed circles (●) indicate the experimental data in relation to gene expression.

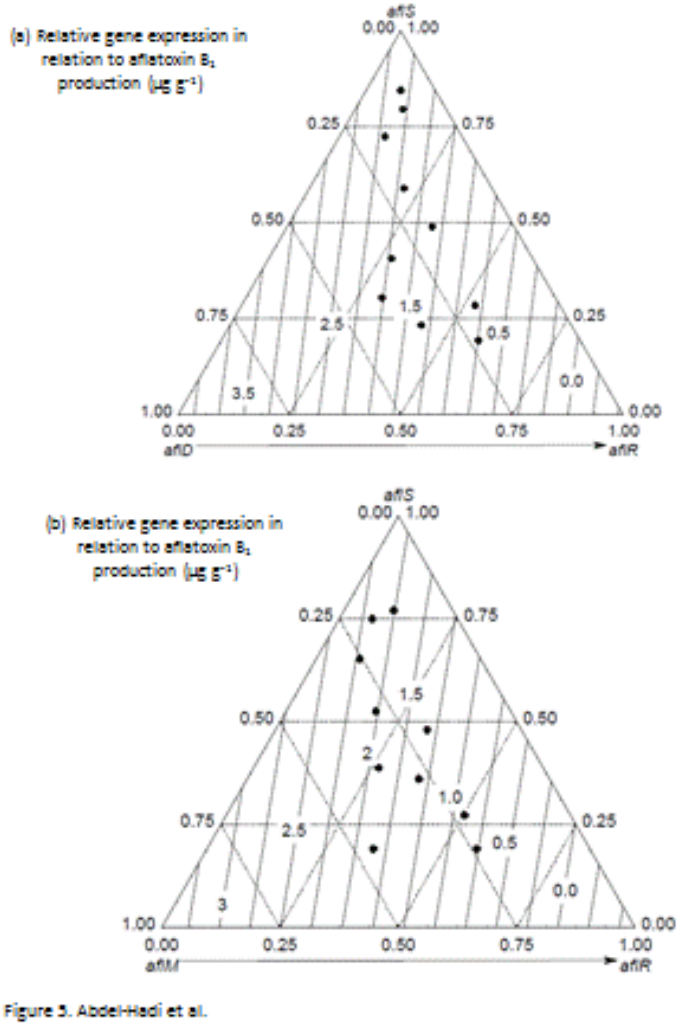

Figure 5. Abdel-Hadi et al.

Figure 5. Ternary diagram of relative expression of (a) *aflD*, *aflR*, and *aflS* and (b) *aflM*, *aflR* and *aflS* on aflatoxin B_1 production (µg g^{-1}). Legends indicate relative expression of each gene. The closed circles (●) represent the experimental data in relation to gene expression.