Unveiling the effect of interacting forecasted abiotic factors on growth and Aflatoxin B_1 production kinetics by *Aspergillus flavus*

Esther GARCIA-CELA[1,2]† and Carol VERHEECKE-VAESSEN[1,†], Maria GUTIERREZ-POZO[1,3], Elisavet KIAITSI[1], Alessandra MARCON-GASPERINI[1], Naresh MAGAN[1], Angel MEDINA*

[1]Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, Beds. MK43 0AL, UK

[2]Biological and Environmental Sciences, School of Life and Medical Sciences, University of Hertfordshire, Hatfield, AL109AB, UK

[3]Surface Engineering and Precision Institute, Cranfield University, Cranfield, Beds. MK43 0AL, UK

† the two authors contributed equally to the work.

*Corresponding authors: Dr Angel Medina, Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, Beds. MK430AL, U.K. E-mail: a.medinavaya@cranfield.ac.uk

ABSTRACT (200 words)

The aim was to decipher the temporal impact of key interacting climate change (CC) abiotic factors of temperature (30 vs 37 °C), water activity (a_w; 0.985 vs 0.930) and CO_2 exposure (400 vs 1000 ppm) on (a) growth of *Aspergillus flavus* and effects on (b) gene expression of a structural (*aflD*) and key regulatory gene (*aflR*) involved in aflatoxin B_1 (AFB_1) biosynthesis and (c) AFB_1 production on a yeast extract sucrose medium over a period of 10 days. *A. flavus* grew and produced AFB_1 very early with toxin detected after only 48 hours. Both growth and toxin production were significantly impacted by the interacting abiotic factors. The relative expression of the *aflD* gene was significantly influenced by temperature; *aflR* gene expression was mainly modulated by time. However, no clear relationship was observed for both genes with AFB_1 production over the experimental time frame. The optimum temperature for AFB_1 production was 30°C. Maximum AFB_1 production occurred between days 4-8. Exposure to higher CO_2 conditions simulating forecasted CC conditions, the amount of AFB_1 produced in elevated temperature (37 °C) was higher than with the optimum temperature (30 °C) showing a potential for increased risk for human/animal health due to higher accumulation of AFB_1.

KEYWORDS:

mycotoxins, water activity, toxin gene expression, elevated CO_2, *aflD*, *aflR*.

INTRODUCTION

Aspergillus flavus is an important primary and secondary pathogen of maize, tree nuts and peanuts and can contaminate these commodities with the class 1 carcinogenic mycotoxin: Aflatoxin B_1 (AFB_1) (IARC, 2012). It has now been shown that in some Lower Middle Income Countries (LMICs), especially in West Africa, consumption of a predominantly maize-based diet can lead to stunting in children due to exposure to AFB_1 (IARC, 2012).

Recent forecasts of climate change prediction are suggesting different scenarios of temperature and CO_2 changes depending on socio-economic behaviour which could lead to increases from +3.5 to 7.5°C and from 500 to 1200 ppm (Gidden *et al.*, 2018). Moreover, there have been concerns that extreme weather events could exacerbate the contamination of staple commodities with mycotoxins including AFB_1. Some studies have suggested that environmental stress may lead to increases in mycotoxin production, or indeed a switch to other toxigenic secondary metabolites (Garcia-Cela et al., 2014; Gilbert et al., 2017; Medina et al., 2014; Medina et al., 2015 a,c; Verheecke-Vaessen et al., 2019). It has been previously shown that on a conducive yeast extract sucrose (YES) medium, individual abiotic factors such as temperature, water availability, or exposure to elevated CO_2 alone have less modifying effects on the ecology of mycotoxigenic fungi than when combined (Medina et al., 2015a). However, interactions between these factors may have a synergistic impact on either growth and/or production of mycotoxins. Recent studies have shown variable effects of such interacting abiotic factors with little effect on growth or mycotoxin production (e.g. *A. carbonarius*, ochratoxin A) while for others such as *A. westerdijkiae* and *A. flavus* species colonising maize and coffee there was a stimulation of mycotoxin production, although less effects on growth (Medina et al., 2015a; Akbar et al., 2016). However, most of these studies have involved a single time point for analyses of impacts of the interacting abiotic climate-related factors. There is little knowledge of the temporal changes which

may occur in growth or mycotoxin production under existing or future climate-related interacting abiotic factors.

The objective of this study was to examine the kinetics of the impact of interacting climate-related abiotic factors of temperature (30 vs 37 °C), water activity (a_w; 0.985 vs 0.930) and CO_2 exposure (400 vs 1000 ppm) on (a) lag times prior to growth and colonisation rates, (b) relative temporal expression of structural (*aflD*) and regulatory (*aflR*) biosynthetic genes involved in toxin production and (c) effects on phenotypic AFB_1 production by *A. flavus* on a conducive YES medium over a period of 10 days.

MATERIALS AND METHODS

Fungal strains

An aflatoxigenic type strain of *A. flavus* (NRRL 3357; Northern Regional Research Laboratories (NRRL) of the US Department of Agriculture USDA, New Orleans) was used in these experiments. The strain was maintained in glycerol:water (70:30, v/v) at -20 °C in the culture collection of the Applied Mycology Group, Cranfield University,UK.

Media preparation and inoculation

The water activity (a_w) of the media was modified by the addition of different amounts of glycerol to the water which was mixed and used as water to prepare the treatment media (0.985, 0.930). A standard curve was prepared of the amounts of glycerol/water solutions necessary to modify the YES medium to the target treatment a_w levels. This was added to the YES media (2% yeast extract, 15% sucrose, 0.05% $MgSO_47H_2O$). The accuracy of the modifications was confirmed using an Aqualab 4TE instrument (Decagon, Pullman, WA, US) and found to be within ±0.01 of the target a_w levels.

Spore suspensions of conidia were prepared from 7-days old colonies of *A. flavus* on MEA (3% malt extract, 5% mycological peptone, 1.5% agar) incubated at 25 °C. The conidial concentration was measured using a haemocytometer (Thoma Cell, Fisher Scientific) and the concentration diluted with sterile water to obtain a concentration of 10^6 conidia mL^{-1}.

The different a_w agar media were poured into sterile Petri dishes (Ø 9 cm) in a sterile safety cabinet and allowed to cool. The treatments and replicates were centrally inoculated with *A. flavus* conidial spore suspensions (5 µl; 10^6 spores mL^{-1}). Three replicates were destructively sampled

for growth measurements, mycotoxins and gene expression analysis every two days over the 10-days period.

Exposure of* A. flavus *treatments to interacting abiotic factors

The different treatments and replicates were placed in separate 13 L plastic environmental chambers (Verheecke-Vaessen et al., 2019). These chambers also contained an inlet and outlet with valves for flushing with CO_2. The chambers contained glycerol/water solutions to maintain the ERH of the atmosphere within the individual chambers to maintain the target a_w levels. The chambers were flushed with either air or 1000 ppm CO_2 daily for 10 days. The gas cylinders contained either 400 ppm CO_2 (ambient air) or air supplemented with CO_2 to obtain a 1000 ppm CO_2 concentration from a speciality certified gas cylinder (British Oxygen Company, Guildford, Surrey, U.K.). The containers were flushed at 3 L min^{-1} to replace 3x the volume of the incubation chamber. The chambers were incubated at either 30 or 37°C.

To calculate the fungal colonisation rates the diametric size of the colonies was measured daily in two perpendicular directions. Every two days, three replicates were destructively sampled and the fungal biomass harvested by scratching the plate surface and stored at -80 °C for gene expression analysis and 2-5 plugs (using a 5 mm surface-sterilised cork borer) across the *A. flavus* colony and media were collected. The plugs were placed in a pre-weighed 2 mL Eppendorf tube, weighed again and sealed before being stored at -20 °C for mycotoxin analysis.

AFB_1 extraction and quantification

Samples were thawed and extracted by mixing the agar plugs with 750 µL of methanol. The tubes were shaken for 90 min at 400 g at 25 °C in the dark. They were then centrifuged at 13,000 g for 15 min and all of supernatant removed and the plugs were re-extracted again in the same way. Extracts were dried in a miVac evaporator (Genevac, Ipswich, UK) for 6 h. The samples

were resuspended in 1000 µL of methanol:water (50:50, v:v) and filtered through a 0.22 µm Nylon-filter (type) into HPLC vials and stored at −20 °C until analyses.

The samples were injected into a HPLC-FLD Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with post-column derivatisation with a UVE photochemical reactor with UV-Light (LCTech GmbH, Germany) and a fluorescence detector (λ_{exc} 360 nm; λ_{exc} 440 nm). A C_{18} column (Agilent Zorbax® Eclipse Plus, 2.1x100mm, 3.5 µm particle size) preceded by a Phenomenex® Gemini C_{18} guard column cartridge 3 mm x 3 µm (Phenomenex, California, USA) was used for separation. Isocratic elution with methanol:water:acetonitrile (30:60:15, v/v/v) mobile phase and the flow rate at 1.0 mL min^{-1} were used. AFB_1 was quantified on the base of the HPLC fluorimetric response compared with a range of mycotoxin standards supplied by Romer Labs (Romer Labs Ltd, UK) (0.5-2,010 ng mL^{-1}, R^2=0.99). Samples outside of the range were appropriately diluted with methanol:water (50:50, v:v). AFB_1 retention time was 9.5 min and the limit of quantification (LOQ) was 0.5 ng mL^{-1} based on a signal-to-noise ratio 10:1. Signals were processed using the Agilent Chem-Station software Ver. B Rev: 03.01 [317] (Agilent Technologies, Palo Alto, CA, USA). Method validation and recovery were determined by directly spiking both media with AFB_1 standard solutions. Once autoclaved, the media was cooled down to 56 °C, then AFB_1 standard was added onto the media, mixed vigorously and poured in Petri plates (Ø 5 mm). After media solidification three agar plugs were collected across the plate and the mycotoxins extracted as described previously. The recovery for AFB_1 for three replicates was 88.3%± 4.9.

Relative expression of the* aflD *and* aflR *genes

Fungal RNA was extracted from the fungal biomass using the method previously described (Verheecke-Vaessen et al., 2019). The RNA obtained was checked for integrity by electrophoresis

(a ratio of intensity 25S/18S higher than 2 was visually observed) and purity ($ratio_{260/280}$=2-2.2, $ratio_{260/230}$>2) by Genova Nano (Jenway, Stone, UK), and stored at –80 ºC. Reverse transcription was performed using the Omniscript® Reverse Transcription Kit (Qiagen, Hilden, Germany) using $Oligo(dT)_{18}$ following the manufacturers' instructions. The qPCR targeting *aflD*, *aflR* and *βtub* (reference gene) was performed as described previously (Medina et al., 2015c).

Statistical Analysis

JMP® 14 (SAS Institute Inc., 2016. Cary, NC, USA) was used to perform statistical analyses. Normality and homoscedasticity of each dataset (lag time, growth rate, aflatoxins and gene expression) was checked using Shapiro-Wilk test and Brown-Forsythe test. Lag time, gene expression and growth rate (square root transformed) data showed both normality and homoscedasticity; thus ANOVA was used to investigate significant differences. A Student *t*-test was done to compare each pair (*p-value* ≤ 0.05).

For AFB_1 production data and gene expression, non-normality or variance homogeneity was confirmed even after multiple transformation trials were performed. Thus, a non-parametric test, Kruskal-Wallis, was performed. When significant differences were found (*p-value* ≤ 0.05), each pair were compared by a *post-hoc* Wilcoxon method. For gene expression and toxin production comparison, the Spearman test was used.

RESULTS

Effect of three-way interacting climate change environmental factors on **A. flavus** *growth on YES media*

The lag phases prior to growth were shorter as a_w was increased (x 2 to x 4 fold; Table 1). The exposure to higher CO_2 generally reduced these lag times. This was significant at 37 °C, 0.930 with a 3.6-fold reduction in lag phase due to the higher CO_2 exposure. Colonisation was significantly higher at 0.985 a_w. The optimal growth occurred at 30 °C and 0.985 a_w. Generally, the elevated CO_2 significantly reduced the colonisation rate, except at 37 °C and 0.985 a_w. Under drier conditions (0.93 a_w) and elevated CO_2 the growth rates were the lowest at both temperatures.

The statiscal analysis compared the effect of interacting abiotic climate-related factors on the lag phases prior to growth and growth rates. Statistically, temperature (30 vs 37 °C), a_w (0.985 vs 0.930) and CO_2 exposure (400 vs 1000 ppm) effects on growth rates were compared. Both lag times prior to growth and growth rates were significantly affected (*p-value* = <0.0003 and *p-value* = <0.0001, respectively). Temperature had a significant impact on the lag phase (*p-value* = <0.0086) and a_w had a significant effect on growth rate (*p-value* = <0.0001) and CO_2 to a lesser extent (*p-value* = 0.081).

Influence of climate change related abiotic factors on kinetics of the expression of the AFB_1 *biosynthetic genes* **aflD** *and* **aflR**

The effect of temperature (30 and 37 °C), a_w (0.930 and 0.985) and CO_2 exposure (400 and 1000 ppm) on the kinetics of *aflD* and *aflR* gene expression was studied and the results are presented in Figure 1 and Supplementary Figure 1. Overall, the gene expression pattern of both genes was significantly impacted by climate change interacting abiotic factors.

For the structural gene *aflD*, an increase in temperature led to a general decrease of gene expression over the experimental period, independently of the a_w studied. Comparisons between current conditions (30 °C; 0.985; 400 ppm; Figure 1b) and future scenarios (37 °C; 0.93; 1000 ppm; Figure 1c) showed a general decrease in *aflD* gene expression especially after 8 and 10 days. For the biosynthetic gene *aflR*, encoding the aflatoxin transcription factor, gene expression was generally down regulated through time except at 37 °C/0.985 in elevated CO_2 (Supplementary Figure 1). The down regulation was higher at 0.930 a_w independently of the temperature tested. Generally, the downregulation increased over time at 30 °C, while this decreased over time at 37 °C (Figure 1). When looking into the effects of time, the gene expression of both the *aflD* and *aflR* genes was highest on day 4, regardless of the treatment (see Figure 1). By day 4, under current CO_2 concentration (400 ppm) and 30 °C and 0.930 a_w, there was a significant increase in the expression of the *aflR* gene (Figure 1a). In contrast, the structural *aflD* gene expression was generally higher after 6 to 10 days growth when compared with day 4.

Interestingly, exposure of *A. flavus* colonies to elevated CO_2 resulted in a significant reduction in the *aflR* gene expression after 4 days, with no subsequent temporal changes. Conversely, *aflD* gene expression was optimum after day 4-6. At 37 °C in the 0.930 a_w (see Figure 1c) treatment, the gene expression was higher after 4 days for both genes.

Further analysis was performed to study the impact of the three-way interacting climate change factors on gene expression on the YES media (Table 2). *aflD* gene expression was significantly impacted by temperature (*p-value* = <0.0001) and *aflR* gene expression might be mainly modulated by time (*p-value* = 0.0729).

Effect of three-way interacting climate change environmental factors on the AFB_1 temporal production on YES

Figure 2 shows the effect of the interacting abiotic climate-related abiotic factors on AFB_1 production. Overall, maximum production occurred at 30 °C. Under current CO_2 conditions (400 ppm), maximum production was found after 6 days at 30 °C and 0.930 a_w (435.7 µg g^{-1}). When *A. flavus* was exposed to elevated CO_2 conditions, the maximum production (117.5 µg g^{-1}) was also found under the same temperature and a_w levels but was lower and delayed until 8 days incubation.

Generally, it was observed that elevated CO_2 enhanced AFB_1 production at 37 °C in the dryer treatment (0.930 a_w). AFB_1 synthesis appeared to occur very early in the development of the colonies of *A. flavus* from conidial inoculum as levels of the toxins were found under all conditions after 2 days incubation.

Over time, at 30 °C, the accumulation of AFB_1 with 400 ppm of atmospheric CO_2 was consistently higher than the accumulation of toxin under elevated CO_2 conditions with a few exceptions (day 8, 0.985 a_w). Interestingly, this trend was completely reversed when the fungus was exposed to temperature stress conditions (37 °C), where additional atmospheric CO_2 led to higher (at 0.930 a_w) and earlier (at day 2, 0.985 a_w) accumulation of AFB_1 at both a_w levels tested.

DISCUSSION

This study has addressed one of the current gaps in knowledge with regards to the impact of interacting abiotic factors on the kinetics of growth and AFB_1 production by *A. flavus*. Most of the previous studies have only analysed effects after a single time point which may not provide the necessary insights into the mechanisms involved in growth, biosynthetic gene expression and mycotoxin production (Gilbert et al., 2017; Medina et al., 2015a,b,c; 2017; Verheecke et al., 2015).

In this work, it was found that an increase in atmospheric CO_2 modified the overall lag time values by 0.1 to 0.5 days and maximum growth rates by <1 mm diameter/day. It was clear that the lag times were significantly influenced, especially by drier conditions (0.930 a_w) and under high temperature (37 ^oC) where the addition of CO_2 shortened the lag times significantly. However, these conditions did not influence growth significantly and the difference in growth rate between elevated and non-elevated CO_2 conditions was only 0.66 mm diameter day^{-1}. This suggests that under combined water and temperature stress conditions elevated CO_2 induces earlier growth of *A. flavus* and hence will confer this species with a competitive advantage under such environments.

Previous studies in relation to three-way interacting climate-related abiotic changes on both YES and maize-based media and maize kernels have suggested very little effect on colonisation rates. However, in ripening maize cobs it has been shown that colonisation by *A. flavus* is similar at both 30 and 37 ^oC and indeed that AFB_1 production may be even higher at 37 ^oC in the milky ripe and dough stages (a_w range: 0.958-0.985; Marcon-Gasperini et al., 2019). However, interaction with elevated CO_2 was not included in these specific studies. Previous studies with *A. flavus* on YES media under 3-way (temperature, a_w and CO_2 concentration) climate-related abiotic conditions showed relatively minimal effect on growth rates (6.5-12 Ø mm day^{-1}; a_w 0.97-0.92 at 34 and 37 °C) (Medina et al., 2015c).

The relative expression of an early structural gene and a regulatory gene (*aflD*, encoding the norsolorinic acid reductase; *aflR*, encoding the main aflatoxin cluster transcription factor, respectively) were found to be very variable in the present study with little direct correlation with phenotypic AFB_1 production. Overall, the relative expression of the *aflD* gene was mostly modulated by temperature, with little effect of elevated CO_2 or drought stress. For the *aflR* gene expression, this was predominantly influenced by time of analysis over the 10 day period, regardless of the climate-related abiotic factors. Usually, biosynthesis of aflatoxins requires the switching on of the early structural genes (including *aflD*) and the biosynthetic gene regulators (*aflR, aflS*). The results suggest that *aflD* is switched on very early during the growth process facilitating subsequent downstream biosynthetic genes being activated. This hence suggests that the activation of *aflR*, which is the transcription factor of the cluster, and hence *aflS*, might occur even earlier than the first sampling point in this work and thus resulting in aflatoxins production by day 2. We have observed a general trend indicating that over several days, there is a reduction in expression of both *aflD* and *aflR* with a concomitant subsequent increase in toxin production when the fungus was exposed to the higher CO_2 levels. Thus, complementary studies including earlier time points and a larger set of biosynthetic genes would be beneficial for further elucidation of how AFB_1 production is modulated.

Previous studies have shown that early biosynthetic gene expression prior to mycotoxin production occurs in mycotoxigenic fungi generally (Geisen et al., 2004). Studies have also suggested that the impact of two-interacting abiotic factors influences the *aflR/aflS* regulatory gene ratio and this would indicate the effect on aflatoxins production (Schmidt-Heydt et al., 2010)

The present study has shown that AFB_1 production kinetics is initiated very early in the growth phase (2 days). Subsequently, there is an increase in AFB_1 production over the 10 day incubation period. At elevated temperature and CO_2 levels, under water stress conditions there was

a stimulation of AFB_1 production. This suggests that under combined stress conditions there may be a different temporal effect on mycotoxin production as suggested previously by Schmidt-Heydt et al. (2011). In addition, previously Medina et al. (2015c) showed that there was a stimulation of AFB_1 production under elevated CO_2 and 37 °C when compared to 30 °C and 400 ppm CO_2. At 0.930 a_w, maximum production values were observed after 4-8 days, after which there was a decrease. With freely available water, results are different with little difference between climate-related treatments.

The results presented in this study suggest that earlier colonisation and the early production of AFB_1 in the life cycle could be important for their survival, especially the saprophytic component, where abiotic stresses are often extreme allowing effective competitive exclusion of other microorganisms and subsequent production of both asexual and sexual spores for infection of ripening maize crops under conducive environmental conditions.

In conclusion, this kinetic study has shown that genes involved in the biosynthesis of aflatoxins are switched on very early in the growth phase of *A. flavus* under both existing and future climate-related abiotic factors. Although generally there was an increase in the lag phase under 3-way interacting stress conditions growth rates were relatively unaffected. This suggests a very good resilience of *A. flavus* under climate-stress abiotic factors and will allow this species to produce afltoxins over a long period of time, even at 37 °C, elevated CO_2 and drought-stress conditions. There was no direct correlation between biosynthetic gene expression for a structural and biosynthetic gene and AFB_1 production over the 10 day experimental period. This suggests a complex interaction between interacting climate-related abiotic factors and the ecology of *A. flavus*. The resilience of *A. flavus* under such stress conditions suggests that there may be increased risks of mycotoxins under such conditions. Such datasets will be useful to inform predictions of

the relative risk of aflatoxins contamination of staple cereals such as maize and potential impacts on the food safety agenda.

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Figure 1 – Impact of three-way interacting Climate change factors on the gene expression of *aflD* and *aflR* in *Aspergillus flavus*. For each gene, the data with different letters are significantly different.

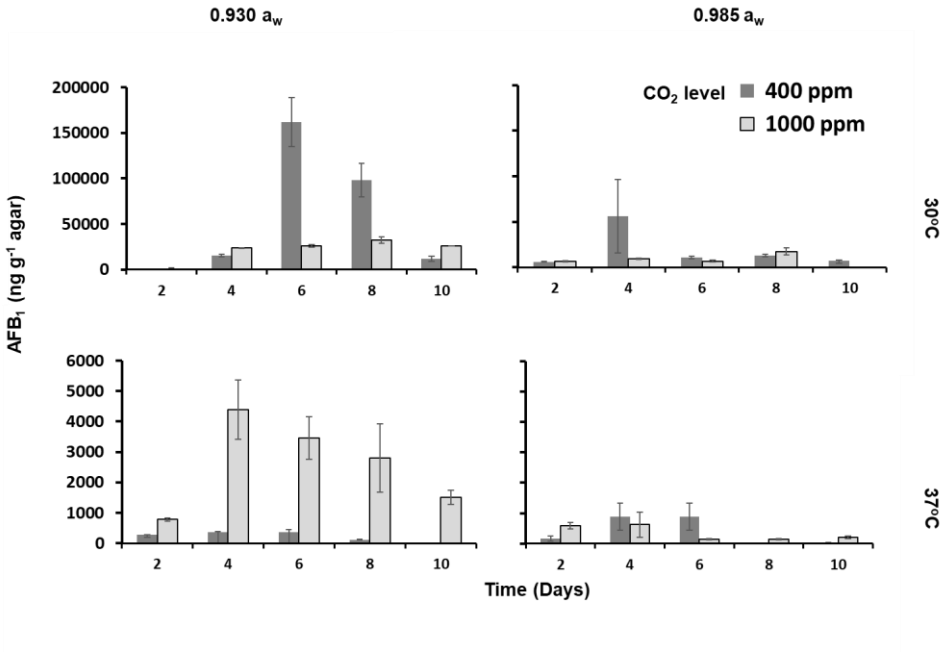

Figure 2 – Impact of three-way interacting Climate change factors on the AFB_1 production on YES media for 10 days. Bars indicate standard error of 3 replicates. X indicates missing values.

Table 1. Effect of interacting abiotic factors including temperature, water activity and CO_2 on *A. flavus* lag times prior to growth and the growth rates.

Temperature	Water activity (a_w)	CO_2 (ppm)	Lag phase (days)	Diametric growth rate (mm day^{-1})
30°C	0.985	400	$0.55 \pm 0.04^{c,d}$	12.33 ± 0.76^{a}
		1000	$0.44 \pm 0.11^{d,e}$	11.25 ± 0.21^{b}
	0.93	400	1.02 ± 0.03^{a}	$6.76 \pm 0.32^{f,g}$
		1000	$0.84 \pm 0.11^{a,b}$	$6.64 \pm 0.46^{f,g}$
37°C	0.985	400	$<0.1^{g}$	8.87 ± 0.83^{d}
		1000	$0.21 \pm 0.06^{e,f,g}$	10.1 ± 0.17^{c}
	0.93	400	$0.72 \pm 0.07^{b,c}$	$7.18 \pm 0.21^{e,f}$
		1000	$0.20 \pm 0.14^{e,f,g}$	6.52 ± 0.10^{g}

<0.1 denotes that no Lag time was calculated. Different letters indicate statistically significant differences between conditions.

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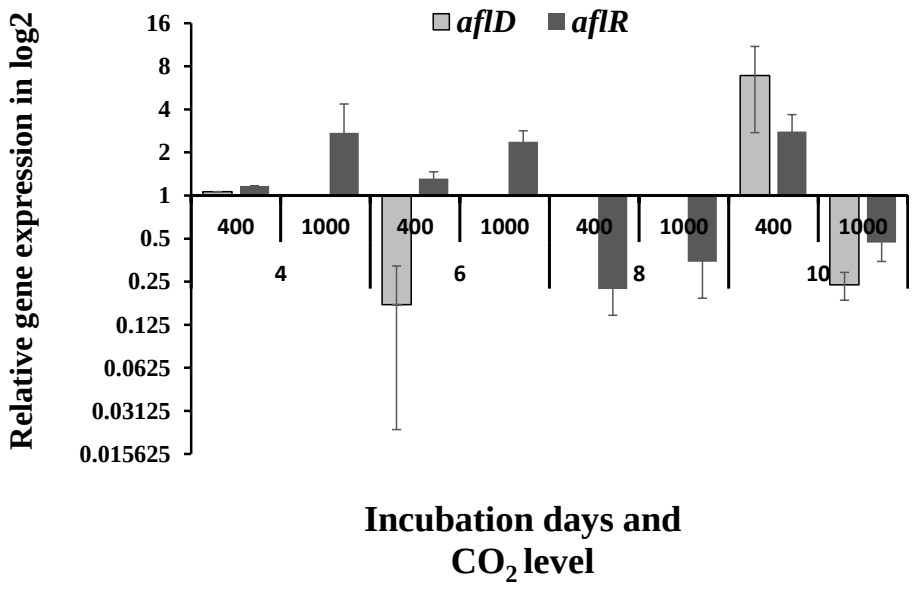
Table 2. Comparison of the effect of interacting environmental factors on *aflD* and *aflR* gene expression of *Aspergillus flavus.* *denotes significant values (*p-value*<0.05).

Source	*aflD*		*aflR*	
	ChiSquare	Prob>ChiSquare	ChiSquare	Prob>ChiSquare
Time	1.4761	0.6878	6.9685	0.0729
Temperature	18.1896	<0.0001*	2.2509	0.1335
Water activity	2.7360	0.0981	1.3864	0.2390
CO_2	1.0561	0.3041	0.4194	0.5172



Supplementary Figure 1: Supplementary Figure 1 – Impact of CO_2 (1000 ppm) exposure on the gene expression of *aflD* and *aflR* in *Aspergillus flavus* at 37 °C , 0.985 a_w. For each gene, the data with different letters are significantly different.