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# Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature

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**Abstract**

A microarray analysis was performed to study the effect of varying combinations of water activity and temperature on the activation of aflatoxin biosynthesis genes in *A. flavus* grown on YES medium. Generally *A. flavus* showed expression of the aflatoxin biosynthetic genes at all parameter combinations tested. Certain combinations of $a_w$ and temperature, especially combinations which imposed stress on the fungus resulted in a significant reduction of the growth rate. At these conditions induction of the whole aflatoxin biosynthesis gene cluster occurred, however the produced aflatoxin $B_1$ was low. At all other combinations (25 ℃/0.95 and 0.99; 30 ℃/0.95 and 0.99; 35 ℃/0.95 and 0.99) a reduced basal level of cluster gene expression occurred. At these combinations a high growth rate was obtained as well as high aflatoxin production. When single genes were compared, two groups with different expression profiles in relation to water activity/temperature combinations occurred. These two groups were co-ordinately localized within the aflatoxin gene cluster. The ratio of *afl*R/*afl*J expression was correlated with increased aflatoxin biosynthesis.

**Keywords**: aflatoxin; gene expression; microarray; water activity; temperature; stress; *afl*S; *afl*R; aflatoxin cluster

## 1. Introduction

Aflatoxins are produced by *Aspergillus flavi* group species and are thought to be one of the most cancerous natural substances known. Economically and biologically the most important fungal species able to produce the aflatoxins are *A. flavus* and *A. parasiticus* (Bhatnagar et al., 2002). The aflatoxin biosynthesis gene cluster of *A. parasiticus* has been completely elucidated (Yu et al., 2004). Moreover the genome of *A. flavus* is known and a whole genome microarray of *A. flavus* is available and has been used to study the regulation of aflatoxin biosynthesis genes (O´Brian et al., 2003). Generally, the aflatoxin biosynthesis genes of *A. flavus* and *A. parasiticus* are highly homologous and the order of the genes within the cluster being the same (Yu et al., 1995). *A. flavus* usually produces only aflatoxin $B_1$ and $B_2$. Not all of the strains of *A. flavus* isolated from the natural habitat are able to produce these metabolites at least under laboratory conditions. On the other hand, *A. parasiticus* is a strong and consistent aflatoxin producing species (Bennett and Christensen, 1983; Vaamonde et al., 2003). Most strains of this species are able to produce the aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$.

The biosynthesis of aflatoxins, as all secondary metabolites, is strongly dependent on growth conditions such as substrate composition (Luchese and Harrigan, 1993) or physical factors like pH, water activity, temperature or modified atmospheres (Ellis et al., 1993; Molina and Gianuzzi, 2002; Ribeiro et al., 2006; Giorni et al., 2008). Depending on the particular combination of external growth parameters the biosynthesis of aflatoxin can either be completely inhibited, albeit normal growth is still possible or the biosynthesis pathway can be fully activated. Knowledge about these relationships enables an assessment of which parameter combinations can control aflatoxin biosynthesis or which are conducive to phenotypic

aflatoxin production. A mathematical model which delineates the relationship between pH, propionic acid concentration and temperature on aflatoxin biosynthesis by *A. parasiticus* was described by Molina and Giannuzzi (2002). Sweeney et al. (2000) developed a reverse transcription polymerase chain reaction to analyse the expression of the *afl*R and *afl*Q gene of the aflatoxin pathway in relation to various nutritional media. Xu et al. (2000) monitored the expression of the *afl*D gene after growth of *A. parasiticus* on peanut pods by a reporter gene approach. Mayer et al. (2003) showed that expression of the *afl*D gene and the biosynthesis of aflatoxin in wheat are directly coupled. Recently Price et al. (2005) used a whole genome microarray approach to analyse the influence of substrate composition and pH on the activation of aflatoxin biosynthesis genes. With a pathway specific microarray and by Real Time PCR the influence of physical parameters like water activity, temperature and pH on the expression of ochratoxin A-, trichothecene- and aflatoxin biosynthesis genes have been analysed and a common expression profile was found (Schmidt-Heydt and Geisen, 2007). The published data show a correlation between external parameters and gene expression, however no comprehensive systematic analysis of this in relation to aflatoxin production has been performed. Recently Georgianna and Payne (2009) summarized the current knowledge of the regulation of aflatoxin biosynthesis in relation to external factors.

In the current work the influence of various combinations of the most important physical parameters, temperature and $a_w$, on the regulation of the aflatoxin biosynthesis genes of *A. flavus* was systematically analysed. A basic model was generated to visualize the correlation between parameter combinations and cluster gene activation. This type of study is required to elucidate the pivotal role of environmental factors in the activation of the aflatoxin biosynthesis genes.

## 2. Materials and methods

### *2.1 Strains and growth conditions*

*A. flavus* SRKC-G1907 produces aflatoxin $B_1$ when grown under permissive conditions. This strain produces high amounts of aflatoxin after growth on YES agar (20 g/l yeast extract, 150 g/l sucrose, 15 g/l agar) at 25 to 30 ℃ for 5 days. For expression and toxin analysis the agar plates were overlayed with sterile 8.5 cellophane sheets (P400, Cannings, Bristol, U.K.) and then single point inoculated centrally by applying 10 µl of a spore suspension ($10^7$ spores in TWS (0.5 % tween 80, 0.85 g/l NaCl)). The water activity of the media was adjusted with glycerol by using glycerol/water mixtures. The following amounts were used per litre (108 ml, 0.99; 13.1 ml, 0.98; 19.9 ml, 0.95; 24.5 ml, 0.93; 31.3 ml, 0.90). The water activity of the medium was verified by using an Aqua Lab Lite water activity meter (Graintec Pty Ltd). The plates were inoculated at the temperature indicated (15 – 42 ℃).

### *2.2 Growth assessment*

For measurement of the diametric mycelial growth rate, the diameter of the colony was measured in two directions at right angles to each other. The increase in colony radius was plotted and the linear regression lines for the linear phase were used to obtain the relative growth rates (cm/7days). The biomass was removed from the cellophane surface for extraction and molecular and aflatoxin analysis. All experiments were carried out with 3-4 replicates and some temperature treatments repeated for confirmation of results.

*2.3 Isolation of RNA from samples*

To perform microarray experiments RNA was isolated using the RNAeasy Plant Mini kit (Qiagen, Hilden, Germany). An amount of 1 g of the mycelium was ground with a mortar and pestel in liquid nitrogen. About 250 mg of the resulting powder was used for isolation of total RNA. The powder was resuspended in 750 µl lysis buffer, mixed with 7.5 µl β-mercaptoethanol and 100 glass beads with a diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) in a 2 ml RNase free micro reaction tube. The extracts were mixed thoroughly and incubated for 15 min at 55 ℃ and 42 kHz in an S10H ultrasonic bath (Elma, Singen, Germany). All further procedures were essentially the same as suggested by the manufacturer of the kit.

*2.4 Microarray experiments*

cDNA synthesis and labelling were performed using the Micromax Direct Labeling kit (Perkin Elmer Life And Analytical Sciences, Inc. Boston, USA). For this purpose an amount of 50 µg of the DNase I treated total RNA was used according to the specifications of the kit. After cDNA synthesis and labelling, the cDNA was purified with the QiaQuick Min Elute kit (Qiagen, Hilden, Germany). The labelled and purified cDNA was brought to dryness in a vacuum concentrator (Speed Vac, Savant Instruments, Farmingdale, USA), resuspended in 60 µl hybridization buffer (Scienion, Berlin, Germany), heated for 2 min. at 95 ℃, placed on ice to prevent strand rearrangement and hybridized for 18 h at 42 ℃ to the microarray by using an automatic hybridization station (Perkin Elmer, Boston, USA). After hybridization the array was scanned with a confocal laser system (Scanarray lite, Perkin Elmer) at a

resolution of 5 µm. The analysis of the results was performed using the Scanarray software (Perkin Elmer, Boston, USA). The results were normalized using the Lowess algorithm (locally weighted scatter plot smoothing) together with subtraction of the background signal. As control, the β-tubulin gene was used.

*2.5 Quantitative determination of aflatoxin $B_1$ by HPLC*

Detection and quantitative determination of aflatoxin $B_1$ from fungal colonies were performed on a high-pressure gradient system from Shimadzu (Duisburg, Germany) equipped with an auto injector and a fluorescence detector. For this purpose 100 mg of the fungal colony were extracted under shaking conditions in 500 µl chloroform at room temperature for 30 min. The residue was discarded and the chloroform was evaporated to dryness in a vacuum concentrator (Speed Vac, Savant Instruments, Farmingdale, USA). The residue was then redissolved in 200 µl methanol and subjected to HPLC analysis (20 µl).

Separation was carried out on a Prontosil (250 mm x 4.6 mm i.d., particle size 3 µm) reversed-phase column (Bischoff, Leonberg, Germany). The solvent system consisted of 0.1 % formic acid in water (pH = 3) (A) and acetonitrile (B). Elution was effected using a linear gradient from 30 % to 90 % B in 30 min. The flow rate was 0.8 ml/min. The fluorescence detector settings were an excitation wavelength of 365 nm and an emission wavelength of 440 nm.

## 3. Results

### *3.1 Expression profile of the aflatoxin biosynthesis genes of* A. flavus *in relation to the parameter combinations temperature x water activity*

In order to analyse the influence of combinations of the external parameters temperature x $a_w$ on aflatoxin gene activation *A. flavus* was grown for 7 days on YES medium adjusted to the respective $a_w$ at the temperatures as indicated. After this time the activation of the aflatoxin biosynthesis gene cluster was determined by microarray analysis. In parallel at each parameter combination the colony growth as well as the aflatoxin $B_1$ produced was determined. The results obtained are shown in Figure 1.

The growth rates follow a general pattern. At each temperature growth increased as $a_w$ was increased from 0.90 to 0.99. The growth optimum for this strain was at 37℃. At 25, 30 and 35 ℃ the biosynthesis p attern of aflatoxin followed the growth rate and was high at 0.99 $a_w$ and low at 0.90 $a_w$. Highest amounts of aflatoxin were produced between 25 and 30 ℃, but also at 20 ℃. Very low amounts were produced at ≥37 ℃ and at a $_w$ of 0.90.

The expression profiles of the aflatoxin cluster genes were co-ordinately changed in response to the environmental conditions and were partly inversely related to the growth rate and also to aflatoxin biosynthesis. At intermediate combinations of temperature x $a_w$ (25 ℃/0.95 and 0.99; 30 ℃/0.95 and 0.99; 35 ℃/0.95 and 0.99) a high production of aflatoxin $B_1$ was observed despite the fact that the cluster genes are transcribed only to a certain basal level. At these

combinations growth was moderate or close to the optimum. At combinations of temperature x $a_w$ which are less favourable for growth due to low $a_w$ or high temperature (25 ℃/0.90; 30 ℃/0.90; 37 ℃/0.90 and 0.95 and 0.99; 40 ℃/0.90 and 0.95 and 0.99; 42 ℃/0.99) the overall cluster genes are induced when compared to the basal level. Interestingly most of the cluster genes are activated nearly to the same level for all these combinations except of some single genes which are expressed at a significantly higher level. Generally growth was significantly decreased at these stress conditions with an exception at 37℃/0.99 where growth was at an intermediate level. Under these inducing conditions only very low amounts of aflatoxin were produced. Even after prolonged incubation of *A. flavus* no increase in aflatoxin concentration occurred (data not shown). An even more pronounced induction of several genes of the cluster could be observed at only one specific condition, e.g. 20℃/0.99. At this condition, although growth is moderate high amounts of aflatoxin were produced. At combinations of 20 ℃/0.95 and 0.90 growth was practically inhibited and analysis was not possible.

*3.2 Expression of specific genes*

It was noticeable that the expression of some genes was much higher than the general cluster gene expression level under certain parameter combinations. This was especially true for the *afl*S gene. The *afl*S (formerly *afl*J) gene is a regulatory gene which interacts and activates *afl*R, the major regulatory gene, which than activates the aflatoxin structural genes (Chang, 2003). At most conditions with a temperature >37℃ the *afl*S gene expression was highly increased especially at 37℃/0.90. At certain other parameter combinations at 20, 25 and 30℃ the expression of the *afl*S gene was also increased when compared to the other genes of

the cluster. This was often accompanied by an increased biosynthesis of aflatoxin compared to the neighbouring conditions. At conditions <37°C a reduction of the $a_w$ to 0.90 did not lead to an increased expression of the *afl*S gene compared to the other cluster genes. This lower activity of the *afl*S gene was always accompanied by a lower biosynthesis of aflatoxin (see Figure 1). At 20°C/0.99 $a_w$ the *afl*S gene was very high expressed relative to the other genes and accompanied by a similar activation of the *afl*J (formerly *est*A) gene. Under these conditions growth was moderate and aflatoxin biosynthesis high. At all analysed conditions the *afl*R gene was not prominently expressed and followed the general expression of the cluster genes. However the ratio of expression between the *afl*S and *afl*R gene seemed to be critical for aflatoxin biosynthesis (Table 1). At conditions with low $a_w$ (0.90) the ration is far below 1. At these conditions low amounts of aflatoxin are produced. At moderate conditions the ratio is more than 1. At these conditions high amounts of aflatoxin are produced.

*3.3 Differences in the regulation of the single cluster genes in relation to combinations of temperature and water activity*

In order to compare the expression profiles of single genes in relation to both parameters all expression data of the single genes were plotted against temperature and $a_w$ (Figure 2). Based on the expression profiles, two groups of genes could be identified. The expression profile of group 1 is shown in Figure 2A. The following genes belonged to this group and all showed a very similar profile: *afl*M, *afl*E, *afl*H, *afl*J, *afl*N, *afl*R, *afl*S, *afl*U, *afl*Q, *afl*X. The expression profile of the second group (group 2) of genes is shown in Figure 2B. The following genes belong to this group: *afl*G, *afl*T, *afl*P, *afl*D, *afl*L, *afl*O, *afl*C, *afl*F, *afl*A, *afl*B, *afl*K, *afl*V, *afl*W, *afl*Y. Also in this

group the expression patterns of all the genes were very similar and resembled the profile which is shown in Figure 2B. The genes *afl*S and *afl*J, which were highly expressed under certain conditions, were both in group 1. When the two gene groups are assigned to the cluster all of the group 1 genes are adjacently located covering the region from *afl*R to *afl*N. The other group is split and comprises the regions from the *afl*F to the *afl*A gene and from the *afl*G to the *afl*Y gene. The only exception is the *afl*U gene, which belongs to the expression profile of group 1. The data suggests that different mechanisms are involved in the regulation of the two groups in relation to environmental changes and that co-localization within the cluster might facilitate this different behaviour (Fig. 3).

*3.4 Matrix of aflatoxin cluster gene activation in relation to water activity and temperature*

Based on the expression data obtained during this systematic analysis a matrix of aflatoxin cluster gene expression in relation to $a_w$ and temperature could be generated (Fig. 4). This matrix plots the level of induction versus certain combinations of $a_w$ levels and temperatures. Three sets of parameter combinations could be identified in this analysis. First parameter combinations which allow a basal level of expression (Fig. 3, white squares) in the temperature range of 25-37℃ at $a_w$ levels >0.90 (e. g. $a_w$ 0.95 and 0.99). These conditions support moderate to good growth of *A. flavus* and are usually accompanied by high production levels of aflatoxin. A general activation level of the whole gene cluster with high expression of certain genes could be identified at all temperatures in combination with low $a_w$ (0.90) or at temperatures >37℃ (Fig. 3, light grey squares). These conditions impose stress on the fungus, growth is significantly reduced and very low amounts of aflatoxin are

287 produced. Finally an activation level at which certain genes are very highly expressed
288 (*afl*S) could be identified at one unique combination only (20 ℃/0.99; Fig. 3, dark
289 grey square). At this condition moderate growth is possible suggesting moderate
290 stress. Also at this condition high amounts of aflatoxin are produced. The reason why
291 the expression behaviour at the combination 37℃/0. 90 was not included in this last
292 group is because despite the quite high induction of the *afl*S gene, which was about
293 half of that at 20℃/0.99, no increase in aflatoxin production occurred. These results
294 suggest that the stress level applied is due to these differences. Both situations are at
295 the margins of the window for growth because adjacent combinations are no longer
296 supportive for growth (e.g. 20℃/0.95 and 40℃/0.90 ).
297
298 **4. Discussion**
299
300 In this study the regulation of the aflatoxin cluster genes of *A. flavus* in relation
301 to changing environmental parameters, especially temperature x $a_w$ combinations
302 have been analysed using a toxin gene cluster microarray. Three types of cluster
303 gene expression could be observed, which appeared to be related to the level of
304 abiotic stress imposed. The involvement of abiotic stress in the activation of
305 mycotoxin biosynthetic genes have been described in several studies (Schmidt-Heydt
306 et al., 2008; Jayashree and Subramanyam, 2000; Jurado et al., 2008; Kim et al.,
307 2005; Ochiai et al., 2007; Kohut et al., 2009). In conditions favourable for growth of *A.*
308 *flavus* the cluster genes are expressed at a basal level with the strain producing high
309 amounts of aflatoxin. Under mild stress conditions (20 ℃/0.99) several of the cluster
310 genes, in particular *afl*S and *afl*J were very highly induced concomitant with high
311 levels of phenotypic aflatoxin production. Under increased stress conditions (0.90 $a_w$
312 and >37 ℃) the whole cluster was induced, with the *afl*S expression being prominent

313 at certain parameter combinations (>37 ℃), however much less compared to the
314 former situation. Under these conditions, surprisingly very low amounts of aflatoxin
315 were produced. Similar behaviour was recently obtained for patulin biosynthesis
316 (Baert et al., 2007). These authors showed that mild stress imposed by abiotic factors
317 induced patulin production, but higher stress was inhibitory. Similar effects were also
318 reported by Schmidt-Heydt et al. (2008) and Jurado et al. (2008) for mycotoxigenic
319 *Fusarium* and *Penicillium* species. It may be that although the gene cluster is
320 expressed above the basal level other posttranscriptional mechanisms impair
321 aflatoxin biosynthesis. This mechanism might act at the stage of transcription, e. g. a
322 reduction of transcription or at the protein level, e. g. an inhibition of enzyme activity
323 by some kind of protein modification. Alternatively at the metabolic level the
324 concentration of precursors or other metabolites required might be suboptimal. For
325 example, even at the combination of 37℃/0.90 where a high activation of the *afl*S
326 gene occurred, similar to that at 20 ℃/0.99, only very low aflatoxin production was
327 observed. Interestingly, both conditions are at the growth-no growth boundary where
328 conditions are very marginal for growth (no growth at 20℃/0.95 and 40℃/0.90).
329 Similar observations were made in other studies, in which low production of aflatoxin
330 was observed, despite the fact that the cluster genes were actively transcribed
331 (Georgianna and Payne, 2009).
332
333 O´Brian et al. (2007) recently used a microarray analysis to study the influence
334 of temperature on expression of the aflatoxin biosynthesis genes. They found a
335 reduced expression and no production at 37℃ for th eir strain. However, a strong
336 induction of the genes occurred at 28℃ which was p aralleled by high aflatoxin
337 biosynthesis. Most of their data fits very nicely to the data presented here. Also in the
338 current analysis a high production between 25 and 30 ℃ could be seen. At

339 temperatures beyond or at 37 ℃ very scarce amounts of aflatoxin were produced.
340 However differences were observed for the expression of the aflatoxin cluster genes.
341 O´Brian et al. (2007) found a high level of expression at optimal production
342 temperature (28 ℃) and a low level at 37 ℃. Durin g this analysis only a basal level
343 of expression was found between 25 and 30 ℃ as lon g as the water activity was
344 above 0.90, but an induced level at temperatures above 37 ℃. In the current study
345 the fungus were incubated on solid YES medium for 7 days. In the study of O'Brian et
346 al. (2007) *A. flavus* was grown in liquid A & M medium for 7 days which might
347 account for these differences. Moreover the water activity was not controlled in the
348 former study.
349
350 O'Brian et al. (2007) observed a difference between the level of *afl*R and *afl*S
351 expression and aflatoxin biosynthesis. They found that both regulatory genes were
352 highly expressed at 37℃, conditions under which th e strain of *A. flavus* produced no
353 aflatoxin. The present study also found a strong expression of the *afl*S gene at
354 temperatures >37℃ at nearly all $a_w$ values tested. Despite this high activation of the
355 *afl*S gene, low amounts of aflatoxin were produced under these conditions, which is
356 supported by the data of O,Brian et al. (2007). A slight increased expression of the
357 *afl*S gene was also found at certain moderate parameter combinations at 30, 25 and
358 20 ℃ where the cluster is expressed at the basal l evel. Interestingly, at all these
359 combinations, high amounts of aflatoxin were produced when the *afl*S expression
360 was elevated. At temperatures <37 ℃, when $a_w$ leads to cluster gene activation (at
361 0.90 $a_w$) no enhanced expression of the *afl*S was observed, paralleled by a low
362 biosynthesis of aflatoxin. These results indicate that the increased activation of this
363 regulatory gene is inhibited by low $a_w$ levels. No increased *afl*R expression could be
364 observed under the current conditions, which however was shown by O´Brain et al

(2007). Instead the *afl*R expression paralleled the general cluster gene expression. Interestingly the ratio of expression of *afl*S:*afl*R is high when high amounts of aflatoxin are produced, except at temperatures above 37 ℃ where the ratio is still high, but only scarce amounts of toxin are produced. The expression ratio however is low at water activities of 0.90 when only minor amounts of toxin are produced. The fact that under certain conditions only low amounts of aflatoxin were produced might be due to an unknown post-transcriptional mechanism as discussed above.

Two groups of cluster genes could be identified based on plotting of the expression profile of the single genes against temperature and $a_w$. Interestingly, when these groups were allocated to the cluster, they suggest a division into three homogenous parts of the cluster consisting of genes of the same group. Genes with expression profile 2 are located within the cluster region between *afl*F and *afl*A followed by a region completely covered by genes of the expression profile 1 (*afl*R to *afl*N) again followed by group 2 genes from *afl*G to *afl*Y. It has long been observed that the physical order of the genes is similar to the order of enzyme reactions catalysed by their gene products (Trail et al., 1995; Roze et al., 2007). This organization seems to be reflected here, dividing the cluster into early, middle and late genes based on the expression profile. Based on these results it seems that the middle genes may be regulated differently compared to the early and late groups of genes. The only exception is the *afl*U gene, which lies in the group of early genes, but has the same expression profile as group 1. According to Ehrlich et al. (2004) this gene is important in *A. parasiticus* for the biosynthesis of the G type aflatoxins and this gene together with the *afl*F gene is often non-functional in *A. flavus* due to deletions. In a recent publication Carbone et al. (2007) showed that the aflatoxin cluster of *A. parasiticus* is comprised of 5 distinct recombination blocks which seem to play a role in the evolution of the cluster. Interestingly block 2 and 3 of the analysis

of Carbone et al. (2007) contains the same genes as group 1 of the current analysis of *A. flavus*. This suggests that also the cluster of *A. flavus* consist of evolutionary building blocks and that the blocks determine differential expression in relation to environmental parameters.

The results described here demonstrate activity of the aflatoxin gene cluster over the whole range of analysed abiotic factors. It appears that at the basal level of expression, high amounts of aflatoxin are produced. Under mild stress conditions (20 ℃/0.99) a burst of gene induction occurred whi ch at the end resulted in the same amounts of aflatoxin. This may seem contradictory, however it has to be kept in mind that the aflatoxin production values are accumulated values and the microarray data are snapshots of total expression activity over time. Moreover the aflatoxin reaches a saturation level, e. g., the saturation can either be reached due to growth under basal level expression for a longer time or under high expression levels over a shorter time. This possibility might explain the fact that the amount of aflatoxin produced is the same for both conditions. Additionally regulatory events beyond transcription can explain the facts described here. This possibility is also discussed by Georgianna and Payne (2009).

The influence of *afl*S seems to be different under various abiotic stress conditions. In the case of temperature stress and at moderate temperatures and $a_w$ levels *afl*S is highly expressed when compared to the other cluster genes and high amounts of aflatoxin are produced if temperature stress is not too high (< 37 ℃). In the case of $a_w$ stress *afl*S expression was not increased over the other genes of the cluster and aflatoxin biosynthesis greatly reduced. The ratio between *afl*S expression and general cluster gene expression or *afl*R seems to be more important for aflatoxin

biosynthesis than the overall expression level. At temperatures >37℃ this might also be true but a unknown post-transcriptional mechanisms might prevent aflatoxin biosynthesis.

Again, in this analysis what was previously shown for ochratoxin A biosynthesis in *P. verrucosum*, trichothecene biosynthesis in *F. culmorum* and aflatoxin biosynthesis in *A. parasiticus* has been demonstrated now for *A. flavus*. High amounts of the toxin are produced at conditions slightly lower than those at which growth is optimal, but in addition biosynthesis is also activated at the edges of the growth window, when growth is retarded (Schmidt-Heydt et al., 2008).

From a food safety point of view the data can be used to optimize certain food technological processes, e. g. the drying of susceptible products like for example fruits like figs or dates. These products are very often sun dried at temperatures between 30 – 37 ℃ which is inducive for aflatoxin biosynthesis. If the products are instead immediately dried in a heater at temperatures above 37 ℃ (repressive for ochratoxin A biosynthesis) until a water activity of 0.9 is reached (repressive for ochratoxin biosynthesis) the process should be safe according the data presented here. The water activity of 0.9 is about the water activity of the final product which corresponds to a moisture content of < 25 %.

The current study shows that stress applied by external abiotic factors has a complex influence on aflatoxin gene regulation. This study using a mycotoxin gene cluster array gives a first insight into this sophisticated regulatory network.

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**Figure Legends:**

**Figure 1.** Comparison of (a) relative cluster gene expression determined by microarray (upper row), (b) aflatoxin biosynthesis determined by HPLC and (c) growth determined by diameteric measurement (lower row) of *A. flavus* SRKC-G1907 grown for 7 days on YES medium agar plates for 7 days. The various combinations of temperature x water activity are indicated at the top. The numbers in the upper row indicate the relative expression levels of the highly expressed genes *afl*S and *afl*J at the combinations where expression exceeds the level of the y axis. Increased *afl*S expression is indicated by an asterisk (*), increased *afl*J expression is indicated by a cross (+).

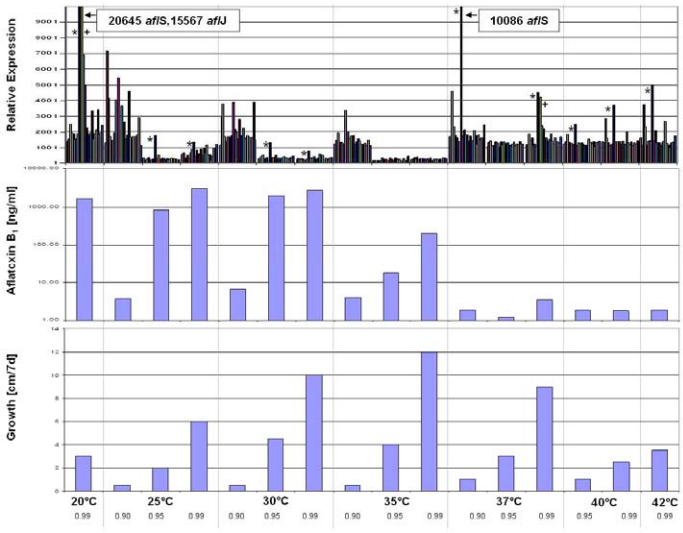
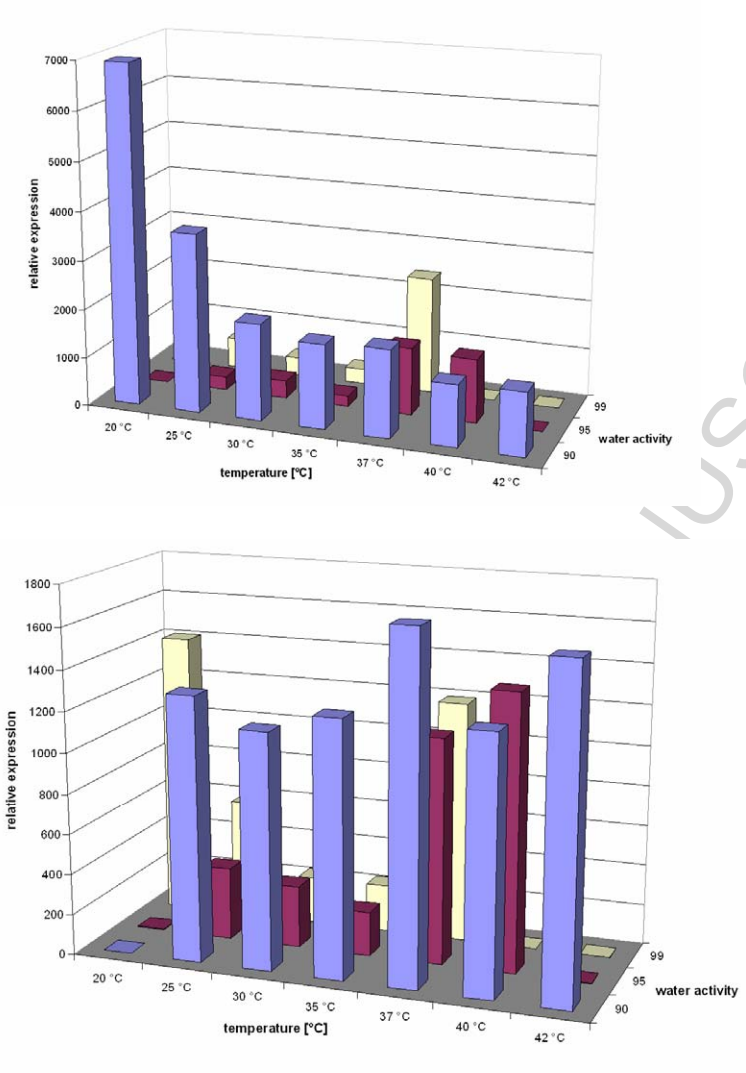


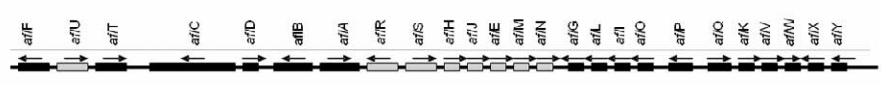

**Figure 2.** Typical expression profiles of one example gene of group 1 (A) and group 2 (B). The example gene for group 1 is *afl*E and for group 2 *afl*F.

564




565

566 **Figure 3.** Allocation of the biosynthesis genes, grouped according to their expression
567 profile at various temperature x water activity combinations, to the gene cluster.
568 Genes of expression profile group 1 are light grey; genes of expression group 2 are
569 dark grey.

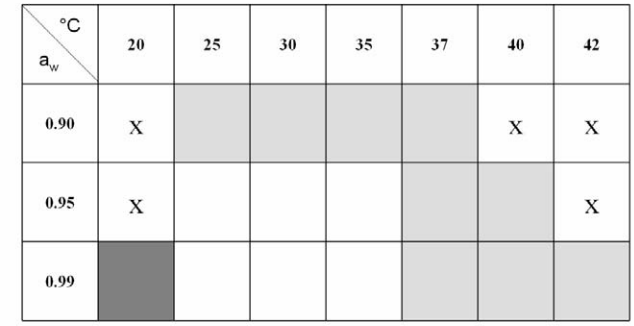



570

571 **Figure 4.** Matrix of the expression level of the cluster genes in relation to various

| Parameter combination | Ratio *afl*S/*afl*R | Aflatoxin [ng/g] |
|---|---|---|
| 25℃/0.90 | 0.5 | 3.66 |
| 35℃/0.90 | 0.3 | 4.67 |
| 25℃/0.95 | 7.4 | 830.24 |
| 30℃/0.95 | 7.1 | 3016.94 |
| 25℃/0.99 | 1.5 | 1957.25 |
| 30℃/0.99 | 2.7 | 2758.74 |

572 combinations of temperature x water activity. Basal expression level, white squares;
573 medium induced expression level, light gray squares; highly induced expression level
574 (dark gray squares). The crosses indicate marginal growth. It was not possible to use
575 these colonies for further analysis.




576

577 **Table 1: Ratio of *afl*S/*afl*R in relation to aflatoxin biosynthesis and different**
578 **parameter combinations**
579

# Complex regulation of the aflatoxin biosynthesis gene cluster of Aspergillus flavus in relation to various combinations of water activity and temperature

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