

Accepted Manuscript

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

Markus Schmidt-Heydt, Ahmed Abdel-Hadi, Naresh Magan, Rolf Geisen

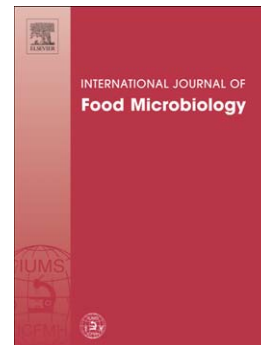
PII: S0168-1605(09)00369-9
DOI: doi: [10.1016/j.ijfoodmicro.2009.07.026](https://doi.org/10.1016/j.ijfoodmicro.2009.07.026)
Reference: FOOD 4826

To appear in: *International Journal of Food Microbiology*

Received date: 17 March 2009
Revised date: 15 July 2009
Accepted date: 19 July 2009

Please cite this article as: Schmidt-Heydt, Markus, Abdel-Hadi, Ahmed, Magan, Naresh, Geisen, Rolf, Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature, *International Journal of Food Microbiology* (2009), doi: [10.1016/j.ijfoodmicro.2009.07.026](https://doi.org/10.1016/j.ijfoodmicro.2009.07.026)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

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

Markus Schmidt-Heydt^a, Ahmed Abdel-Hadi^b Naresh Magan^b and Rolf Geisen^a

^a Max Rubner Institute, Haid-und-Neu-Str. 9, 76131 Karlsruhe, Germany

^b Applied Mycology Group, Cranfield Health, Cranfield University, Silsoe, Bedford, U.K. MK45 4DT, U.K.

Corresponding author

Rolf Geisen

Max Rubner Institute Karlsruhe

Haid und Neu Str. 9, 76131 Karlsruhe, Germany

tel.:0049(0)721-6625-450; fax:0049(0)721-6625-453

rolf.geisen@mri.bund.de;

27 **Abstract**

28

29 A microarray analysis was performed to study the effect of varying combinations of
30 water activity and temperature on the activation of aflatoxin biosynthesis genes in *A.*
31 *flavus* grown on YES medium. Generally *A. flavus* showed expression of the aflatoxin
32 biosynthetic genes at all parameter combinations tested. Certain combinations of a_w
33 and temperature, especially combinations which imposed stress on the fungus
34 resulted in a significant reduction of the growth rate. At these conditions induction of
35 the whole aflatoxin biosynthesis gene cluster occurred, however the produced
36 aflatoxin B₁ was low. At all other combinations (25 °C/0.95 and 0.99; 30 °C/0.95 and
37 0.99; 35 °C/0.95 and 0.99) a reduced basal level of cluster gene expression occurred.
38 At these combinations a high growth rate was obtained as well as high aflatoxin
39 production. When single genes were compared, two groups with different expression
40 profiles in relation to water activity/temperature combinations occurred. These two
41 groups were co-ordinately localized within the aflatoxin gene cluster. The ratio of
42 *afIR/afIJ* expression was correlated with increased aflatoxin biosynthesis.

43

44 **Keywords:** aflatoxin; gene expression; microarray; water activity; temperature; stress;
45 *afIS*; *afIR*; aflatoxin cluster

46

47

48

49

50

51

52

53 **1. Introduction**

54

55 Aflatoxins are produced by *Aspergillus flavi* group species and are thought to
56 be one of the most cancerous natural substances known. Economically and
57 biologically the most important fungal species able to produce the aflatoxins are *A.*
58 *flavus* and *A. parasiticus* (Bhatnagar et al., 2002). The aflatoxin biosynthesis gene
59 cluster of *A. parasiticus* has been completely elucidated (Yu et al., 2004). Moreover
60 the genome of *A. flavus* is known and a whole genome microarray of *A. flavus* is
61 available and has been used to study the regulation of aflatoxin biosynthesis genes
62 (O'Brian et al., 2003). Generally, the aflatoxin biosynthesis genes of *A. flavus* and *A.*
63 *parasiticus* are highly homologous and the order of the genes within the cluster being
64 the same (Yu et al., 1995). *A. flavus* usually produces only aflatoxin B₁ and B₂. Not all
65 of the strains of *A. flavus* isolated from the natural habitat are able to produce these
66 metabolites at least under laboratory conditions. On the other hand, *A. parasiticus* is
67 a strong and consistent aflatoxin producing species (Bennett and Christensen, 1983;
68 Vaamonde et al., 2003). Most strains of this species are able to produce the
69 aflatoxins B₁, B₂, G₁, and G₂.

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

79 aflatoxin production. A mathematical model which delineates the relationship
80 between pH, propionic acid concentration and temperature on aflatoxin biosynthesis
81 by *A. parasiticus* was described by Molina and Giannuzzi (2002). Sweeney et al.
82 (2000) developed a reverse transcription polymerase chain reaction to analyse the
83 expression of the *afIR* and *afIQ* gene of the aflatoxin pathway in relation to various
84 nutritional media. Xu et al. (2000) monitored the expression of the *afID* gene after
85 growth of *A. parasiticus* on peanut pods by a reporter gene approach. Mayer et al.
86 (2003) showed that expression of the *afID* gene and the biosynthesis of aflatoxin in
87 wheat are directly coupled. Recently Price et al. (2005) used a whole genome
88 microarray approach to analyse the influence of substrate composition and pH on the
89 activation of aflatoxin biosynthesis genes. With a pathway specific microarray and by
90 Real Time PCR the influence of physical parameters like water activity, temperature
91 and pH on the expression of ochratoxin A-, trichothecene- and aflatoxin biosynthesis
92 genes have been analysed and a common expression profile was found (Schmidt-
93 Heydt and Geisen, 2007). The published data show a correlation between external
94 parameters and gene expression, however no comprehensive systematic analysis of
95 this in relation to aflatoxin production has been performed. Recently Georgianna and
96 Payne (2009) summarized the current knowledge of the regulation of aflatoxin
97 biosynthesis in relation to external factors.

98

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

105 2. Materials and methods

106

107 2.1 Strains and growth conditions

108

109 *A. flavus* SRKC-G1907 produces aflatoxin B₁ when grown under permissive
110 conditions. This strain produces high amounts of aflatoxin after growth on YES agar
111 (20 g/l yeast extract, 150 g/l sucrose, 15 g/l agar) at 25 to 30 °C for 5 days. For
112 expression and toxin analysis the agar plates were overlaid with sterile 8.5
113 cellophane sheets (P400, Cannings, Bristol, U.K.) and then single point inoculated
114 centrally by applying 10 µl of a spore suspension (10⁷ spores in TWS (0.5 % tween
115 80, 0.85 g/l NaCl)). The water activity of the media was adjusted with glycerol by
116 using glycerol/water mixtures. The following amounts were used per litre (108 ml,
117 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
118 the medium was verified by using an Aqua Lab Lite water activity meter (Graintec Pty
119 Ltd). The plates were inoculated at the temperature indicated (15 – 42 °C).

120

121 2.2 Growth assessment

122

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

130

131 *2.3 Isolation of RNA from samples*

132

133 To perform microarray experiments RNA was isolated using the RNAeasy
134 Plant Mini kit (Qiagen, Hilden, Germany). An amount of 1 g of the mycelium was
135 ground with a mortar and pestel in liquid nitrogen. About 250 mg of the resulting
136 powder was used for isolation of total RNA. The powder was resuspended in 750 μ l
137 lysis buffer, mixed with 7.5 μ l β -mercaptoethanol and 100 glass beads with a
138 diameter of 1 mm (B. Braun Biotech International GmbH, Melsungen, Germany) in a
139 2 ml RNase free micro reaction tube. The extracts were mixed thoroughly and
140 incubated for 15 min at 55 $^{\circ}$ C and 42 kHz in an S10H ultrasonic bath (Elma, Singen,
141 Germany). All further procedures were essentially the same as suggested by the
142 manufacturer of the kit.

143

144 *2.4 Microarray experiments*

145

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

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

161

162 *2.5 Quantitative determination of aflatoxin B₁ by HPLC*

163

164 Detection and quantitative determination of aflatoxin B₁ from fungal colonies
165 were performed on a high-pressure gradient system from Shimadzu (Duisburg,
166 Germany) equipped with an auto injector and a fluorescence detector. For this
167 purpose 100 mg of the fungal colony were extracted under shaking conditions in 500
168 μl chloroform at room temperature for 30 min. The residue was discarded and the
169 chloroform was evaporated to dryness in a vacuum concentrator (Speed Vac, Savant
170 Instruments, Farmingdale, USA). The residue was then redissolved in 200 μl
171 methanol and subjected to HPLC analysis (20 μl).

172 Separation was carried out on a Prontosil (250 mm x 4.6 mm i.d., particle size
173 3 μm) reversed-phase column (Bischoff, Leonberg, Germany). The solvent system
174 consisted of 0.1 % formic acid in water (pH = 3) (A) and acetonitrile (B). Elution was
175 effected using a linear gradient from 30 % to 90 % B in 30 min. The flow rate was 0.8
176 ml/min. The fluorescence detector settings were an excitation wavelength of 365 nm
177 and an emission wavelength of 440 nm.

178

179

180

181

182

183 3. Results

184

185 3.1 Expression profile of the aflatoxin biosynthesis genes of *A. flavus* in relation to the 186 parameter combinations temperature x water activity

187

188 In order to analyse the influence of combinations of the external parameters
189 temperature x a_w on aflatoxin gene activation *A. flavus* was grown for 7 days on YES
190 medium adjusted to the respective a_w at the temperatures as indicated. After this time
191 the activation of the aflatoxin biosynthesis gene cluster was determined by
192 microarray analysis. In parallel at each parameter combination the colony growth as
193 well as the aflatoxin B₁ produced was determined. The results obtained are shown in
194 Figure 1.

195

196 The growth rates follow a general pattern. At each temperature growth
197 increased as a_w was increased from 0.90 to 0.99. The growth optimum for this strain
198 was at 37°C. At 25, 30 and 35 °C the biosynthesis pattern of aflatoxin followed the
199 growth rate and was high at 0.99 a_w and low at 0.90 a_w . Highest amounts of aflatoxin
200 were produced between 25 and 30 °C, but also at 20 °C. Very low amounts were
201 produced at ≥37 °C and at a w of 0.90.

202

203 The expression profiles of the aflatoxin cluster genes were co-ordinately
204 changed in response to the environmental conditions and were partly inversely
205 related to the growth rate and also to aflatoxin biosynthesis. At intermediate
206 combinations of temperature x a_w (25 °C/0.95 and 0.99; 30 °C/0.95 and 0.99;
207 35 °C/0.95 and 0.99) a high production of aflatoxin B₁ was observed despite the fact
208 that the cluster genes are transcribed only to a certain basal level. At these

209 combinations growth was moderate or close to the optimum. At combinations of
210 temperature x a_w which are less favourable for growth due to low a_w or high
211 temperature (25 °C/0.90; 30 °C/0.90; 37 °C/0.90 and 0.95 and 0.99; 40 °C/0.90 and
212 0.95 and 0.99; 42 °C/0.99) the overall cluster genes are induced when compared to
213 the basal level. Interestingly most of the cluster genes are activated nearly to the
214 same level for all these combinations except of some single genes which are
215 expressed at a significantly higher level. Generally growth was significantly
216 decreased at these stress conditions with an exception at 37°C/0.99 where growth
217 was at an intermediate level. Under these inducing conditions only very low amounts
218 of aflatoxin were produced. Even after prolonged incubation of *A. flavus* no increase
219 in aflatoxin concentration occurred (data not shown). An even more pronounced
220 induction of several genes of the cluster could be observed at only one specific
221 condition, e.g. 20°C/0.99. At this condition, although growth is moderate high
222 amounts of aflatoxin were produced. At combinations of 20 °C/0.95 and 0.90 growth
223 was practically inhibited and analysis was not possible.

224

225 3.2 Expression of specific genes

226

227 It was noticeable that the expression of some genes was much higher than the
228 general cluster gene expression level under certain parameter combinations. This
229 was especially true for the *af/S* gene. The *af/S* (formerly *af/J*) gene is a regulatory
230 gene which interacts and activates *af/R*, the major regulatory gene, which than
231 activates the aflatoxin structural genes (Chang, 2003). At most conditions with a
232 temperature >37°C the *af/S* gene expression was highly increased especially at
233 37°C/0.90. At certain other parameter combinations at 20, 25 and 30°C the
234 expression of the *af/S* gene was also increased when compared to the other genes of

235 the cluster. This was often accompanied by an increased biosynthesis of aflatoxin
236 compared to the neighbouring conditions. At conditions $<37^{\circ}\text{C}$ a reduction of the a_w
237 to 0.90 did not lead to an increased expression of the *afIS* gene compared to the
238 other cluster genes. This lower activity of the *afIS* gene was always accompanied by
239 a lower biosynthesis of aflatoxin (see Figure 1). At $20^{\circ}\text{C}/0.99 a_w$ the *afIS* gene was
240 very high expressed relative to the other genes and accompanied by a similar
241 activation of the *afIJ* (formerly *estA*) gene. Under these conditions growth was
242 moderate and aflatoxin biosynthesis high. At all analysed conditions the *afIR* gene
243 was not prominently expressed and followed the general expression of the cluster
244 genes. However the ratio of expression between the *afIS* and *afIR* gene seemed to
245 be critical for aflatoxin biosynthesis (Table 1). At conditions with low a_w (0.90) the
246 ration is far below 1. At these conditions low amounts of aflatoxin are produced. At
247 moderate conditions the ratio is more than 1. At these conditions high amounts of
248 aflatoxin are produced.

249

250 *3.3 Differences in the regulation of the single cluster genes in relation to*
251 *combinations of temperature and water activity*

252

253 In order to compare the expression profiles of single genes in relation to both
254 parameters all expression data of the single genes were plotted against temperature
255 and a_w (Figure 2). Based on the expression profiles, two groups of genes could be
256 identified. The expression profile of group 1 is shown in Figure 2A. The following
257 genes belonged to this group and all showed a very similar profile: *afIM*, *afIE*, *afIH*,
258 *afIJ*, *afIN*, *afIR*, *afIS*, *afIU*, *afIQ*, *afIX*. The expression profile of the second group
259 (group 2) of genes is shown in Figure 2B. The following genes belong to this group:
260 *afIG*, *afIT*, *afIP*, *afID*, *afIL*, *afIO*, *afIC*, *afIF*, *afIA*, *afIB*, *afIK*, *afIV*, *afIN*, *afIY*. Also in this

261 group the expression patterns of all the genes were very similar and resembled the
262 profile which is shown in Figure 2B. The genes *afIS* and *afIJ*, which were highly
263 expressed under certain conditions, were both in group 1. When the two gene groups
264 are assigned to the cluster all of the group 1 genes are adjacently located covering
265 the region from *afIR* to *afIN*. The other group is split and comprises the regions from
266 the *afIF* to the *afIA* gene and from the *afIG* to the *afIY* gene. The only exception is the
267 *afIU* gene, which belongs to the expression profile of group 1. The data suggests that
268 different mechanisms are involved in the regulation of the two groups in relation to
269 environmental changes and that co-localization within the cluster might facilitate this
270 different behaviour (Fig. 3).

271

272 *3.4 Matrix of aflatoxin cluster gene activation in relation to water activity and* 273 *temperature*

274

275 Based on the expression data obtained during this systematic analysis a
276 matrix of aflatoxin cluster gene expression in relation to a_w and temperature could be
277 generated (Fig. 4). This matrix plots the level of induction versus certain
278 combinations of a_w levels and temperatures. Three sets of parameter combinations
279 could be identified in this analysis. First parameter combinations which allow a basal
280 level of expression (Fig. 3, white squares) in the temperature range of 25-37°C at a_w
281 levels >0.90 (e. g. a_w 0.95 and 0.99). These conditions support moderate to good
282 growth of *A. flavus* and are usually accompanied by high production levels of
283 aflatoxin. A general activation level of the whole gene cluster with high expression of
284 certain genes could be identified at all temperatures in combination with low a_w (0.90)
285 or at temperatures $>37^\circ\text{C}$ (Fig. 3, light grey square s). These conditions impose stress
286 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 (*af/S*) could be identified at one unique combination only (20 °C/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°C/0.90 was not included in this last
292 group is because despite the quite high induction of the *af/S* gene, which was about
293 half of that at 20°C/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°C/0.95 and 40°C/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 °C/0.99) several of the cluster
310 genes, in particular *af/S* and *af/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 °C) the whole cluster was induced, with the *af/S* expression being prominent

313 at certain parameter combinations ($>37\text{ }^{\circ}\text{C}$), 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 $37^{\circ}\text{C}/0.90$ where a high activation of the *afIS*
326 gene occurred, similar to that at $20\text{ }^{\circ}\text{C}/0.99$, 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 $20^{\circ}\text{C}/0.95$ and $40^{\circ}\text{C}/0.90$).
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°C for their strain. However, a strong
336 induction of the genes occurred at 28°C which was paralleled 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 $30\text{ }^{\circ}\text{C}$ could be seen. At

339 temperatures beyond or at 37 °C 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 °C) and a low level at 37 °C. During this analysis only a basal level
343 of expression was found between 25 and 30 °C as long as the water activity was
344 above 0.90, but an induced level at temperatures above 37 °C. 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 *afIR* and *afIS*
351 expression and aflatoxin biosynthesis. They found that both regulatory genes were
352 highly expressed at 37°C, conditions under which the strain of *A. flavus* produced no
353 aflatoxin. The present study also found a strong expression of the *afIS* gene at
354 temperatures >37°C at nearly all a_w values tested. Despite this high activation of the
355 *afIS* 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 *afIS* gene was also found at certain moderate parameter combinations at 30, 25 and
358 20 °C where the cluster is expressed at the basal level. Interestingly, at all these
359 combinations, high amounts of aflatoxin were produced when the *afIS* expression
360 was elevated. At temperatures <37 °C, when a_w leads to cluster gene activation (at
361 0.90 a_w) no enhanced expression of the *afIS* 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 *afIR* expression could be
364 observed under the current conditions, which however was shown by O'Brain et al

365 (2007). Instead the *af/R* expression paralleled the general cluster gene expression.
366 Interestingly the ratio of expression of *af/S:af/R* is high when high amounts of
367 aflatoxin are produced, except at temperatures above 37 °C where the ratio is still
368 high, but only scarce amounts of toxin are produced. The expression ratio however is
369 low at water activities of 0.90 when only minor amounts of toxin are produced. The
370 fact that under certain conditions only low amounts of aflatoxin were produced might
371 be due to an unknown post-transcriptional mechanism as discussed above.

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

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

395

396 The results described here demonstrate activity of the aflatoxin gene cluster
397 over the whole range of analysed abiotic factors. It appears that at the basal level of
398 expression, high amounts of aflatoxin are produced. Under mild stress conditions
399 (20 °C/0.99) a burst of gene induction occurred which at the end resulted in the same
400 amounts of aflatoxin. This may seem contradictory, however it has to be kept in mind
401 that the aflatoxin production values are accumulated values and the microarray data
402 are snapshots of total expression activity over time. Moreover the aflatoxin reaches a
403 saturation level, e. g., the saturation can either be reached due to growth under basal
404 level expression for a longer time or under high expression levels over a shorter time.
405 This possibility might explain the fact that the amount of aflatoxin produced is the
406 same for both conditions. Additionally regulatory events beyond transcription can
407 explain the facts described here. This possibility is also discussed by Georgianna and
408 Payne (2009).

409

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

417 biosynthesis than the overall expression level. At temperatures $>37^{\circ}\text{C}$ this might also
418 be true but a unknown post-transcriptional mechanisms might prevent aflatoxin
419 biosynthesis.

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

426 From a food safety point of view the data can be used to optimize certain food
427 technological processes, e. g. the drying of susceptible products like for example
428 fruits like figs or dates. These products are very often sun dried at temperatures
429 between $30 - 37^{\circ}\text{C}$ which is inducive for aflatoxin biosynthesis. If the products are
430 instead immediately dried in a heater at temperatures above 37°C (repressive for
431 ochratoxin A biosynthesis) until a water activity of 0.9 is reached (repressive for
432 ochratoxin biosynthesis) the process should be safe according the data presented
433 here. The water activity of 0.9 is about the water activity of the final product which
434 corresponds to a moisture content of $< 25\%$.

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

438

439 **Acknowledgements**

440

441 Parts of this work were supported by the EU-project "Development of cost-
442 effective control and prevention strategies for emerging and future foodborne

443 pathogenic microorganisms throughout the food chain" (Pathogen Combat), FOOD-
444 CT-2005-07081 and by the EU-project "Novel integrated strategies for worldwide
445 mycotoxin reduction in the food and feed chains" (No. 222690). The *A. flavus* strain
446 was a gift from Deepak Bhatnagar. We would like to thank Katja Kramer for skilful
447 technical assistance. A. Abdel-Hadi gratefully thanks the Assuit Al-Azhar University
448 and the Egyptian Higher Education Ministry for support.

449

450 References

451

452 Baert, K., Devlieghere, F., Flyps, H., Oosterlinck, M., Ahmed, M.M., Verlinden, B.,
453 Nicolai, B., Debevere, J., De Meulenaer, B., 2007. Influence of storage
454 conditions of apples on growth and patulin production by *Penicillium*
455 *expansum*. International Journal of Food Microbiology 119, 170-181.

456 Bennett, J.W., Christensen, S.B., 1983. New perspectives on aflatoxin biosynthesis.
457 Advances in Applied Microbiology 29, 53-92.

458 Bhatnagar, D., Yu, J., Ehrlich, K.C., 2002. Toxins of filamentous fungi. In Fungal
459 Allergy and Pathogenicity, pp. 167-206. (Eds M. Breitenbach, R. Cramer and
460 S. B. Lehrer). Basel, Karger: Chem. Immunol.

461 Carbone, I., Jakobek, J. L., Ramirez-Prado, J. H., Horn, B., 2007. Recombination,
462 balancing selection and adaptive evolution in the aflatoxin gene cluster of
463 *Aspergillus parasiticus*. Molecular Ecology 16, 4401-4417

464 Chang., P. K., 2003. The *Aspergillus parasiticus* protein AFLJ interacts with the
465 aflatoxin-pathway specific regulator AFLR. Molecular and General Genomics
466 268, 711-719

- 467 Ehrlich, K.C., Chang, P.-K., Yu, J., Cotty, P.J., 2004. Aflatoxin biosynthesis cluster
468 gene *cypA* is required for G aflatoxin formation. Applied and Environmental
469 Microbiology 70, 6518-6524.
- 470 Ellis, W.O., Smith, P.J., Simpson, B.K., Khanizadeh, S., Oldham, J.H., 1993. Control
471 of growth and aflatoxin production of *Aspergillus flavus* under modified
472 atmosphere packaging conditions. Food Microbiology 10, 9-21.
- 473 Georgianna, D.R., Payne, G., 2009. Genetic regulation of aflatoxin biosynthesis:
474 From gene to genome. Fungal Genetics and Biology 26, 113-125.
- 475 Giorni, P., Battilani, P., Pietri, A., Magan, N., 2008. Effect of a_w and CO_2 level of
476 *Aspergillus flavus* growth and aflatoxin production in high moisture maize post-
477 harvest. International Journal of Food Microbiology 122, 109-113.
- 478 Jayashree, T., Subramanyam, C., 2000. Oxidative stress as a prerequisite for
479 aflatoxin production by *Aspergillus parasiticus*. Free Radicals in Biology and
480 Medicine 29, 981-985.
- 481 Jurado, M., Marín, P., Magan, N., González-Jaén, M.T., 2008. Relationship between
482 solute and matric potential stress, temperature, growth, and *FUM1* gene
483 expression in two *Fusarium verticillioides* strains from Spain. Applied and
484 Environmental Microbiology 74, 2032-2036.
- 485 Kim, J.H., Campbell, B.C., Yu, J., Mahoney, N., Chan, K.L., Molyneux, R.J.,
486 Bhatnagar, D., Cleveland, T.E., 2005. Examination of fungal stress response
487 genes using *Saccharomyces cerevisiae* as a model system: targeting genes
488 affecting aflatoxin biosynthesis by *Aspergillus flavus* Link. Applied
489 Microbiology and Biotechnology 67, 807-815.

- 490 Kohut, G., Ádám, A. L., Fazekas, B., Hornok, L. 2009. N-starvation stress induced
491 *FUM* gene expression and fumonisin production is mediated via the HOG-type
492 MAPK pathway in *Fusarium proliferatum*. International Journal of Food
493 Microbiology, 130: 65-69
- 494 Luchese, R.H., Harrigan, W.F., 1993. Biosynthesis of aflatoxin-the role of nutritional
495 factors. Journal of Applied Bacteriology 74, 5-14.
- 496 Mayer, Z., Bagnara, A., Färber, P., Geisen, R., 2003. Quantification of the copy
497 number of *nor-1*, a gene of the aflatoxin biosynthetic pathway by real-time
498 PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. International
499 Journal of Food Microbiology 82, 143-151.
- 500 Molina, M., Giannuzzi, L., 2002. Modelling of aflatoxin production by *Aspergillus*
501 *parasiticus* in a solid medium at different temperatures, pH and propionic acid
502 concentrations. Food Research International 35, 585-594.
- 503 Ochiai, N., Tokai, T., Nishiuchi, T., Takahashi-Ando, N., Fujimura, M., Kimura, M.,
504 2007. Involvement of the osmo sensor histidine kinase and osmotic stress-
505 activated protein kinases in the regulation of secondary metabolism in
506 *Fusarium graminearum*. Biochemical and Biophysical Research
507 Communication 363, 639-644.
- 508 O'Brian, G.R., Fakhoury, M., Payne, G.A., 2003. Identification of genes differentially
509 expressed during aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus*
510 *parasiticus*. Fungal Genetics and Biology 39, 118-127.
- 511 O'Brian, G.R., Georgianna, D.R., Wilkinson, J.R., Yu, J., Abbas, H.K., Bhatnagar, D.,
512 Cleveland, T.E., Nierman, W., Payne, G.A., 2007. The effect of elevated

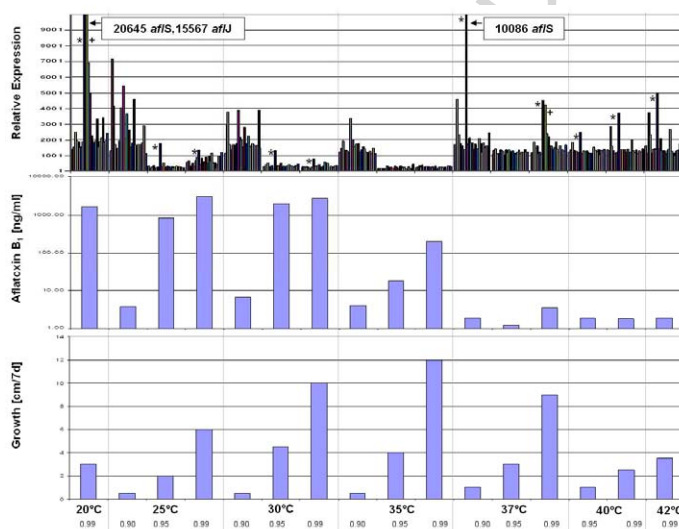
- 513 temperature on gene transcription and aflatoxin biosynthesis. *Mycologia* 99,
514 232-239.
- 515 Price, M.S., Conners, S.B., Tachdjian, S., Kelly, R.M., Payne, G.A., 2005. Aflatoxin
516 conducive and non-conductive growth conditions reveal new gene associations
517 with aflatoxin production. *Fungal Genetics and Biology* 42, 506-518.
- 518 Ribeiro, J.M.M., Cavaglieri, L.R., Fraga, M.E., Direito, G.M., Dalcero, A.M.,
519 Rosa, C.A.R., 2006. Influence of water activity, temperature and time on
520 mycotoxins production on barley rootlets. *Letters of Applied Microbiology* 42,
521 179-184.
- 522 Roze, L.V., Arthur, A.E., Hong, S.J.; Chanda, A., Linz, J.E., 2007. The initiation and
523 pattern of spread of histone H4 acetylation parallel the order of transcriptional
524 activation of genes in the aflatoxin cluster. *Molecular Microbiology* 66, 713-726
- 525 Schmidt-Heydt, M., Geisen, R., 2007. A microarray for monitoring the production of
526 mycotoxins in food. *International Journal of Food Microbiology* 117, 131-140.
- 527 Schmidt-Heydt, M., Magan, N., Geisen, R., 2008. Stress induction of mycotoxin
528 biosynthesis genes by abiotic factors. *FEMS Microbiology Letters* 284, 142-
529 149.
- 530 Sweeney, M.J., Pamies, P., Dobson, A.D.W., 2000. The use of reverse transcription-
531 polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in
532 *Aspergillus parasiticus* 439. *International Journal of Food Microbiology* 56, 97-
533 103.
- 534 Trail, F., Mahanti, N., Linz, J., 1995. Molecular biology of aflatoxin biosynthesis.
535 *Microbiology* 141, 755-765.

- 536 Vaamonde, G., Patriarca, A., Fernández Pinto, V., Comerio, R., Degrossi, C., 2003.
537 Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section
538 *flavi* from different substrates in Argentina. International Journal of Food
539 Microbiology 79-84.
- 540 Xu, H., Annis, S., Linz, J., Trail, F., 2000. Infection and colonization of peanut pods
541 by *Aspergillus parasiticus* and the expression of the aflatoxin biosynthetic
542 gene, *nor-1*, in infection hyphae. Physiological and Molecular Plant Pathology
543 56, 185-196.
- 544 Yu, J., Bhatnagar, D., Cleveland, T.E., 2004. Completed sequence of aflatoxin
545 pathway gene cluster in *Aspergillus parasiticus*. FEBS Letters 564, 126-130.
- 546 Yu, J., Chang, P.K., Cary, J.W., Wright, M., Bhatnagar, D., Cleveland, T.E., Payne,
547 G.A., Linz, J., 1995. Comparative mapping of aflatoxin pathway gene clusters
548 in *Aspergillus parasiticus* and *Aspergillus flavus*. Applied and Environmental
549 Microbiology 61, 2365-2371.

550 **Figure Legends:**

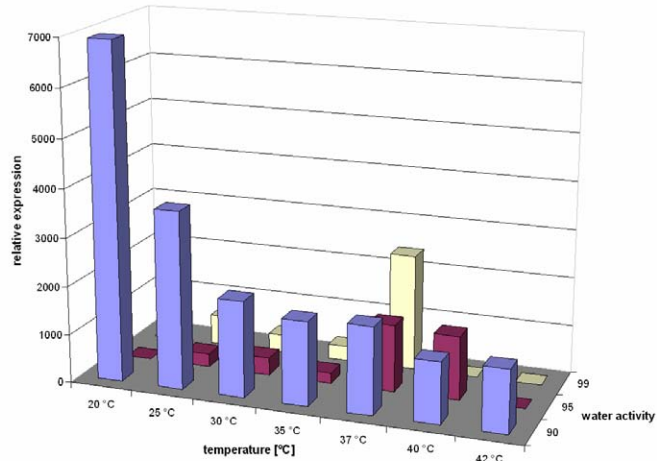
551

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

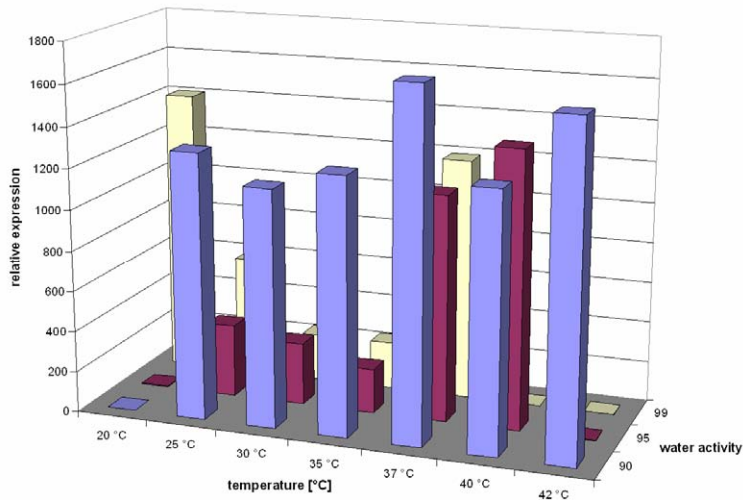


561

562 **Figure 2.** Typical expression profiles of one example gene of group 1 (A) and group
 563 2 (B). The example gene for group 1 is *afIE* and for group 2 *afIF*.



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 <i>af/S/af/R</i>	Aflatoxin [ng/g]
25°C/0.90	0.5	3.66
35°C/0.90	0.3	4.67
25°C/0.95	7.4	830.24
30°C/0.95	7.1	3016.94
25°C/0.99	1.5	1957.25
30°C/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.

$\frac{^{\circ}\text{C}}{a_w}$	20	25	30	35	37	40	42
0.90	X					X	X
0.95	X						X
0.99							

576

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

579