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Temporal monitoring of the *nor-1* (*aflD*) gene of *Aspergillus flavus* in relation to aflatoxin B_1 production during storage of peanuts under different water activity levels

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Abstract

Aims: A relative quantification system (RQ-PCR) was used to monitor the correlations between the activity of the *nor-1* (=*aflD*) gene of *Aspergillus flavus* using real time PCR in relation phenotypic aflatoxin B_1 (AFB_1) production and populations of *A. flavus* in stored peanuts at three water activity levels (a_w, 0.95, 0.90 and 0.85) for six weeks. **Methods and Results:** Real time PCR was used to amplify the *nor-1* gene (target gene) and *benA56* (β-tubulin gene), used as a control gene. Expression of three structural genes, *nor-1 (=aflD*), ver-1 (=*aflM*), and omtA (=*aflP*), and the regulatory gene *aflR* of the aflatoxin biosynthetic pathway were also assayed. There were significant differences between *nor-1* gene expression at the three a_w levels; higher expression at 0.90 a_w in weeks 1-3, when compared to 0.95. In contrast, in the driest treatment (0.85 a_w) none or very low *nor-1* expression occurred. The populations of *A. flavus* (CFUs g^{-1}) increased over time with the highest at 0.95 a_w. Highest AFB_1 production was at 0.90 and 0.95 a_w from weeks 3-6. A_w had a significant effect on *aflR* transcription at 0.95 a_w over the 6 week period, while at 0.90 a_w, only in the last two weeks.

Conclusions: Correlations between different factors showed that log AFB_1 x log CFUs, log AFB_1 x a_w, and log CFUs x a_w were statistically significant; while log CFUs x RQ-PCR and RQ-PCR x a_w were not. The *AflR* gene may not have an important role in regulation of *nor-1* expression in food matrices (e.g. peanuts).

Significance and Impact of the study: Determination of correlations between *nor-1* expression and aflatoxin production by *A. flavus* in raw peanuts under different a_w levels could be helpful to predict potential risk of aflatoxin production during storage of this hygroscopic food product and minimise contamination with the AFB_1.

Introduction

Aflatoxin (AFA) contamination continues to be a serious problem in many parts of the world. *A. flavus* and *A. parasiticus* are known as pathogens of cotton, corn, peanuts and other oilseed crops, producing toxins both in the field and during storage under various environmental conditions (Pittet 1988; Llewellyn *et al*. 1992; Cotty 1997; Payne and Brown 1998; Bhatnagar *et al*. 2000; Horn 2007). The key environmental determinants pre- and post-harvest are water availability and temperature (Magan *et al.* 2003; Magan and Aldred 2007). The biosynthesis of secondary metabolites, including mycotoxins, is significantly influenced by environmental conditions such as pH, water activity (a_w) and temperature (Belli *et al*. 2004; Hope *et al*. 2005).

Previously, Moubasher *et al*. (1980) examined the effect of different moisture contents (8.5-21 % on a dry-weight basis) and temperatures (5-45^{o}C) on *A. flavus* infection of peanuts stored for up to 6 months. Highest population counts of A. flavus was found in peanuts stored at 13.5 % moisture content (approx. 0.90 a_w) at 15 °C for 1 month. Recently, a survey of Egyption peanuts by Sultan and Magan (2010) showed that *Aspergillus* section *Flavi* was consistently the most frequent genus in in-shell peanuts and was the dominant mycotoxigenic component of the mycobiota. However, in this two year survey, there was no direct correlation between the moisture content of the samples and the fungal populations on peanut seeds from different regions. The major mycotoxins found in Egyptian peanuts are aflatoxins (El-Maghraby and El-Maraghy 1987).

Molecular techniques have been applied for the detection of aflatoxigenic fungi in food samples (Geisen 1996; Shapira *et al*. 1996; Mayer *et al*. 2003; Somashekar *et al*. 2004). Traditional methods used to assess the presence of mycotoxigenic fungi in food are dependent on selective media, which are only available for some mycotoxigenic species. However, knowledge of the ability of the fungus to activate mycotoxin biosynthesis genes

under different environmental conditions may be a better indicator for determining the risk from specific mycotoxigenic species. Few studies have attempted to relate the expression of specific mycotoxin biosythesis genes with phenotypic mycotoxin production under different environmental conditions. Some studies have attempted to integrate the correlation of ecophysiological conditions with gene expression and phenotypic toxin production (Schmidt-Heydt *et al*. 2007; Geisen *et al*. 2008; Jurado *et al*. 2008; Schmidt-Heydt *et al*. 2009).

It has been reported that at least 25 identified genes are clustered within a 70-kb DNA region in the chromosome involved in aflatoxin biosynthesis (Yu *et al*. 2004). One of these is the nor-1 (=*AflD*) gene which encodes an enzyme that catalyses the ketoreduction of norsolorinic acid (the first stable pathway intermediate) to averantin (Chang *et al*. 1992; Trail *et al*. 1994). Disruption of this gene in *A. parasiticus* resulted in norsolorinic acid (NA) accumulation (Chang *et al*. 1992), confirming the important function of the nor-1 (=*aflD*) in AFA synthesis and suggesting that NA is a substrate for this protein.

Several studies have measured the expression of genes involved in the AFA biosynthetic pathway to distinguish between AFA producers and non-producers (Scherm *et al*. 2005; Degola *et al*. 2007; Rodrigues *et al.* 2009). Real-time RT-PCR is highly sensitive and allows quantification of rare transcripts and small changes in gene expression. Recently, Price *et al*. (2005) used a whole genome microarray approach to analyse the influence of substrate composition and pH on the activation of AFA biosynthesis genes. Yu *et al.* (2004) described the whole biosynthetic pathway and renamed the genes in the cluster. We have used the new names except for the *nor-1* (=*aflD*) expression for comparison with previous studies.

Schmidt-Heydt and Geisen (2007) developed and used a mycotoxin gene microarray and Real-Time PCR to study the influence of physical parameters like water activity (a_w), temperature and pH on the expression of ochratoxin A, trichothecenes and AFA gene

clusters. Schmidt-Heydt *et al*. (2008) studied the effect of temperature and a_w on growth and mycotoxin gene expression of several fungal species, including the AFA cluster of *Aspergillus parasiticus*. Recently, the influence of both parameters on AFA gene expression and aflatoxin B_1 (AFB_1) production by *A. flavus* was analysed (Schmidt-Heydt *et al*. 2009). The ecology and regulation of AFA biosynthesis by *A. flavus* in relation to external factors have also recently been summarized (Abbas *et al*. 2009; Georgian and Payne 2009). Recently, Passone *et al*. (2010) applied a real-time PCR system to detect and quantify the *nor-1* gene of the aflatoxin biosynthetic pathway based on DNA analyses in relation to *Aspergillus* section *Flavi* populations in stored peanuts.

The objectives of this study were to apply molecular tools and compare this with traditional assessment methods and quantitative AFB_1 analyses in monitoring temporal changes in stored peanuts. Experiments were carried out with *A. flavus* inoculated peanuts stored at three a_w levels (0.95, 0.90, 0.85) to measure (a) asexual reproduction of *A. flavus* (CFUs), (b) quantification of *nor-1* (*aflD*) gene expression, (c) AFB_1 production and (d) transcription of four AFA genes *nor-1* (*aflD*),*ver-1* (*aflM*), *omtA* (*aflP*), and *AflR* over a period of six weeks at 25^oC.

Materials and methods

Fungal strain and growth conditions: In this study, an aflatoxigenic strain of *Aspergillus flavus* (EGP9) isolated from Egyptian peanuts has been used. This was compared with a type strain of *A. flavus* SRRC G1907 provided by Dr. D. Bhatnagar, USDA and confirmed to be taxonomically similar, and an aflatoxin B_1 and B_2 producer. The strain was sub-cultured on Malt Extract Agar (20.0 g Malt extract (Difco), 2.0 g Peptone (Difco), 15.0 g Agar (Sigma) for 7 days at 25 °C in the dark.

Inoculation of peanut samples: A moisture adsorption curve was prepared for shelled peanuts in order to accurately determine the amount of water required to add to obtain the target a_w levels. This curve was obtained by adding different quantities of water to peanuts, equilibration overnight, and then determining moisture content by drying at 130°C for 12 hrs, and comparing this with the a_w level measured with an AQUALAB ® 3TE, USA. This showed that 90 µl, 53 µl and 25.4 µl water per gram peanuts were required to reach the target a_w levels of 0.95, 0.90 and 0.85 respectively.

One hundred g sub-samples of peanuts (three replicates per treatment) were put in glass jars covered with lids containing a microporous membrane and autoclaved at 121 ºC for 20 min. After cooling, the water was added and after equilibration the peanut samples were inoculated with 1 ml of a 10^6 spores ml^{-1} of *A. flavus* and vigorously shaken to coat the peanuts with spores and incubated at 25°C for six weeks in polyethylene sandwich boxes containing glycerol/water solutions to maintain the equilibrium relative humidity conditions. Samples were destructively sampled every 7 days (approx 15 g of contaminated peanuts) and divided into three parts: (a) 10 g for aflatoxin extraction, (b) 1 g for CFU determination and (c) 1 g for RNA extraction followed by RT-PCR and real-time PCR.

Determination of colony forming units (CFUs): The *A. flavus* total colony forming units (CFUs) were determined by serial dilution and spread plating the different dilutions on MEA and incubating for 4-5 days before counting numbers of colonies.

Aflatoxin extraction and HPLC analysis: 10 g of peanuts was extracted for AFA analyses using an immunoaffinity column (Neogen, Europe Ltd). The residue was derivatized using TFA (Triflouroacetic acid) as decribed by the AOAC (2000). Sample extracts were analyzed using an Agilent 1200 series HPLC (Agilent, Berkshire, UK) using a 470 fluorescence detector (FLD, G1321A, Agilent) (λ_{exc} 360 nm; λ_{em} 440 nm) and a C_{18} column (Phenomenex Luna ODS2 150 x 4.6 mm, 5 µm). The analysis was performed using a mobile

phase of methanol:water:acetonitrile (30:60:10) at a flow rate of 1 ml/min and a run time of 25 mins.

Isolation of RNA from the samples and RT-PCR: Total RNA was extracted from inoculated peanuts using the RNeasy and Plant Mini Kit (Qiagen GmbH, Hilden, Germany) with minor modifications. 1 g peanuts was ground in a mortar with a pestle in the presence of liquid nitrogen, and 500µl of lysis buffer from the RNeasy kit and 0.5 g of polyvinylpolypyrrolidone (PVPP). Insoluble PVPP binds to both polysaccharide and phenolic compounds and prevents the undesirable binding between nucleic acids and these compounds (Chen *et al*. 2000). RNA extraction was then performed according to the instructions provided by the manufacturer. RNA was treated with DNase I (RNase free DNase I, Amplification Grade, Sigma) to digest residual DNA in the samples.

The expression of three structural genes *nor-1* (*aflD*), *ver-1*(*aflM*), and *omtA* (*aflP*), and the regulatory gene *aflR* of the aflatoxin biosynthetic pathway were assayed in all treatments and replicates. The expression of the housekeeping gene (β-tubulin) was used as a control (see Table 1). RT was performed with a Qiagen sensiscript ® kit (Qiagen, UK) using oligo-dT primers to amplify the mRNA. The reaction was assembled in a 20 µl tube as follows: 1 µM Oligo(dT) primer, 1 x reaction buffer, 4U sensiscript Reverse Transcriptase, 2 µM dNTPs, 10 U RNase inhibitor , and 40 ng RNA sample in 12 µL H_2O (RNase free). The mixtures was incubated at 37 °C for 60 min followed by 93°C for 5 min in a thermal cycler (Peltier Thermal cycler PTC-200 MJ Research), followed by rapid cooling on ice.

Each 25 µl PCR reaction contained 800 µM dNTPs, 1 x reaction buffer , 1.25 U Taq DNA polymerase I, 0.2 µM of each primer, 1 µl cDNA mixture, 12 µL H_2O (RNase and Dnase free). PCR conditions were an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 60 s at 65 °C and 90 s at 72 °C, with a final extension at 72 °C for 7

min (Scherm *et al*. 2005). Primer sequences are detailed in Table 1. PCR products were visualized on a UV transilluminator (Gene Genius Bio Imaging system).

TaqMan probes and primer design: Real Time RT-PCR was used to amplify the *nor-1* gene (target gene) and *benA56* (β-tubulin gene) as a control gene (Mayer *et al*. 2003). The two primers and an internal fluorescence labelled probe used in the reaction were nortaq-1 5 -GTCCAAGCAACAGGCCAAGT-3 ; nortaq-2 5 -TCG TGCATGTTGGTGATGGT-3 ; norprobe 6FAM TGTCTTGATCGC GCCCG- BHQ2; bentaq-1 5 -CTTGTTGACCAGGTTGTGGAT-3 ; bentaq-2 5 -GTCGCAGCCCTCAGCCT-3 , benprobe CY5-CGATGTTGTCCGTCGCGAGGCT-BHQ2.

Real-time PCR conditions: Amplification was performed using a total reaction volume of 25 µl in a MicroAmp optical 96-well reaction plate (Applied Biosystems). For each reaction 12.5 µl of TaqMan Universal Master Mix (Applied Biosystems), 2.5 µl cDNA, 3 µl of primer and probe mix (0.5 nM primer and 0.2 nM probe), and 7 µl of free RNases water. Real Time reactions were performed using the Bio Rad CFX96 platform (Bio Rad) with the following conditions: an initial step at 95 ºC for 10 min, and all 40 cycles at 95ºC for 15 s, 55ºC for 20s and 72ºC for 30s.

Relative quantification method: The efficiency of PCR (E) was calculated from each linear regression of standard curves of each target and control gene which was calculated from the formula $E= [10^{(-1/slope)} -1] X 100$ (Figure 1). This method compares the relative amount of the target gene (*nor-1*) to control gene (*benA56*). The target and control amplification were carried out in separate tubes in triplicate. Normalized relative quantity $(NRQ) = E^{Ct\ nor\text{-}1}/E^{Ct\ benA56}$ where E is the PCR efficiency for each target, Ct is the threshold cycle (Pfaffl 2001). Only the linear range was used for quantification.

Contour map of responses: The three dimensional (3D) response contour plot was employed to determine the relationship between RQ-PCR data and a_w in relation to the

temporal storage period. The data was analysed using Statistica version 8 (StatSoft, Inc, 1984-2007) software.

Statistical analysis: All experiments were carried out with 3-4 replicates and repeated twice with similar results. Statistical tests were performed using Statistica version 8 (StatSoft, Inc, 1984-2007) for three-way ANOVA and LSD Fisher was determined at the 95% confidence limits.

Results

Effect of water activity on populations of *A. flavus* on stored peanuts: Figure 2 shows the temporal changes in *A. flavus* isolated from the three a_w treatments. There was a rapid increase in viable propagules produced at 0.95 a_w reaching a maximum at the end of the experiment. At 0.90 a_w, CFU numbers were <0.95 a_w with a maximum total after 4 weeks incubation. As water stress was imposed, the populations of *A. flavus* isolated were significantly decreased (P=0.05). Statistical analysis of the effect of single, and interaction conditions of a_w, time and a_w x time were statistically significant (Table 2a).

Effect of a_w on aflatoxin production: Figure 2 also compares the temporal AFB_1 production by *A. flavus* in the stored peanuts under different a_w regimes. Overall, *A. flavus* produced maximum amounts of AFB_1 at 0.90 a_w and 0.95 a_w after 3 weeks storage. The production of AFB_1 was detected after 1 week storage at 0.95 and 0.90 a_w. No increase in AFB_1 production occurred at 0.85 a_w when compared to the controls over the storage period. Table 2b summarises the statistical significance of the single and two way interaction factors for a_w, time and a_w x time which were all statistically significant.

RQ-PCR of *nor-1* in relation to water activity: The normalized relative quantity (NRQ) of *nor-1* aflatoxin gene with the β-tubulin gene (housekeeping gene) of *A. flavus* in peanuts was analysed (see Figure 2). There was a significant differences between *nor-1*

expression at the three a_w levels, There was higher expression at 0.90 a_w especially during weeks 1-3, after which expression decreased. At 0.95, the expression was lower than at 0.90 a_w and the highest expression was after 3 weeks and then decreased further. At 0.85 a_w, there was no *nor-1* expression during the first two weeks and very low expression subsequently. Single and interacting factors were all significant (Table 2c).

The data for *nor-1* gene relative expression was analysed to examine whether there was any pattern to production over time. Figure 3 shows the contour map for relative expression of the *nor-1* gene at different a_w levels in relation to time. There is a clear optimum of expression at 0.90 during the first 2 weeks of storage, with less expression at 0.95 and 0.85 a_w and over time. This suggests some pattern with regard to relative expression of the *nor-1* gene when *A. flavus* colonises peanuts.

Analysis of aflatoxin gene transcription in relation to water activity: In this study the transcription of four genes, *nor-1* (*aflD*), *ver-1*(*aflM*), and *omtA* (*aflP*), and *aflR* in the biosynthetic pathway for AFB_1 production were assessed (Figure 4). The expression of the house keeping gene (β-tubulin) was used as a control. Transcription of the genes was assayed by RT-PCR. To ensure there was no DNA contamination in the RNA, for each sample PCR was performed following an RT reaction in the presence (+RT) or absence (-RT) of the reverse transcriptase enzyme. RT-PCR results revealed that at 0.95 a_w, all four genes were transcribed from the beginning to the end of the storage period. At 0.90 a_w, *aflD* was expressed from the start of the experiments while *aflR* only from 4 weeks onwards. The genes *aflP* and *aflM* were expressed from the 2^{nd} week onwards. In the driest conditions tested (0.85 a_w) only two genes were transcribed (*aflD* and *aflM*) after 3 weeks.

Correlation co-efficients for comparing different factors: Table 3 shows the results from examining the possible correlations between different treatment factors. There was a good correlation between *A. flavus* CFUs and a_w (R= 0.75: P= 0.00), AFB_1 correlated

significantly with a_w (R= 0.68; P= 0.00) and AFB_1 x log CFUs (r=0.85. However, for other factors there were no significant correlations.

Discussion

This is one of the first studies to compare the influence of a_w on relative quantification of the *nor-1* gene in relation to CFUs, phenotypic aflatoxin production, and aflatoxin gene transcription of *A. flavus* during storage of peanuts. Quantification of the *nor-1* gene expression, based on the relative expression of this gene, versus a reference gene or housekeeping gene (β-tubulin gene) provided useful information to relate molecular changes to ecophysiological parameters. Previously, Mayer *et al*. (2003) reported that the β-tubulin gene was constitutively expressed and constant during the subsequent growth phases when compared with the expression of *nor-1* gene of *A. flavus.*

Our results showed that temporal changes in asexual reproduction (CFUs) in relation to storage a_w showed a good correlation (r=0.75; P =0.00). In the wettest condition tested more rapid colonization and sporulation occurred reflecting the high $\log_{10}$ CFUs found. No statistical correlation between *A. flavus* CFUs and the quantified *nor-1* gene expression levels (r=0.175; P=0.09) was found. This may partially be because of the fact that at lowered a_w levels (e.g., 0.90) there was an increase in *nor-1* gene expression although populations (CFUs) of *A. flavus* increased at a slower rate over the 6 week storage period. Recently, Passone *et al*. (2010) reported a good correlation (r=0.613; P<0.0001) between *nor-1* gene expression and CFUs in naturally stored peanuts over a period of 4 months for *Aspergillus* section *Flavi*. However, their study was based on DNA analyses of the *nor-1* gene, not RNA expression. Since all propagules contain the gene, the presence of *nor-1*, per se, may not accurately reflect expression and phenotypic production of aflatoxins.

The only other recent study was that by Schmidt-Heydt *et al*. (2007) who examined *Penicillium verrucosum* populations and ochratoxin A (OTA) and the OTA polyketide synthesis gene expression (*otapks*Pv) in wheat stored at three water contents (14, 19 and 24%) for up to three months. They showed good correlations between the *otapks*Pv expression, phenotypic OTA production and in some cases that this was paralleled by CFUs of *P. verrucosum*.

Overall, similar results were obtained by real time PCR at both 0.90 a_w and 0.95 a_w; with high expression especially during the first three weeks, before expression slowed down. The contour map of expression of *nor-1* shows these changes clearly over storage time with the optimum during the first few weeks of storage. There was thus a poor correlation between RQ-PCR data and AFB_1 production (r= 0.488; p=0.000). This poor correlation is probably due to the *nor-1* expression being initiated very early, prior to phenotypic aflatoxin production being synthesised. Thus expression of the *nor-1* transcripts may already be decreasing as the increase in toxin production is detected (Mayer *et al*. 2003).

The high sensitivity of the nor-1 (=*aflD*) gene expression in relation to changes in a_w levels during storage of peanuts can be easily determined by the real-time PCR system. This could be a useful tool to improve food safety of peanuts and predict environmental condition that we can use to inhibit or reduce expression of this important gene as well as aflatoxin production. Previously, Mayer *et al.* (2003) used a real-time reverse transcription-PCR system to monitor the expression of the nor-1 gene of *A. flavus* in wheat. They found that the described real-time PCR system was able to completely characterize the mycological status of wheat as a model food matrix.

Several genes code for proteins involved in the aflatoxin biosynthesis pathway. Among the 25 genes involved in aflatoxin biosynthesis, we selected three structural genes *aflD* (early stage), *aflM* (middle stage), *aflP* (late stage) and the regulatory gene *aflR* that

plays a role in controlling the level of structural gene expression (Woloshuk *et al*. 1994; Chang 2004; Price *et al*. 2005) to measure their transcriptional status in relation to changes in a_w level during peanut storage.

Water activity had a significant effect on *aflR* transcription, especially at 0.90 a_w, where it was transcripted from the 4^{th} week, while during the initial three weeks there was high expression of *nor-1* gene, transcription of the structural genes and high aflatoxin production. This suggests that *aflR* may not have a role in regulation of structural gene expression in food matrices such as peanuts. This contrasts with Degola *et al*. (2007) who reported that structural gene expression follows regulatory genes *aflR* and *aflS* transcription. Incomplete induction of these genes does not permit the detection of the structural gene expression, even by RT- PCR. This may confirm the fact that gene expression may be variable depending on physiological and environmental conditions.

Our results support those obtained by Schmidt-Heydt *et al*. (2009). They demonstrated that at lowered a_w (0.90) levels, the ratio of *aflS/aflR* was decreased compared to the other genes of the cluster. Thus, although expression was high (including that of *aflD*), low amounts of AFB_1 were produced in vitro. In contrast, in peanuts a high amount of AFB_1 was produced. This may partially be because the present study was carried out directly on the food matrix which may give different results from those on a conducive in vitro medium. However, in situ studies are critical to enable a better understanding of the ecophysiological and functional importance of specific regulatory genes to develop effective control approaches to minimise mycotoxin contamination of a range of important staple food commodities.

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Table 1: Details of primer sequences, target gene, annealing temperature and expected PCR/RT-PCR product length in base pairs (bp).

Primer pair	Gene	Primer sequence (5'- 3')	Optimal Annealing Temp. (°C)	RT-PCR product size(bp)
Tub1-F	**Tub 1**	GTCCGGTGCTGGTAACAACT	65	837
Tub1-R		GGAGGTGGAGTTTCCAATGA		
NOR1-F	**aflD**	ACCGCTACGCCGGCACTCTCGGCAC	65	400
NOR1-R		GTTGGCCGCCAGCTTCGACACTCCG		
VER1-F	**aflM**	GCCGCAGGCCGCGGAGAAAGTGGT	65	487
VER1-R		GGGGATATACTCCCGCGACACAGCC		
OmtA-F	**aflP**	GTGGACGGACCTAGTCCGACATCAC	65	624
OmtA-R		GTCGGCGCCACGCACTGGGTTGGGG		
AflR-F	**aflR**	CGAGTTGTGCCAGTTCAAAA	55	999
AflR-R		AATCCTCGCCCACCATACTA		

Table 2. (a) Analysis of Variance of the effect of $a_{w,}$ time and their interactions on CFUs of *A. flavus* in stored peanut peanuts; (b) single and two way interactions on aflatoxin production, and (c) single and two way interactions on RQ-PCR of *A. flavus* in peanuts.

(a)

	DF	MS	F	P
Factor				
a_w	2	207.367	1064.18	0.00
Time	6	35.263	180.96	0.00
Interaction factors				
a_w x Time	12	8.635	44.31	0.00
Error	42	0.195		

(b)

	DF	MS	F	P
Factor				
a_w	2	643315	1064.18	0.00000
Time	6	895126	180.96	0.00000
Interaction factors				
a_w x Time	12	243808	44.31	0.00000
Error	42	31469		

(c)

	DF	MS	F	P
Factor				
a_w	2	10.49419	157.7194	0.00000
Time	5	1.61667	24.2973	0.00000
Interaction factors				
a_w x Time	10	1.4641	22.0044	0.00000
Error	36	0.06654		

DF: Degree of freedom, MS: mean square, P: Probability

Table 3. Statistical correlations between *A. flavus* populations (CFUs), aflatoxin B_1 production, and RQ-PCR of nor-1 gene of *A. flavus* in stored peanuts at different a_w levels for up to 6 weeks storage.

Correlations	R value	F	P
log aflatoxin & log CFUs	0.849	157.44	0.000
log aflatoxin & water activity	0.68	78.22	0.000
log aflatoxin & RQ-PCR	0.488	19.03	0.000
log CFUs & water activity	0.75	78.22	0.000
log CFUs & RQ-PCR	0.175	1.919	0.09
RQ-PCR & water activity	0.08	0.416	0.0051

R: correlation coefficient. P: Probability

Figure legends

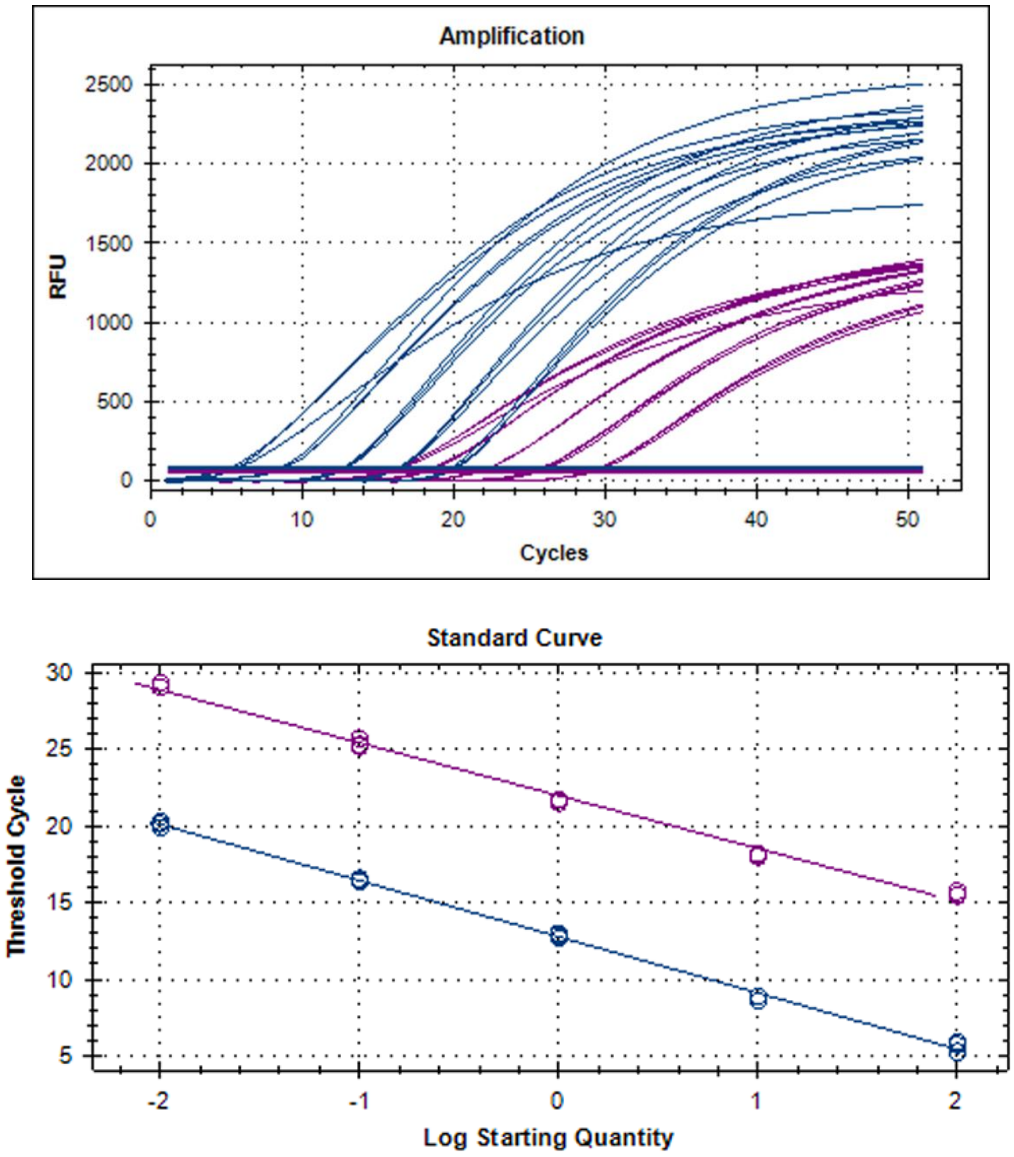

Figure 1. Amplification plot and standard curve of nor-1 gene (target gene) labelled with FAM and β-tubulin gene (control gene) labelled with Cy5. Where (RFU) is Relative fluorescent unit, E: The efficiency of PCR, R^2 value: correlation coefficient.

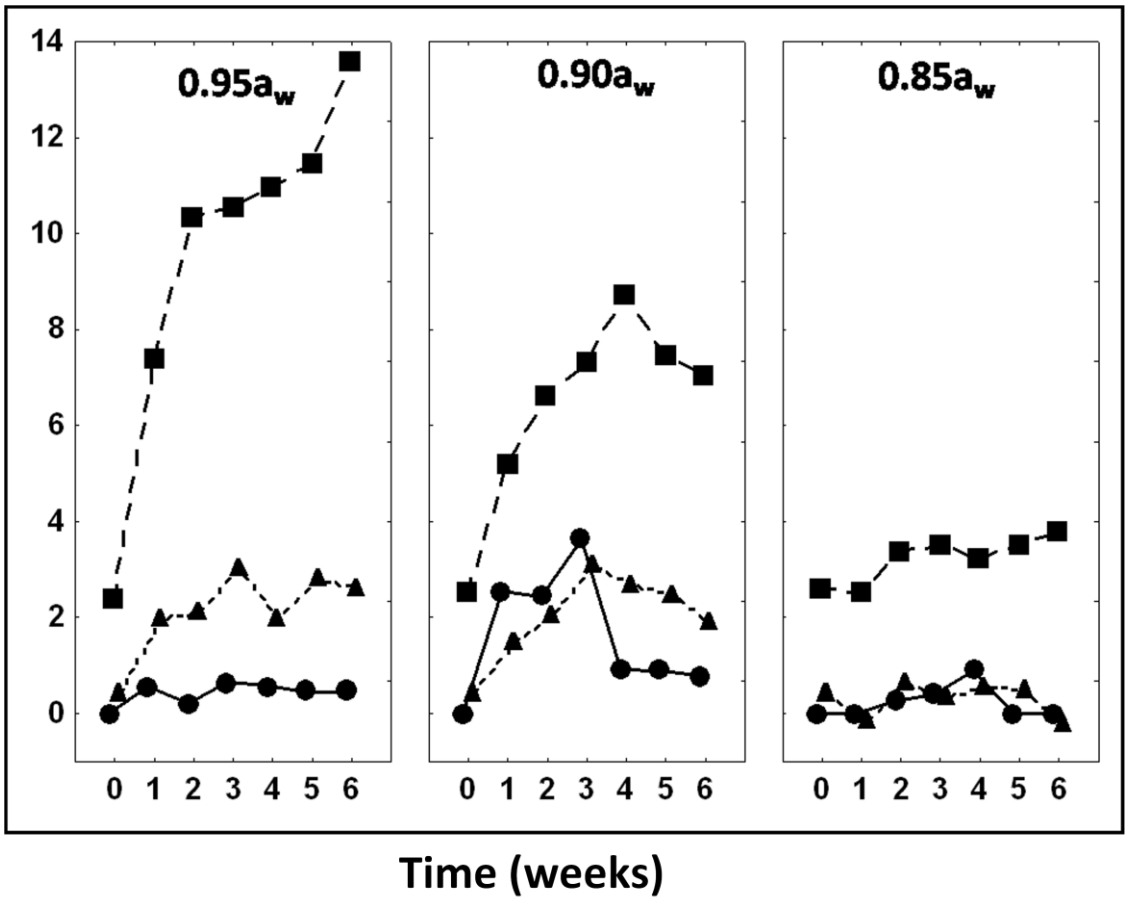

Figure 2. Comparison of CFU values , Aflatoxin B_1 production and RQ-PCR of nor-1 gene of *A. flavus* in peanut at different a_w levels and different incubation intervals at 25 °C. Vertical bar indicates 95% confidence limits.

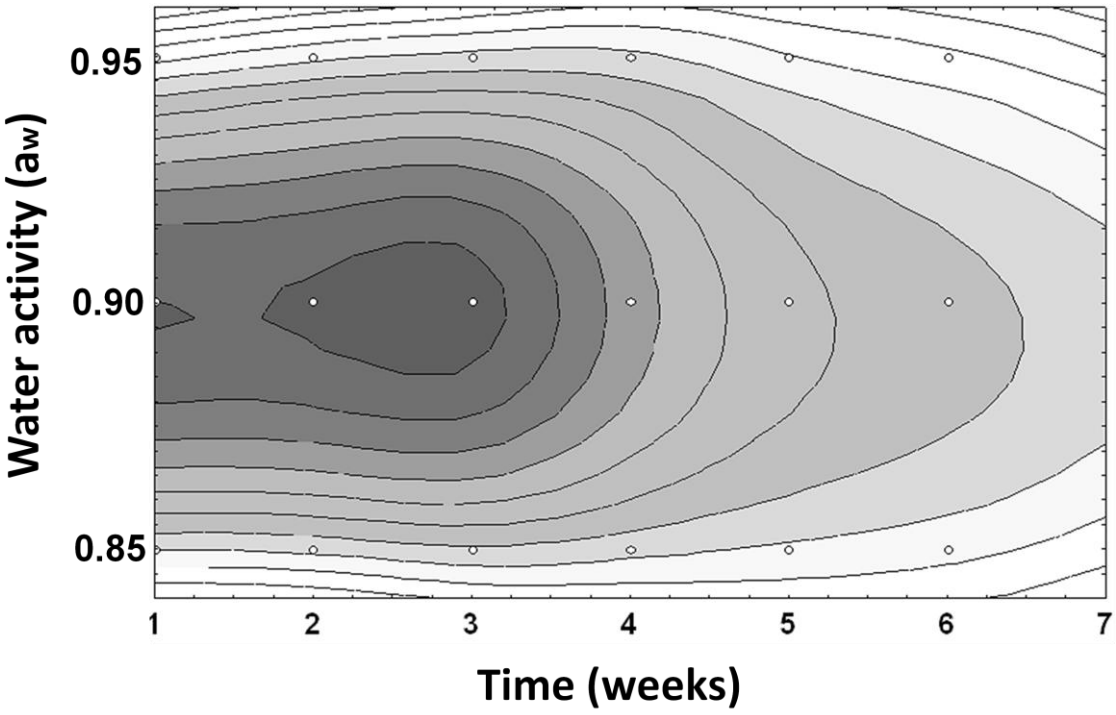

Figure 3. 3D contour plot for comparison of effect of water activity (a_w) and time on RQ-PCR of nor-1 (=*aflD*) gene of *A. flavus* in peanuts at 25°C. The categories are for relative expression of this gene.

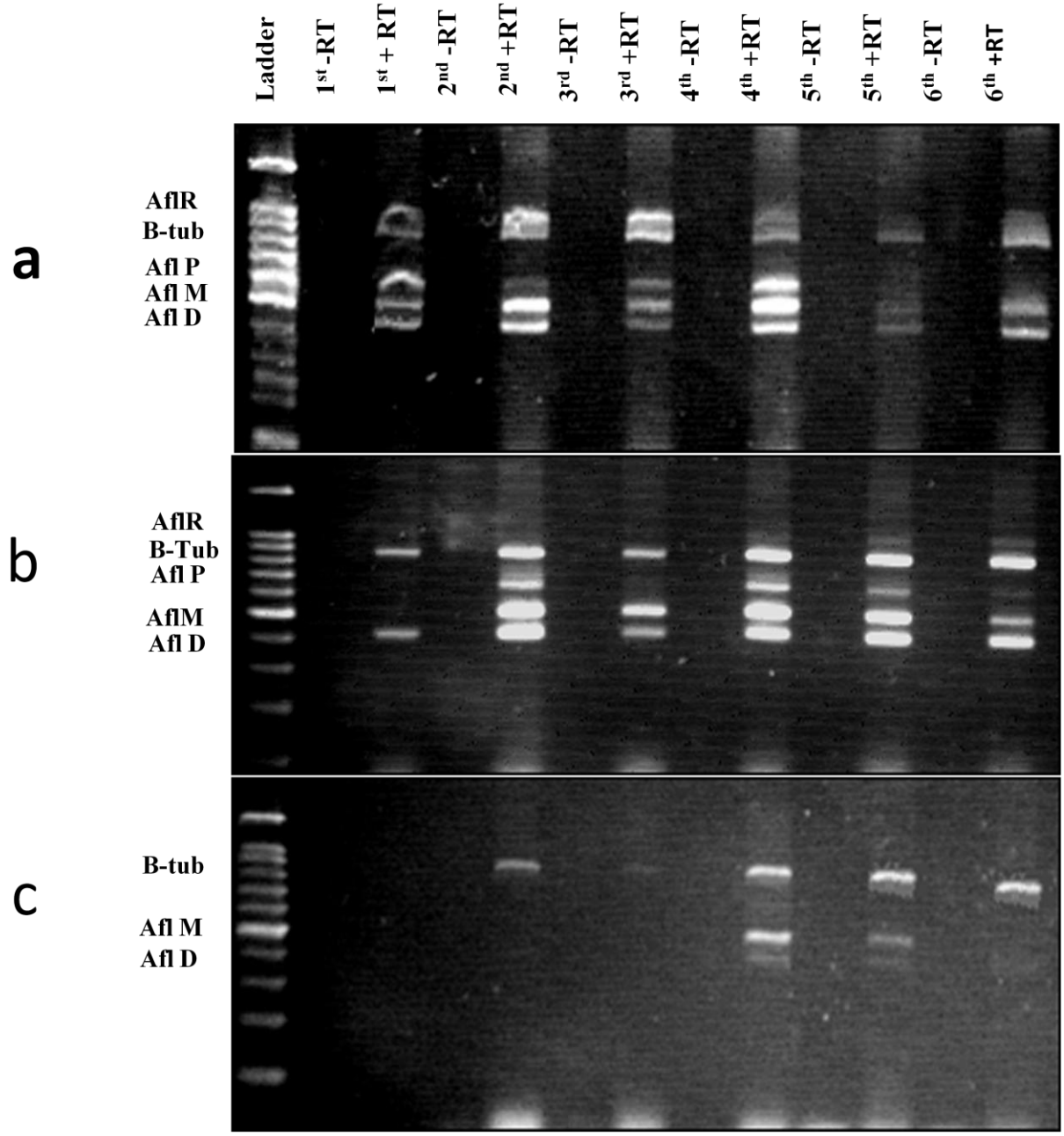

Figure 4. Transcription of primer set (β- tubulin, AflD, AflM, AflP AflR) detected by RT-PCR in *A. flavi* EGP9 at three a_w levels (a) 0.95, (b) 0.90 and (c) 0.85 for six weeks. First lane 100bp ladder; RNA from each treatment was amplified by PCR following reverse transcription in the absence (-RT) or presence (+RT) of the RT enzyme. PCR products were separated on a 2 % agarose gel, stained with ethidium bromide and visualized under UV.