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

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Running title: Environmental stress and transcriptomics of *Aspergillus flavus*

Key words: Water activity, temperature, RNA-sequencing, *Aspergillus flavus*, aflatoxin B_1, transcriptome

Highlights

- Temperature (30/37°C) and water stress (0.99/0.91 a_w) affected aflatoxin B_1 on maize
- RNAseq showed significant impacts of environmental factors on gene categories
- Temperature/water stress changed differential expression of genes
- Secondary metabolite gene clusters were differentially expressed by the imposed stress
- This RNAseq study helps to identify functional gene groups involved in stress tolerance and toxin production

ABSTRACT

Effects of *Aspergillus flavus* colonization of maize kernels under different water activities (a_w; 0.99 and 0.91) and temperatures (30, 37°C) on (a) aflatoxin B_1 (AFB_1) production and (b) the transcriptome using RNAseq were examined. There was no significant difference (P=0.05) in AFB_1 production at 30 and 37°C and 0.99 a_w. However, there was a significant (P=0.05) increase in AFB_1 at 0.91 a_w at 37°C when compared with 30°C/0.99 a_w. Environmental stress effects using gene ontology enrichment analysis of the RNA-seq results for increasing temperature at 0.99 and 0.91 a_w showed differential expression of 2224 and 481 genes, respectively. With decreasing water availability, 4,307 were affected at 30°C and 702 genes at 37°C. Increasing temperature from 30 to 37°C at both a_w levels resulted in 12 biological processes being upregulated and 9 significantly downregulated. Decreasing a_w at both temperatures resulted in 22 biological processes significantly upregulated and 25 downregulated. The interacting environmental factors influenced functioning of the secondary metabolite gene clusters for aflatoxins and cyclopiazonic acid (CPA). An elevated number of genes were co-regulated by both a_w and temperature. An interaction effect for 4 of the 25 AFB_1 genes, including regulatory and transcription activators occurred. For CPA, all 5 biosynthetic genes were affected by a_w stress, regardless of temperature. The molecular regulation of *A. flavus* in maize is discussed.

1. INTRODUCTION

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

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

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

Different molecular approaches including reverse transcriptase real-time PCR (RT-qPCR) (Abdel-Hadi *et al.,* 2010, 2012; Jurado *et al.,* 2008; Marín *et al.,* 2010a, 2010b, Rodríguez *et al.,* 2014) and microarrays (Schmidt-Heydt and Geisen, 2007) have been used to elucidate the relationship between these interacting environmental factors and *A. flavus* growth and AFB_1 production. The genes involved in biosynthesis of aflatoxins are clustered together on the genome and the expression of the key regulatory (*aflR*; *aflS*) and related structural genes (e.g. *aflD*) have been shown to be influenced by interacting conditions of a_w x temperature. Indeed, Schmidt-Heydt *et al.* (2010) showed that the ratio of *aflR/aflS* expression was

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

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

2. MATERIALS AND METHODS

2.1 Fungal Strain

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

2.2 Modification of maize grain water activities

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

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

2.3 Aflatoxin analyses

(a) Extraction of aflatoxins from maize

AFB_1 extraction was performed using AflaStar™® - Immunoaffinity Columns (IAC, Romer Labs Inc., MO, USA), following the manufacturers' protocol. Briefly, 5 g of the sample were dried overnight at 80°C and stored at

room temperature. The samples were ground, 4 g weighed into a 50 ml falcon tube and 16 ml of a solution of methanol:water (60/40 v/v) added. The samples were shaken for 1 hr at room temperature, and then filtered through qualitative filter paper (QL 110, Fisher Scientific UK Ltd, Loughborough, UK). The extract (1 ml) was diluted in a falcon tube (15 ml) with 9 ml of 1x PBS buffer (0.05M/0.15M NaCl, pH 7.4, Fisher Bioreagents®, Fisher Scientific UK Ltd, Loughborough, UK) and the pH checked with pH strips. The diluted extract was applied to the IAC, and allowed to drip through. After further cleaning, 3 ml of Methanol (HPLC grade) was used to elute the aflatoxins. The eluent was dried in the fume *cupboard for derivatisation.*

(b) Preparation of standards

200 µl aflatoxin (R-Biopharm Rhône Ltd., Darmstadt, Germany) of stock solution comprising of 200 ng AFB_1 was prepared. The stock solution was pipetted into 2 ml Eppendorf tubes and left to evaporate to dryness overnight inside a fume cupboard, and thereafter derivatised.

(c) Derivatization, detection and quantification of aflatoxins by HPLC

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

A reversed-phase Agilent 1200 series HPLC system with fluorescence detection was used to confirm the identity and quantify AFB_1.. This consisted of an in-line degasser, auto sampler, binary pump and a fluorescence detector (excitation and emission wavelength of 360 and 440 nm, respectively). Separation was achieved through the use of a C_{18} column (Agilent Zorbax Eclipse plus C_{18} 4.6 mm × 150 mm, 3.5 µm particle size; Agilent, Berks, UK) preceded by guard cartridge with the same packing material. Isocratic elution with methanol:water:acetonitrile (30:60:10, v/v/v) as mobile phase was performed at a flow rate of 1.0 ml/min. The injection volume was 20 µl. A set of standards was injected (1 to 5 ng AFB_1, aflatoxin B_2, G_1 and G_2 per injection) and standard curves generated by plotting the area underneath the peaks against the amounts of AFB_1 standard injected. Linear regression was performed in order to establish a correlation relationship (correlation coefficient, R^2=0.99).

2.4 Total RNA extraction

For gene expression studies, sampling was performed after 10 days using three replicates This time frame was chosen because previous studies with both *A. flavus* and *A. parasiticus* suggested that gene expression of many of the biosynthetic genes was optimal after 8-10 days growth, although there does appear to be a sequential expression of groups of the aflatoxin biosynthetic genes (Schmidt-Heydt *et al.,* 2008, 2009, 2010). Studies on stored maize grain have also shown optimum AFB_1 production occurs at between 5-7 days at 0.98 a_w) and 10-12 days at 0.95-0.93 a_w (Mohale *et al.,* 2013). We have compromised and used 10 days in this study so that we can obtain both molecular information and relevant mycotoxin data.

1 g of frozen milled maize was ground to powder in a mortar with a pestle in the presence of liquid nitrogen and placed into a 2 mL extraction tube. The resulting powder was used for isolation of total RNA. Total RNA was extracted using the RNAeasy Plant Mini kit (Qiagen, Hilden, Germany). About 100 mg of the resulting powder was used for isolation of total RNA. This was resuspended in 1 ml lysis buffer supplemented with 10 µl β-mercaptoethanol in a 2 ml RNase free micro reaction tube. After vortexing, the tube was quickly frozen in liquid nitrogen. The sample was then thawed on ice. All further procedures were essentially the same as recommended by the manufacturer of the kit.

2.5 RNA sequencing

RNA sequencing was conducted using the NEB Ultra Directional RNA Library Prep Kit. The sequencing was performed on an Illumina HiSeq 2000. Sequencing reads were quality trimmed with Trimmomatic and mapped to the *A. flavus NRRL3357* (assembly JCVI-afl1-v2.0, http://www.ncbi.nlm.nih.gov/genome/360?genome_assembly_id=28730) reference sequence using Tophat (Trapnell *et al.,* 2010). Reads mapping to exons were counted using htseq-count (Anders *et al.,* 2015) followed by differential expression testing with DESeq2 (Love *et al.,* 2014). Genes were considered differentially expressed if they had an adjusted p-value < 0.05. The average effects of temperature and water were calculated by adding half of the interaction term to each main effect, and the interaction effect was calculated according to the DESeq2 documentation. GO enrichment was done using the GOSeq R Bioconductor package.

All Data has been deposited with the SRA repository at www.ncbi.nih.gov, accession ID: PRJNA380582

3. Results and Discussion

3.1 Effects of water activity x temperature effects on aflatoxin B_1 (AFB_1) production

Figure 1 shows the effect of the interacting treatment conditions on AFB_1 production. Generally, more AFB_1 was produced with freely available water (0.99 a_w) than under water stress (0.91 a_w). There was no significant difference in AFB_1 production at 30 and 37°C with freely available water. However, there was significantly more AFB_1 produced at 37°C and 0.91 a_w than at 30°C.

3.2 Impact of water stress x temperature regimes on gene expression

(a) General transcriptomic analysis

The reads obtained from samples exposed to the four environmental conditions (2 temperatures x 2 a_w levels) were mapped against the *A. flavus* genomic sequence and the total number of differentially expressed genes (DEGs) was determined (see Experimental Procedures). DESeq2 was then used to determine the differential expression of genes between the different conditions. To determine which genes were either co-regulated by both environmental factors or only influenced by one, gene datasets for both temperature and a_w were compared. The number of genes with a $\log_2$ fold change of 0.5 or greater, and $p<0.05$ are shown in Figure 2. Genes were considered affected by temperature if the gene increased or decreased in the 37°C sample relative to the 30°C treatment. Figure 2A shows that 321 genes were upregulated by both 37°C and by water stress (0.91 a_w). Similar findings were obtained with the downregulated genes (Figure 2A, right side) where 267 genes were affected by both conditions. Relatively large numbers of genes were uniquely affected by the a_w treatment but not the temperature, with 1,763 being upregulated and 1,875 being downregulated. Within these groups, gene ontology term enrichment analysis (see Experimental Procedures) determined categories of genes enriched due to either a_w, temperature, or both. Genes that were up regulated by low a_w comprised the largest number of biological processes (Figure 2B). Furthermore, 118 biological processes were significantly represented among genes down regulated by temperature. The remaining treatment categories had approx. 50 or less biological processes affected.

The complete listing of GO categories that were significantly enriched are shown in Supplementary file 1. Of note, the biological processes that were upregulated by high temperatures showed significant enrichment of different DNA-replication–associated ontological categories and mitosis. Processes that were increased by water stress (0.91 a_w) included, high numbers of protein synthesis, and protein modification processes (see Supp. file 1). These include translation (GO:0006412), methylation (GO:0032259), and proteolysis processes (GO:0006508). Biological categories of genes downregulated by high temperatures include metabolic processes (GO: 0008152) and in particular oxidation-reduction processes (GO:0055114). Processes downregulated by low a_w included proteasome assembly (GO:0043248) and endocytosis (GO:0006897) (see Supp. file 1). Finally, enrichment of GO categories of genes that were up or down regulated in relation to both water stress and temperature treatment groups were determined (represented

as the overlapping regions in Figure 2A (Supp. file 1). The upregulated genes enriched in these are involved in metabolic processes such as carbohydrate metabolic processes (GO: 0005975) and lipid catabolic processes (GO:0016042), amongst several others (Supp. File 1).

(b) Changing the temperature under steady state a_w conditions

To further investigate the gene regulation at different temperatures, the DEG's in samples incubated at the same a_w but different temperatures were identified. The number of genes affected by temperature at both a_w conditions are illustrated in Figure 3A. Increasing temperature from 30° to 37°C had a much higher impact on the number of DEG's at high a_w as compared to low a_w (2,224 vs 481, respectively). The GO categories of biological processes of the overlapping region and the genes affected by temperature regardless of a_w levels are illustrated in Table 1. Genes that were upregulated were associated with DNA replication and cell division (i.e. GO:0015851 nucleoside transport and GO:0051300 spindle pole body organization), whereas downregulated processes included several metabolic processes (i.e.; GO:0009253, peptidoglycan catabolic process and GO:0006801 superoxide metabolic process.

(c) Effect of changing a_w under steady state temperature regimes

Figure 3B illustrates the effect of decreasing a_w levels at specific temperatures on the number of DEGs. This demonstrated that a decrease in a_w led to a significantly higher number of DEGs when temperatures were lower (30°) than higher (37°). Table 2 illustrates the categories of genes affected by changing a_w at both temperatures tested. Genes were upregulated in diverse categories of genes, including transmembrane transport (i.e., GO:0071577 zinc II ion transmembrane transport). Downregulated categories of genes were rich in genes controlling transcription and DNA synthesis (i.e.; GO:0006368, transcription elongation from RNA polymerase II promoter).

(d) Impact of the interacting a_w x temperature factors on gene regulation

A differential expression test using a multi-factor design was performed using DESeq2 by introducing an interaction term, temperature x a_w (labelled "interaction"), that are differentially expressed due to the interaction of higher temperature and water stress (low a_w). The value of this term showed the difference between the effect of changing a_w levels from 0.99 to 0.91 at 37°C and the effect of changing a_w from 0.99 to 0.91 at 30°C. This analysis revealed that 935 genes were downregulated and 904 genes upregulated. To identify the biological processes affected GO analysis was conducted (Table 3).

(e) *Effects of a_w x temperature on expression of genes involved in secondary metabolite production*

We examined the effect of a_w and temperature on secondary metabolite biosynthetic genes predicted by SMURF (Khaldi *et al.,* 2010). DEGs were identified using DESeq2 with an interaction factor and the $\log_2$ fold changes were calculated (see Experimental Procedures). Results for the aflatoxin and cyclopiazonic acid (CPA) clusters (Cluster 54; 55) are shown in Table 4. The results for all predicted genes clusters are also presented in Supplementary Table 2. Overall, examination of the regulation of secondary metabolite clusters showed that both a_w and temperature had an effect on several clusters. However, a number were not significantly affected including 18, 26, 27, 28, 29, 32, 39, 49, and 53. Variations in a_w led to a more pronounced change in gene regulation than temperature. There were several clusters more affected by changes in a_w then by changes in temperature, e.g. Cluster 9 (production of a siderophore; Tsai *et al.*, 1997) and Cluster 44 (production of 6-hydroxy-7-methyl-3- nonylisoquinoline-5,8-dione; Ahuja *et al.,* 2012). There were also an elevated number of genes which were co-regulated by both a_w and temperature (see Supp. Table 2).

With regard to Cluster 55, responsible for the production of CPA (Duran *et al.* 2007), marked changes (log2 fold) in gene expression of all 4 genes were observed when a_w was changed, regardless of temperature. In contrast, changes in temperature only modified expression of two genes (AFLA_139460, AFLA_139480). For these two genes there was an interaction between both factors. For cluster 54 (aflatoxin cluster) (Duran *et al.,* 2007), which is composed of 34 genes, changes in expression were more prominent when a_w was changed at 30 than at 37°C (25 genes vs 1 gene). For 23 of these genes, increased stress by reducing the a_w from 0.99 to 0.91 led to an upregulation of expression (see Supp. Table 2). When temperature stress was imposed by increasing this factor from 30 to 37°C, and maintaining the same a_w, 20 genes were upregulated at 0.99 a_w. Only one gene significantly changed expression at 0.91 a_w (AFLA_139340). Effects of interactions between temperature and a_w were found for 4 genes (a*flR*, a*flS, aflB* and *aflT)* out of the 25 genes affected by these factors. Interestingly, *aflR* and *aflS* are the two regulatory genes for aflatoxin biosynthesis and transcription activators (Yu *et al.,* 2004).

Isolating the RNA-seq results for the SMURF-identified backbone genes, which includes polyketide synthases, NRPS's, and enzymes involved in terpene, indole, and siderophore synthesis, allowed analysis of how the water and temperature conditions examined may affect the secondary metabolic clusters. Figure 4 illustrates how the effect of lowered a_w (0.91 a_w) and higher temperature (37°C; stress conditions) had a markedly different effect on the backbone genes collectively than other combinations of environmental conditions examined.

Overall, this study has demonstrated that the influence of a_w and temperature on AFB_1 production on stored maize grain was different from that observed on a conducive laboratory medium (YES). On YES media the optimum conditions for AFB_1 production was at 25-30°C and 0.99 a_w, (with similar amounts produced at 35°C and 0.99 and 0.95 a_w (Abdel-Hadi *et al.,* 2013). Furthermore, analyses of *A. flavus* in relation to temperature studies showed no AFB_1 production at 37°C in broth cultures (Yu *et al.,* 2011). However, while this study provided interesting information on the control of gene clusters involved in AFB_1 production, the analyses was carried out after only 24-48 hrs in liquid culture. This short time and the broth culture may account for the lack of mycotoxin production at 37°C. The present study in stored maize kernels indicates significantly higher levels of AFB_1 production at 37°C relative to 30°C. Furthermore, a_w levels affected AFB_1 production, with higher levels occurring in the higher water availability conditions. The RNA-Seq data allowed comparisons to be made of actual toxin levels measured and the corresponding levels of transcripts from the aflatoxin biosynthetic gene cluster. Much of the data is in agreement. There were higher expression levels of most aflatoxin biosynthetic genes in high environmental stress conditions (high temp and low a_w), as well as an increase in AFB_1 levels. Similar effects were observed on transcript levels when only looking at changes in temperature or a_w levels alone. The interaction terms (see above) indicated that 4 genes in the aflatoxin cluster (*aflS*, *aflR*, *aflB*, and *aflT*) showed a 2.2-5.1 log2 fold increase in their response to 0.91 a_w at 37°C then at 30°C, indicating key genes in the aflatoxin cluster are more sensitive to water stress (lower levels) at higher temperatures. This is supported by q-PCR of the *aflD* and *aflR* genes where comparing the relative expression with 30°C/0.99 a_w as the calibrator showed a significant increase in the *aflR* gene relative expression, and a significant reduction in the *aflD* gene expression, an early structural gene in the biosynthetic cluster pathway for secondary metabolite production (Suppl. Figure 1).

A previous study by Zhang *et al.* (2014) examined *A. flavus* activity and the transcriptome at 0.99 and 0.93 a_w on a conducive YES medium at 28°C, which is considered best for growth rather than secondary metabolite production. They found both decreased conidiation and AFB_1 biosynthesis at 0.93 a_w when compared to 0.99 a_w. Overall, their study showed 5362 differentially expressed unigenes identified between 0.99 and 0.93 a_w treatments, including 3156 up-regulated and 2206 down-regulated unigenes. They suggested that *A. flavus* underwent an extensive transcriptome response during a_w variation between freely available water and water stress. However, interactions with temperature were not considered. The

interaction between these two variables have been shown to be critical in determining the impact on the biosynthetic gene cluster for aflatoxins (Abdel-Hadi *et al.,* 2012; Schmidt-Heydt *et al.,* 2008; 2009; 2010).

Interestingly, among the five most highly up-regulated genes, the *aflF*, *aflU* and *aflT* genes are adjacent and located on the very end of the gene cluster, whereas *aflG* and *aflNa* are located next to each other in the middle of the gene cluster. Therefore, the gene *aflF* could be related to turning on/off aflatoxin pathway gene expression, and on chromosomal location these genes may be responsive to the environmental queue of a_w. The *aflR* gene is a Zn2Cys6-type transcription factor that is believed to be necessary for regulating most of the genes in the aflatoxin gene cluster in *A. flavus* (Yu et al., 2004). This previous study also demonstrated that water stress had a significant effect on *aflR* transcription at lower a_w (0.90) compared with higher a_w (0.99).

Several additional developmentally relevant genes showed altered regulation due to either temperature change, water change, or a combination of both factors. An abbreviated list of selected genes is presented in Table 5, and a more comprehensive list is presented in Supplementary Table 2. Genes known to affect conidiation and conidiophore morphology such as *brlA* (Adams, Boylan et al. 1988), *abaA* (Boylan, Mirabito et al. 1987), *flbD* (Wieser and Adams 1995), *vosA* (Ni and Yu 2007) and *wetA* (Boylan, Mirabito et al. 1987) were up regulated in at least two of the comparisons. The *rolA* gene, which encodes a hydrophobin and localizes to conidial surfaces (Takahashi, Maeda et al. 2005) was significantly up regulated by both temperature stress and water stress. In contrast, the genetically related *nosA* and *rosA* have been reported to be up regulated during asexual development (Vienken and Fischer 2006), and exhibit down regulation in all three comparisons presented.

Comparison with previous genomic studies on other species of *Aspergillus*, e.g., *Aspergillus oryzae* (RIB40 strain), the so-called domesticated *A. flavus*, used for food fermentations were difficult. These have predominantly focused on specific blocks of genes involved in synthesis of secondary metabolites such as kojic acid production, those involved in protein secretion, amino acid metabolism and amino acid/sugar uptake transporters. These suggested that there is no induction of key biosynthetic genes in the clusters involved in aflatoxin/cyclopiazonic acid biosynthesis (Liu *et al.,* 2014; Tamano *et al.,* 2008; Terabayashi *et al.,* 2010). Indeed, the effect of individual or interacting environmental stress factors on the transcriptome of *A. oryzae* has not been examined, with a focus more on understanding and optimizing solid substrate/liquid fermentation systems.

Conclusions

In conclusion this study has shown, for the first time, that interacting conditions of key environmental factors of water and temperature stress will have a significant impact on the transcriptome of *A. flavus* and on the biosynthesis of AFB_1 and related toxins. This provides a basis for examining interactions with other interacting environmental stresses, such as climate change factors (temperature x water stress x elevated CO_2) which have been recently demonstrated to impact on biosynthetic structural and regulatory genes such as *aflD* and *aflR* and stimulate AFB_1 production (Medina *et al.,* 2015a; 2015b). A better understanding of the functional response of *A. flavus* to such interacting condition will be beneficial for the development of minimization strategies, including those utilizing RNAi approaches in the pathogen and the host maize plant.

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Figure legends

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

Figure 2. Effect of (A) temperature x water activity on the up and down regulated genes of *A.flavus* NRRL 3357 grown on stored maize grain, and (B) biological processes which were enriched relative to each factor or both.

Figure 3. Relative numbers of up and down regulated genes when changing (A) temperature in relation to water activity (a_w) stress in *A. flavus* NRRL 3357, and (B) a_w levels in relation to elevated temperature stress.

Figure 4. Heat map generated from R log normalized values from DESeq. Reddish brown indicates higher expression values and blue indicates lower values.

Table 1. Gene ontology categories that are enriched due to increased temperature at both 0.99 and 0.91 water activity

GO category	Significance	Annotation
Upregulated		
GO:0015851	0.002	nucleobase transport
GO:0019628	0.004	urate catabolic process
GO:0015856	0.005	cytosine transport
GO:0015861	0.005	cytidine transport
GO:0051300	0.005	spindle pole body organization
GO:0003333	0.006	amino acid transmembrane transport
GO:0035435	0.013	phosphate ion transmembrane transport
GO:0019627	0.013	urea metabolic process
GO:0006863	0.013	purine nucleobase transport
GO:0006144	0.016	purine nucleobase metabolic process
GO:0055114	0.017	oxidation-reduction process
GO:0055085	0.042	transmembrane transport
Downregulated		
GO:0008152	0.006	metabolic process
GO:0055114	0.008	oxidation-reduction process
GO:0008643	0.012	carbohydrate transport
GO:0006952	0.012	defense response
GO:0045454	0.015	cell redox homeostasis
GO:0046439	0.015	L-cysteine metabolic process
GO:0009253	0.027	peptidoglycan catabolic process
GO:0006801	0.037	superoxide metabolic process
GO:0046416	0.037	D-amino acid metabolic process

Table 2. Gene ontology categories that are enriched due to decreased water activity at both 30 and 37°C.

GO category	Significance	Annotation
Upregulated		
GO:0006412	0.000	translation
GO:0032259	0.000	methylation
GO:0006508	0.001	proteolysis
GO:0006830	0.004	high-affinity zinc II ion transport
GO:0071577	0.009	zinc II ion transmembrane transport
GO:0035434	0.012	copper ion transmembrane transport
GO:0030001	0.012	metal ion transport
GO:0018106	0.014	peptidyl-histidine phosphorylation
GO:0045493	0.014	xylan catabolic process
GO:0046084	0.015	adenine biosynthetic process
GO:0009228	0.017	thiamine biosynthetic process

GO:0000413	0.020	protein peptidyl-prolyl isomerization
GO:0000162	0.022	tryptophan biosynthetic process
GO:0009116	0.022	nucleoside metabolic process
GO:0006662	0.029	glycerol ether metabolic process
GO:0006471	0.032	protein ADP-ribosylation
GO:0006836	0.033	neurotransmitter transport
GO:0009092	0.034	homoserine metabolic process
GO:0071555	0.034	cell wall organization
GO:0045490	0.035	pectin catabolic process
GO:0006801	0.043	superoxide metabolic process
GO:0016052	0.049	carbohydrate catabolic process
Downregulated		
GO:0043248	0.002	proteasome assembly
GO:0006897	0.002	endocytosis
GO:0007121	0.003	bipolar cellular bud site selection
GO:0045002	0.003	double-strand break repair via single-strand annealing
GO:0006368	0.003	transcription elongation from RNA polymerase II promoter
GO:0006979	0.003	response to oxidative stress
GO:0000289	0.004	nuclear-transcribed mRNA poly(A) tail shortening
GO:0008654	0.004	phospholipid biosynthetic process
GO:0033260	0.005	nuclear DNA replication
GO:0006277	0.005	DNA amplification
GO:0036228	0.006	protein targeting to nuclear inner membrane
GO:0006892	0.006	post-Golgi vesicle-mediated transport
GO:0000727	0.007	double-strand break repair via break-induced replication
GO:0000084	0.007	mitotic S phase
GO:0045842	0.009	positive regulation of mitotic metaphase/anaphase transition
GO:0042176	0.009	regulation of protein catabolic process
GO:0019751	0.011	polyol metabolic process
GO:0045151	0.011	acetoin biosynthetic process
GO:0000398	0.014	mRNA splicing, via spliceosome
GO:0031578	0.014	mitotic spindle orientation checkpoint
GO:0032968	0.015	positive regulation of transcription elongation from RNA polymerase II promoter
GO:0051056	0.016	regulation of small GTPase mediated signal transduction
GO:0034497	0.018	protein localization to pre-autophagosomal structure
GO:0007120	0.018	axial cellular bud site selection
GO:0045944	0.018	positive regulation of transcription from RNA polymerase II promoter

Table 3. Gene ontology categories that are enriched due to an interaction between 0.91 water activity and 37°C.

GO category	Significance	Annotation
GO:0006555	0.000	methionine metabolic process
GO:0006096	0.000	glycolytic process
GO:0045454	0.000	cell redox homeostasis
GO:0005975	0.000	carbohydrate metabolic process
GO:0055114	0.000	oxidation-reduction process
GO:0006094	0.001	gluconeogenesis
GO:0016226	0.001	iron-sulfur cluster assembly

GO:0006696 0.001 ergosterol biosynthetic process
GO:0015696 0.002 ammonium transport
GO:0019740 0.002 nitrogen utilization
GO:0000096 0.002 sulfur amino acid metabolic process
GO:0044396 0.002 actin cortical patch organization
GO:0019379 0.003 sulfate assimilation, phosphoadenylyl sulfate reduction by phosphoadenylyl-sulfate reductase (thioredoxin)
GO:0009052 0.003 pentose-phosphate shunt, non-oxidative branch
GO:0009051 0.003 pentose-phosphate shunt, oxidative branch
GO:0044774 0.003 mitotic DNA integrity checkpoint
GO:0006221 0.004 pyrimidine nucleotide biosynthetic process
GO:0008612 0.004 peptidyl-lysine modification to peptidyl-hypusine
GO:0000916 0.004 actomyosin contractile ring contraction
GO:0005980 0.005 glycogen catabolic process
GO:0006123 0.005 mitochondrial electron transport, cytochrome c to oxygen
GO:0006098 0.006 pentose-phosphate shunt
GO:0019430 0.006 removal of superoxide radicals
GO:0009086 0.006 methionine biosynthetic process
GO:0031028 0.007 septation initiation signaling
GO:1902600 0.007 hydrogen ion transmembrane transport
GO:0090307 0.007 mitotic spindle assembly
GO:0006865 0.008 amino acid transport
GO:0045901 0.009 positive regulation of translational elongation
GO:0042744 0.010 hydrogen peroxide catabolic process
GO:0009228 0.011 thiamine biosynthetic process
GO:0006106 0.011 fumarate metabolic process
GO:0070534 0.011 protein K63-linked ubiquitination
GO:0006636 0.011 unsaturated fatty acid biosynthetic process
GO:0070814 0.011 hydrogen sulfide biosynthetic process
GO:0072488 0.012 ammonium transmembrane transport
GO:0035999 0.012 tetrahydrofolate interconversion
GO:1902975 0.013 mitotic DNA replication initiation
GO:0045841 0.013 negative regulation of mitotic metaphase/anaphase transition
GO:0007064 0.014 mitotic sister chromatid cohesion
GO:0097428 0.014 protein maturation by iron-sulfur cluster transfer
GO:0042989 0.015 sequestering of actin monomers
GO:0006635 0.016 fatty acid beta-oxidation
GO:0006241 0.017 CTP biosynthetic process
GO:0006216 0.018 cytidine catabolic process
GO:0006749 0.018 glutathione metabolic process
GO:0006207 0.018 'de novo' pyrimidine nucleobase biosynthetic process
GO:0006952 0.019 defense response
GO:0006629 0.019 lipid metabolic process
GO:0019439 0.020 aromatic compound catabolic process
GO:0016052 0.021 carbohydrate catabolic process
GO:0000281 0.021 mitotic cytokinesis
GO:0051225 0.022 spindle assembly
GO:0044208 0.022 'de novo' AMP biosynthetic process
GO:0006437 0.022 tyrosyl-tRNA aminoacylation
GO:0006147 0.022 guanine catabolic process

GO:0044690	0.022	methionine import
GO:0006986	0.023	response to unfolded protein
GO:0033617	0.023	mitochondrial respiratory chain complex IV assembly
GO:0006012	0.023	galactose metabolic process
GO:0000910	0.024	cytokinesis
GO:0006884	0.024	cell volume homeostasis
GO:0006002	0.025	fructose 6-phosphate metabolic process
GO:0006414	0.025	translational elongation
GO:0071507	0.025	MAPK cascade involved in conjugation with cellular fusion
GO:1903695	0.025	MAPK cascade involved in ascospore formation
GO:0006422	0.026	aspartyl-tRNA aminoacylation
GO:0046578	0.027	regulation of Ras protein signal transduction
GO:0005993	0.028	trehalose catabolic process
GO:0005978	0.029	glycogen biosynthetic process
GO:0051276	0.029	chromosome organization
GO:0006979	0.030	response to oxidative stress
GO:0007088	0.030	regulation of mitotic nuclear division
GO:0007155	0.030	cell adhesion
GO:0090522	0.032	vesicle tethering involved in exocytosis
GO:0006879	0.032	cellular iron ion homeostasis
GO:0006801	0.033	superoxide metabolic process
GO:0034599	0.036	cellular response to oxidative stress
GO:0008652	0.037	cellular amino acid biosynthetic process
GO:0009072	0.040	aromatic amino acid family metabolic process
GO:0035434	0.040	copper ion transmembrane transport
GO:0009102	0.042	biotin biosynthetic process
GO:0003333	0.043	amino acid transmembrane transport
GO:0009298	0.043	GDP-mannose biosynthetic process
GO:0006415	0.044	translational termination
GO:0000751	0.046	mitotic cell cycle arrest in response to pheromone
GO:0019509	0.046	L-methionine biosynthetic process from methylthioadenosine
GO:0051258	0.046	protein polymerization
GO:0051014	0.049	actin filament severing
GO:0000165	0.049	MAPK cascade
GO:0007094	0.049	mitotic spindle assembly checkpoint
GO:0000076	0.049	DNA replication checkpoint

Table 4. The $\log_2$ fold changes in gene expression levels for the aflatoxin and cyclopiazonic acid clusters with $p_{adj} < 0.05$.

gene name	AFLA number	37° & 0.91 vs 37° & 99	37°& 0.91 vs 30° & 0.91	37° & 0.91 vs 30° & 0.99	37° & 0.99 vs 30° & 0.91	37° & 0.99 vs 30° & 0.99	30° & 0.91 vs 30° & 0.99	Interaction	Temperature Average (37 ° vs 30°)	Aw Average (0.91 vs 0.99)
SMURF-identified aflatoxin gene cluster										
aflJe/ orf/ Ser -Thr protein phosphatase family protein	AFLA_139100	-	-	-	-	-	-	-	-	-
aflJd/ sugR/ sugar regulator	AFLA_139110	-	-	-	-	-	-	-	-	-
aflJc/ glcA/ glucosidase	AFLA_139120	-	-	-	-	-	-	-	-	-
aflJb/ hxtA/ hexose transporter	AFLA_139130	-	-	-	1.7	-	-	-	-	-0.9
aflJa/ nadA/ NADH oxidase	AFLA_139140	-	-	-	-	-	-	-	-	-
aflJ/ hypA/ hypP/ hypothetical protein	AFLA_139150	-	-	-4.4	-	-3.5	-4.8	-	-	-
aflK/ ordB/ monooxygenase/ oxidase	AFLA_139160	-	-	-7.0	-	-4.6	-5.3	-	-	-
aflW/ moxY/ monooxygenase	AFLA_139170	-	-	-3.5	-	-	-5.1	-	-	-3.6
aflV/ cypX/ cytochrome P450 monooxygenase	AFLA_139180	-	-	-3.7	-	-2.9	-3.6	-	-	-
aflK/ vbs/ VERB synthase	AFLA_139190	-	-	-5.8	-	-5.0	-7.8	-	-	-
aflQ/ ordA/ ord-1/ oxidoreductase/ cytochrome P450 monooxigenase	AFLA_139200	-	-	-2.3	-	-	-3.4	-	-	-
aflP/ omtA/ omt-1/ O-methyltransferase A	AFLA_139210	-	-	-4.5	-	-3.7	-5.5	-	-	-
aflO/ omtB/ dmtA/ O-methyltransferase B	AFLA_139220	-	-	-4.7	-	-4.1	-5.5	-	-	-
aflI/ avfA/ cytochrome P450 monooxygenase	AFLA_139230	-	-	-	-	-	-	-	-	-
aflJa/ hypB/ hypothetical protein	AFLA_139240	-	-	-5.6	-	-4.1	-6.0	-	-	-
aflL/ verB/ desaturase/ P450 monooxygenase	AFLA_139250	-	-	-5.1	-	-4.4	-7.0	-	-	-
aflG/ avnA/ ord-1/ cytochrome P450 monooxygenase	AFLA_139260	-	-	-4.6	-	-3.7	-4.6	-	-	-
aflJIa/ hypD/ hypothetical protein	AFLA_139270	-2.7	-	-4.8	2.1	-2.1	-4.3	-	-1.3	-3.6
aflJN/ verA/ monooxygenase	AFLA_139280	-	-	-3.1	-	-2.9	-3.3	-	-	-
aflJIa/ hypE/ hypothetical protein	AFLA_139290	-	-	-5.7	-	-4.4	-6.4	-	-	-
aflJM/ ver-1/ dehydrogenase/ ketoreductase	AFLA_139300	-	-	-6.0	-	-5.0	-7.7	-	-	-
aflJE/ norA/ aad/ adh-2/ NOR reductase/ dehydrogenase	AFLA_139310	-	-	-6.6	-	-5.2	-7.6	-	-	-
aflJ/ estA/ esterase	AFLA_139320	-	-	-4.7	-	-4.5	-5.2	-	-	-
aflJH/ adhA/ short chain alcohol dehydrogenase	AFLA_139330	-	-	-3.6	-	-3.5	-3.5	-	-	-
aflS/ aflJ pathway regulator	AFLA_139340	-	1.3	-3.4	-	-3.6	-4.6	5.1	-	-2.2
aflR / apa-2 / afl-2 / transcription activator	AFLA_139360	-	-	-2.5	-	-2.1	-3.1	2.8	-	-1.7
aflB / fas-1 / fatty acid synthase beta subunit	AFLA_139370	-	-	-1.1	-	-2.0	-1.7	2.9	-	-
aflA / fas-2 / hexA / fatty acid synthase alpha subunit	AFLA_139380	-	-	-	-	-	-	-	-	-
aflD / nor-1 / reductase	AFLA_139390	-	-	-	-	-	-	-	-	-
aflCa / hypC / hypothetical protein	AFLA_139400	-	-	-	-	-	-	-	-	-
aflC / pksA / pksL1 / polyketide synthase 2109 aa KS-AT-PP-TE	AFLA_139410	-	-	0.8	-	-	1.0	-	-	0.9
aflT / aflT / transmembrane protein	AFLA_139420	-	-	-2.6	-	-1.8	-2.9	2.1	-0.8	-1.9
aflU / cypA / P450 monooxygenase	AFLA_139430	-	-	-2.4	-	-	-2.3	-	-	-1.8
aflF / norB / dehydrogenase	AFLA_139440	-	-	-2.4	-	-	-2.4	-	-	-2.7
SMURF-identified cyclopiazonic acid gene cluster										
MFS multidrug transporter, putative	AFLA_139460	-3.0	-	-6.1	3.5	-3.1	-6.5	3.5	-1.3	-4.9
FAD dependent oxidoreductase,	AFLA_139470	-5.6	-	-5.4	6.8	-	-6.5	-	-	-6.7
dimethylallyl tryptophan synthase, putative	AFLA_139480	-5.1	-	-7.7	5.8	-2.7	-8.5	3.4	-0.9	-7.0
hybrid PKS/NRPS enzyme, 3851 aa; KS_AT_DH_M_KR_PP_C-A-T-NADB	AFLA_139490	-4.7	-	-3.2	5.7	-	-4.2	-	-	-4.9

*Positive log2 value indicates numerator is larger

Table 5. Select developmental genes and their relative log2 fold expression values under various conditions

Gene	BLAST Annotation/function	3357 Annotation	37°C/0.99 a_w vs 30°C/0.99 a_w	30°C/0.91 a_w vs 30°/99 a_w	37°/0.91 aw vs 30°/0.99 aw
abaA	TEA/ATTS domain transcriptional activator	AFLA_029620	2.5	4.3	3.2
brlA	BrlA, C2H2 type conidiation transcription factor	AFLA_082850	2.1	3.0	2.5
flbA	FlbA, developmental regulator	AFLA_134030	-	-	-
flbB	FlbB, bZIP-type transcription factor	AFLA_131490	-	-	-
flbC	FlbC, C2H2 conidiation transcription factor	AFLA_137320	-1.4	-1.1	-1.2
flbD	FlbD, MYB family conidiophore development	AFLA_080170	-	1.5	2.4
flbE	FlbF protein, developmental regulator	AFLA_017380	-	-	-0.8
gprD	Integral membrane protein	AFLA_135680	-	-1.8	-1.7
laeA	LaeA, regulator of secondary metabolism	AFLA_033290	-2.8	-	-
nosA	NosA, C6 transcription factor	AFLA_025720	-1.7	-1.2	-0.8
nsdD	NsdD, GATA-type sexual development transcription factor	AFLA_020210	-1.3	-	-
rolA	Hydrophobin	AFLA_098380	3.5	4.4	4.0
rosA	Repressor of sexual development	AFLA_025720	-1.7	-1.2	-0.8
veA	VeA, sexual development activator	AFLA_066460	-	-	-
velB	VelB, developmental regulator	AFLA_081490	-	1.4	1.8
vosA	VosA, developmental regulator	AFLA_026900	-	1.3	1.1
wetA	WetA, developmental regulator	AFLA_052030	-	2.8	2.4
yA	Laccase-1 Precursor	AFLA_045660	2.3	2.8	1.7

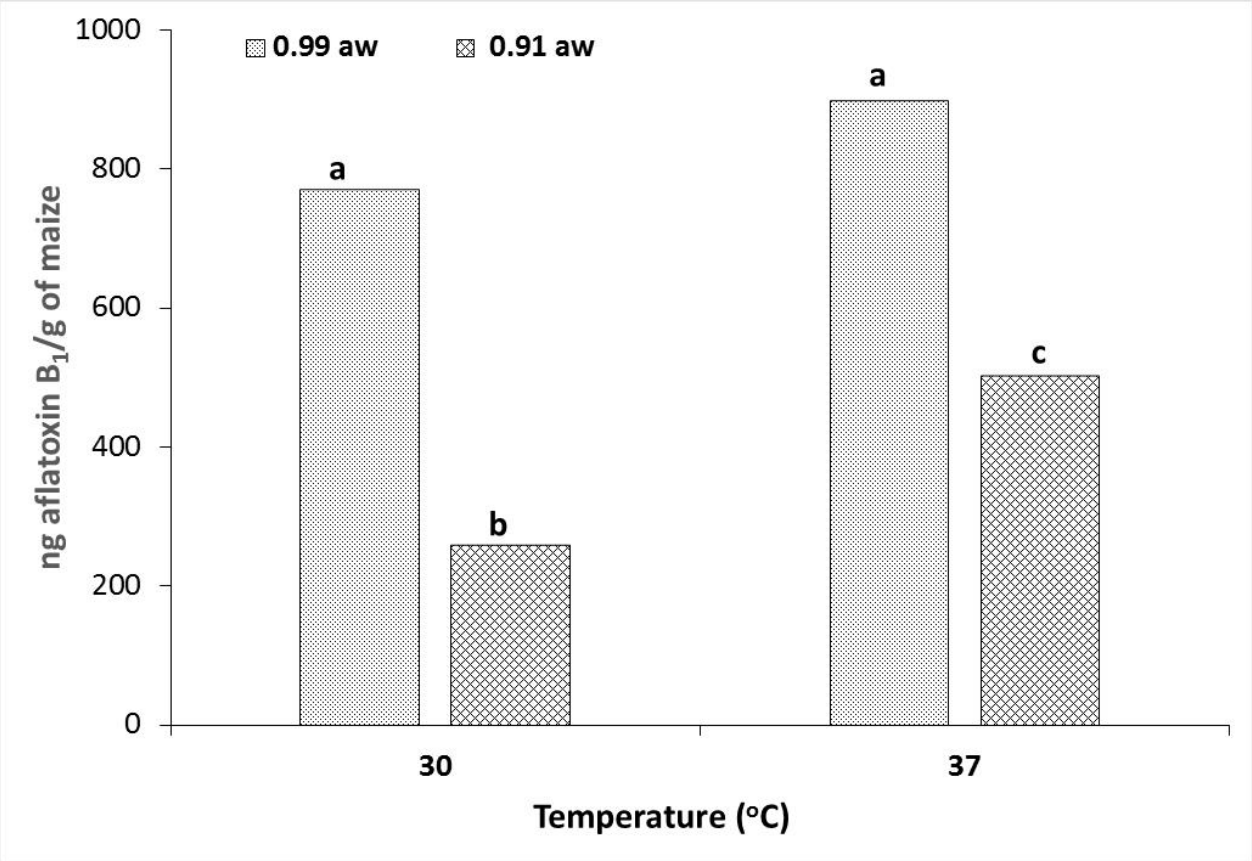

Figure 1

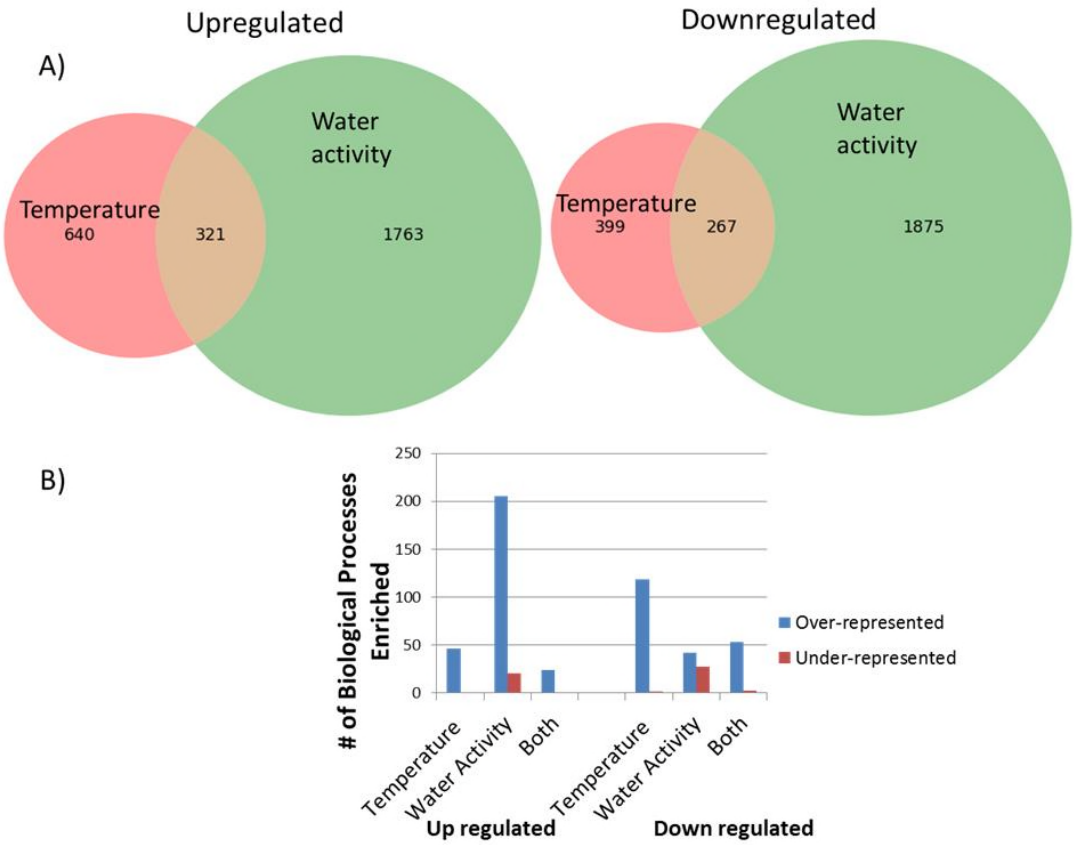

Figure 2. Effect of (A) temperature x water activity on the up and down regulated genes of *A.flavus* NRRL 3357 grown on stored maize grain and (B) biological processes which were enriched relative to each factor or both.

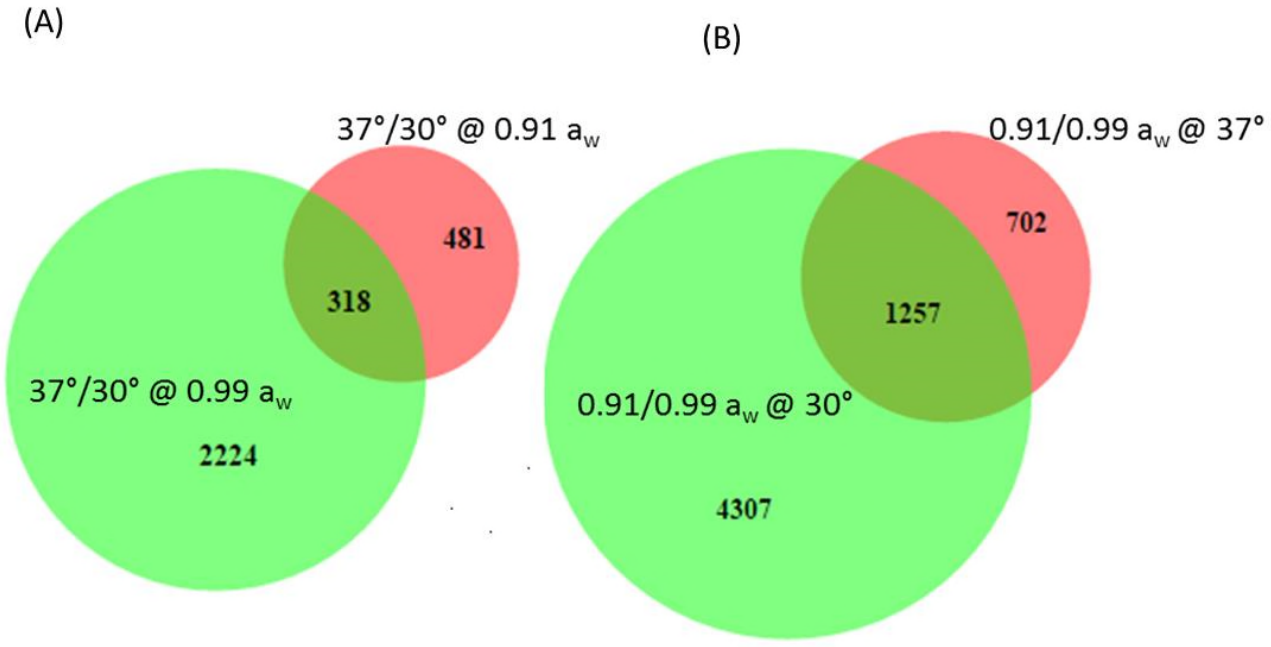

Figure 3. Relative numbers of up and down regulated genes when changing (A) temperature in relation to water activity (a_w) stress in *A. flavus* NRRL 3357 and (B) a_w levels in relation to elevated temperature stress.

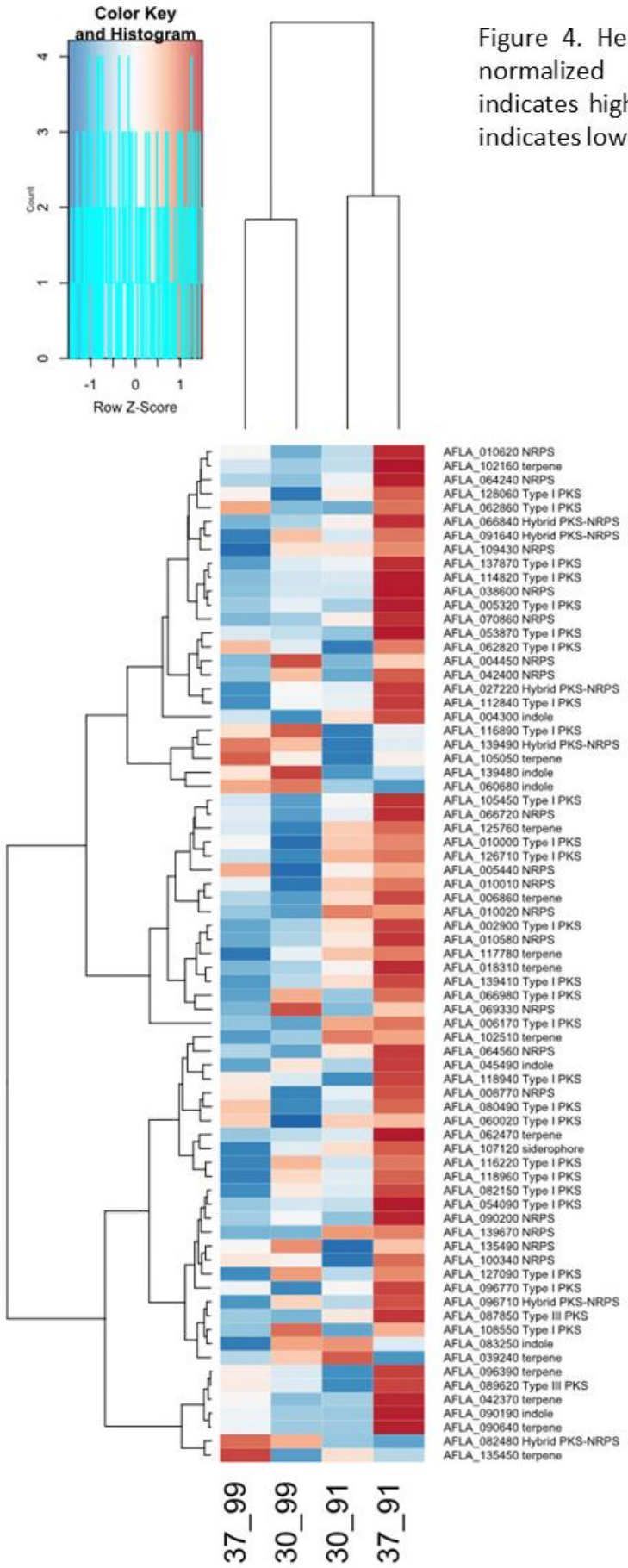

Figure 4. Heat map generated from R log normalized values from DESeq. Brown indicates higher expression values and blue indicates lower values.