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The production of aflatoxin B1 or G1 by *Aspergillus parasiticus* at various combinations of temperature and water activity is related to the ratio of *aflS* to *aflR* expression

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Abstract

The influence of varying combinations of water activity (a_w) and temperature on growth, aflatoxin biosynthesis and *aflR/aflS* expression of Aspergillus parasiticus was analysed in the ranges 17–42°C and 0.90–0.99 a_w. Optimum growth was at 35°C. At each temperature studied, growth increased from 0.90 to 0.99 a_w. Temperatures of 17 and 42°C only supported marginal growth. The external conditions had a differential effect on aflatoxin B1 or G1 biosynthesis. The temperature optima of aflatoxin B1 and G1 were not at the temperature which supported optimal growth (35°C) but either below (aflatoxin G1, 20–30°C) or above (aflatoxin B1, 37°C). Interestingly, the expression of the two regulatory genes aflR and aflS showed an expression profile which corresponded to the biosynthesis profile of either B1 (*aflR*) or G1 (*aflS*). The ratios of the expression data between *aflS:aflR* were calculated. High ratios at a range between 17 and 30°C corresponded with the production profile of aflatoxin G1 biosynthesis. A low ratio was observed at >30°C, which was related to aflatoxin B1 biosynthesis. The results revealed that the temperature was the key parameter for aflatoxin B1, whereas it was water activity for G1 biosynthesis. These differences in regulation may be attributed to variable conditions of the ecological niche in which these species occur.

Introduction

Aflatoxins are toxic metabolites formed predominately by two agronomically important fungi *Aspergillus flavus* and *A. parasiticus*. *A. flavus* produces mainly aflatoxin B1 and B2 whereas *A. parasiticus* forms the 4 aflatoxins, B1, B2, G1 and G2. Generally, *A. parasiticus* produces high concentrations of aflatoxin, and most of the strains isolated from natural habitats (>90%) are able to synthesize aflatoxins. In contrast, only 40–50% of the *A. flavus* isolates are capable of forming the toxin (Bennett and Christensen 1983). The aflatoxin biosynthesis gene cluster has been described (Yu et al. 2004). The structural genes are regulated by the transcription factor *aflR* and the accessory regulatory gene *aflS* (Chang 2003). The habitats of both species overlap, but distinct differences are obvious. The habitat

of *A. flavus* is broader because of being found not only on cereals especially wheat but also on peanuts, tree nuts or high sugar-containing dry fruits.

Both species can also be found in soils; however, this is the predominant ecological niche for *A. parasiticus*. Both species occur in subtropical and tropical regions. According to Pitt and Hocking (1999), *A. parasiticus* is endemic in soil-borne peanuts, and they found more *A. parasiticus* than *A. flavus* strains in Australian peanuts. This situation is the opposite in maize, which is more susceptible to *A. flavus* compared to *A. parasiticus*. Pitt and Hocking (1999) also described intriguing differences in the geographical occurrence of both species. According to their data, *A. parasiticus* can only occasionally be found in southeast Asia, but it is widely distributed in soils and foodstuffs in the United States, Latin America, South Africa, India and Australia. This specific geographical distribution is not known for *A. flavus*, indicating that *A. flavus* may be a more aggressive and more widely distributed species when compared to *A. parasiticus*. Recently, the occurrence of *A. flavus* was described in cereals from north Italy (Giorni et al. 2007) in 2004 and 2005 because of hot dry summer weather. This resulted in significant contamination with aflatoxins. This demonstrates that the ecological distribution of these species is significantly impacted by fluctuations and trends in regional climatic changes. These ecological differences might also be reflected not only by differences in the formation of the aflatoxin structural types B and G but also in the regulation of the biosynthesis of the aflatoxins in response to environmental parameters like temperature and aw. In a recent study, the influence of both parameters on aflatoxin B1 gene expression and phenotypic production by *A. flavus* was analysed (Schmidt-Heydt et al. 2009), and it could be shown that aw was the leading parameter. At moderate temperatures (25–30°C), high aw levels (0.95–0.99) induced aflatoxin B1 production which is in agreement with other data. According to Bhatnagar et al. (2006), aflatoxin biosynthesis in A. flavus is optimal at temperatures between 29 and 30°C, but it is significantly decreased at temperatures <25°C and ≥37°C (O'Brian et al. 2007).

Not much information about the mutual regulation of aflatoxin B1 and G1 in *A. parasiticus* is known at present. In order to understand this aspect, a detailed analysis of the regulation of aflatoxin B1 and G1 biosynthesis in *A. parasiticus* under different combinations of temperature and aw has been carried out. A further objective was to analyse the effect of these environmental parameters on the expression of the aflatoxin-specific regulatory genes (*aflS, aflR*) and their ratio.

Materials and methods

Strains and growth conditions

A. parasiticus, BFE96p produces the B and G group aflatoxins when grown under permissive conditions. This strain forms high concentrations of aflatoxin after growth on YES agar (20 g/l yeast extract, 150 g/l sucrose, 15 g/l agar) at 25–30°C 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 of 107 spores in TWS (0.5% tween 80, 0.85 g/l NaCl). The a_w of the media was adjusted with glycerol by using glycerol/water mixtures. The following amounts were used per litre (10.8 ml, 0.99; 13.1 ml, 0.98; 19.9 ml, 0.95; 24.5 ml, 0.93; and 31.3 ml, 0.90). The water activity of the medium was verified by using an Aqua Lab Lite water activity meter

(Graintec, Toowoomba, Australia). The plates were inoculated at the temperature indicated (15–42°C).

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/7 days). The biomass was removed from the cellophane surface for extraction and molecular as well as aflatoxin analysis. All experiments were carried out with 3–4 replicates and some temperature treatments were repeated for confirmation of results.

Isolation of RNA from samples and transcription in cDNA

To perform Real-Time PCR gene expression analysis, 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 pestle 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, Melsungen, Germany) in a 2-ml RNase free micro reaction tube. The extracts were mixed thoroughly and incubated for 15 min at 55°C and 42 kHz in an S10H ultrasonic bath (Elma, Singen, Germany). All further procedures were essentially the same as recommended by the manufacturer of the kit. For cDNA synthesis, 12 µl of the DNase I-treated total RNA were used along with the Omniscript Reverse Transcription kit (Qiagen). The reaction mixture was composed essentially as described by the manufacturer and incubated at 37°C for 1 h. The cDNA was either directly used for Real-Time PCR or stored at −80°C.

Quantification of aflatoxin B1 and G1 by HPLC/MS/MS

Detection and quantitative determination of aflatoxin B1 and G1 from fungal colonies were performed according to the following method: 100 mg of the fungal colony were extracted under shaking conditions in 500 µl chloroform at room temperature for 30 min. The residues were discarded and the chloroform was evaporated to dryness in a vacuum concentrator (Speed Vac; Savant Instruments, Farmingdale, USA). The residues were then redissolved in 200 µl methanol and 20 µl were subjected to HPLC analysis. HPLC analysis was performed on a 1200 series HPLC system (Agilent, Waldbronn, Germany), equipped with a degaser, a binary pump system, an autosampler and a column oven. The sample was injected into a Prontosil reversed-phase column (150 mm×4 mm, 3 µm particle size; Bischoff, Leonberg, Germany). The mobile phases consisted of 0.1% formic acid in water and acetonitrile. A linear gradient of formic acid from 30% to 0% in 9 min and a 21-min isocratic elution at 0% was employed. The flow rate was set to 0.8 ml/min. The autosampler was cooled to 10°C, whereas the column oven was heated to 25°C.

The HPLC system was directly coupled to a hybrid triple quadrupole linear ion trap mass spectrometer (3200 QTrap; Applied Biosystems, Darmstadt, Germany), equipped with a

TurboIonSpray source. The analytes were detected in the positive ion mode at a vaporiser temperature of 650°C and an ion spray voltage of 5,500 kV. Spectral data were recorded with N2 as curtain and collision gas, at 50 psi and medium, respectively. Data acquisition was performed in the MRM mode monitoring the transition of m/z 313 in Q1 to m/z 285 in Q3 as qualifier and to m/z 241 in Q3 as quantifier for aflatoxin B1. The collision offset energies were 31 and 49 V, respectively, and the declustering potential 76 V. The corresponding transitions m/z 329→ m/z 115 and m/z 329→m/z 243 were monitored for aflatoxin G1 at a declustering potential of 66 V and collision offset energy of 91 and 39 V, respectively.

Quantification of aflatoxin B1 and G1 was performed using commercially available reference compounds. Calibration curves were constructed in the range of 0.1–100 ng/mL in which the linearity of the response was given.

Real-Time PCR

The Real-Time PCR reactions were performed in a GeneAmp 5700® Sequence Detection System (PE Applied Biosystems, Foster City, USA). The SYBR green approach was used. The optimal primers and the internal probe used in the reaction were identified within the aflR_AP (AP=A. parasiticus) with respect to the aflS_AP gene by Primer Express 1.0 software (PE Applied Biosystems). The primer sets for the standard curves to perform absolute quantifications had the following nucleotide sequences: aflR_AP_for (5′ - gcc gcc gtt gag gta cac tg-3′) and aflR_AP_rev (5′ - cga acg tgg tct tgc ctg tc-3′); aflS_AP_for (5′ -cgt cac cgg ggc atg tg-3′) and aflS_AP_rev (5′ -gcg cgg cgt cca tcg-3′). The concentration of these standard PCR products was determined in a fluorometer (DNA Quant 200; Pharmacia, Uppsala, Sweden) and the number of copies was calculated. These stock solutions were diluted serially by a factor of 10 and an aliquot of the dilutions was used as a copy number standard during each setup of the Real-Time PCR reaction. The concentration of unknown samples was calculated by the GeneAmp 5700® system according to the generated standard curve.

For the PCR reaction, the SYBR Green Reagent kit (Eurogentec, Liege, Belgium) was used according to the recommendations of the manufacturer. After an activation step of 10 min at 95°C, all subsequent 40 PCR cycles were performed according to the following temperature regime: 95°C for 20 s, 55°C for 30 s, and 72°C for 40 s. The primer pairs to generate aflR- and aflS-specific internal gene fragments for SYBR Green fluorescence measurement had the following sequences: aflR_SYBR_for (5′ -ggt ccc cac ttc caa aaa cg-3′) and aflR_SYBR_rev- 3_rev (5′ -gct ggt cag gcg caa agc-3′), aflS_SYBR_for (5′ -cgg atg aac tgg caa aac ttg-3′) and aflS_SYBR_rev (5′ -gca ggt atg cca tga tta tcg-3′).

Results

Influence of water activity × temperature interactions on growth of A. parasiticus

The influence of various combinations of aw levels and temperature on growth of *A. parasiticus* is shown in Fig. 1. This strain of *A. parasiticus* displayed a growth optimum at 35°C. The colony diameter was strongly dependent on the aw treatment. The fastest growth was observed at the highest aw level examined (0.99 aw). When aw was decreased there

was a gradual decrease in growth although the optimum remained at 35°C. At 0.90 aw, growth was very slow and only observable between 30 and 37°C.

Influence of water activity × temperature interactions on aflatoxin B1 and G1 biosynthesis

The same samples which were used for the growth measurement were analysed using LC-MS/MS to quantify the amount of aflatoxin B1 and G1 produced under these interacting conditions (Fig. 2). There were different optima for production profiles of the two structural forms of aflatoxin. *A. parasiticus* formed high amounts of aflatoxin B1 at >30°C, with an optimum at 37°C (Fig. 2a). At temperatures between 30 and 37°C, biosynthesis of aflatoxin B1 appeared to be independent of aw as long as it was above 0.90. However, at lower temperatures (e.g. 20 and 25°C), a_w seemed to play an influential role. Under these conditions, aflatoxin B1 was formed in higher amounts at higher a_w levels (0.98–0.99 a_w). At temperatures >37°C, the biosynthesis of aflatoxin B1 decreased sharply. In contrast, aw seems to be important for the optimum production of aflatoxin G1. Large amounts of aflatoxin G1 were produced at aw 0.99 and 0.98 irrespective of the temperature in a range between 20 and 30°C (Fig. 2b). At temperatures above 30 and below 17°C, aflatoxin G1 production drops sharply.

Relationship between aflR and aflS expression to the biosynthesis of aflatoxin B1 and G1

Because it has been shown previously that the expression level of *aflR* and *aflS* is related to aflatoxin B1 biosynthesis in *A. flavus* (Schmidt-Heydt et al. 2009), the expression levels of both genes of *A. parasiticus* were also analysed by Real-Time PCR for the quantification of aflatoxins from the same cultures used for the growth experiments. Surprisingly, the two regulatory genes behaved differently. The expression of the aflR gene tended to be higher at conditions ≥30°C (Fig. 3a), whereas the *aflS* gene expression was higher at conditions ≤30°C (Fig. 3b). This corresponded with the production profile of aflatoxin G1 (*aflS*) or aflatoxin B1 (*aflR*). Generally, the absolute expression of *aflS* was higher than that of *aflR*. The normalised ratio of *aflS:aflR* expression is shown in Fig. 3c. This ratio was high at conditions <30°C, which is within the temperature range optimal for aflatoxin G1 biosynthesis. At temperatures >30°C, supportive for aflatoxin B1 formation, the ratio was low.

Discussion

The influence of a_w × temperature interactions on growth and the biosynthesis of aflatoxin was investigated in this study. Various ecophysiological studies with *A. flavus* (Sorensen et al. 1967; Gqaleni et al. 1997; O'Brian et al. 2007; Schmidt-Heydt et al. 2009) have been done, but much less information is available for *A. parasiticus* (Lin et al. 1980; Faraj et al. 1991; Park and Bullerman 1981). The strain of *A. parasiticus* examined here showed a clear growth responsiveness to interacting aw × temperature conditions. Freely available water (0.99 aw) supported growth at almost all temperatures examined. A clear optimum for growth was found at 35°C, regardless of aw level. A very similar growth surface response curve for *A. parasiticus* in relation to changes in a_w and temperature was shown by Samapundo et al. (2007), although they found an optimum of 30°C for their strain of *A. parasiticus*. For *A.flavus*, previous studies have shown a siilar influence of these parameters on growth behaviour with an optimum at 30°C (Samapundo et al. 2007; Schmidt-Heydt et al.

2009). This suggested that, apparently, growth regulation in response to aw and temperature is similar in both species.

The optimum conditions for biosynthesis of aflatoxins B1 and G1 in *A. parasiticus* differed. Interestingly, optimal conditions for both did not coincide with the growth optimum (35°C), but was either below (G1) or above (B1). Aflatoxin B1 biosynthesis was independent of aw at certain temperatures as long as the a_w was >0.90. In contrast, the optimum production of aflatoxin G1 was more dependent on aw than from temperature in the range 20–30°C. This shift between optimum production and optimum growth has further been observed for ochratoxin production by *Penicillium verrucosum*, trichothecene production by *F. culmorum* (Schmidt-Heydt et al. 2008) or for fumonisin production by *F. verticillioides* (Jurado et al. 2008). A difference in the temperature optima between aflatoxin B1 and G1 was previously found by Lin et al. (1980). They analysed the influence of cycling temperatures on aflatoxin B1 and G1 biosynthesis in *A. parasiticus* and found that more G1 is formed at lower temperatures, e.g. after cycling between 15 and 25°C, whereas more B1 is produced after cycling between 15 and 33°C. This is in agreement with the data reported here and shows the different temperature regulation of the B versus the G aflatoxins.

The expression profiles of the two regulatory genes at the different combinations of aw and temperature showed a relationship with the production profiles of the two different aflatoxin types produced. Both genes were highly expressed at 30°C. At this temperature, moderate to large amounts of both aflatoxins B1 and G1 were formed. Depending on the temperature, the expression of *aflS* or *aflR* changes. The aflR gene was expressed at higher levels at >30°C, whereas the most prominent expression of the aflS gene was at <30°C. This corresponded with the production profile of either aflatoxin B1 (prominent above 30°C) or G1 (prominent below 30°C). When a quotient between the normalised expression data of the *aflS:aflR* genes was generated, high ratios could be found at temperatures <30°C. This is in agreement with the aflatoxin G1 production profile. In a recent study of *A. flavus* (Schmidt-Heydt et al. 2009), it was demonstrated that the ratio between *aflS* and *aflR* expression seems to be an indicator for the activation of aflatoxin B1 biosynthesis. In the current study, a high ratio indicated a high biosynthesis of aflatoxin G1. Interestingly, high production of aflatoxin G1 in *A. parasiticus* occurs under similar conditions (moderate temperatures 20–30°C, high aw of 0.99) as high aflatoxin B1 production in *A. flavus* (Schmidt-Heydt et al. 2009).

The products of both the *aflR* and *aflS* genes are regulatory proteins. AFLR is a transcription factor which directly binds to a consensus sequence in front of the aflatoxin structural genes (Ehrlich and Cary 1995; Ehrlich et al. 1999). The activity of the *aflS* gene on the other hand is not absolutely clear. It is not a DNA binding protein; however, according to Chang (2003), it interacts with *aflR* and somehow supports DNA binding by the latter. Ehrlich (2009) recently discussed that *aflS* may interact with the global secondary metabolite regulatory factor *laeA.* According to a recent analysis of Du et al. (2007), aflS appears to modulate the activity of *aflR*. This activity would coincide with the results reported in the present study. Assuming that the expression of the regulatory genes *aflR* and *aflS* is directly correlated to the amount of gene products, the regulation of aflatoxin B1 or G1 seems to be dependent on the ratio of the transcription of the regulatory genes. The *aflS/aflR* ratio is high under conditions which favour high aflatoxin G1 biosynthesis. As a modulator, aflS may influence

the activity of *aflR*. In the case of *A. parasiticus*, this modulation may shift the affinity of *aflR* towards the G-group-specific genes, *ordA, cypA* and *nadA* (Cai et al. 2008) and activate their expression under these conditions.

This differential production of aflatoxin B1 and G1 surely has an ecological function and may be an adaptation of *A. parasiticus* to a similar but slightly different habitat compared to *A. flavus* (Magan and Aldred 2007a, b). The differentially produced amounts of either aflatoxin B1 or G1 under various environmental conditions is obviously attributable to the adaptation to changing environments. It has been demonstrated that mycotoxins can be one method by which ecological advantage can be obtained by species to improve their competitiveness under fluctuating environmental conditions (Magan and Aldred 2007a, b).

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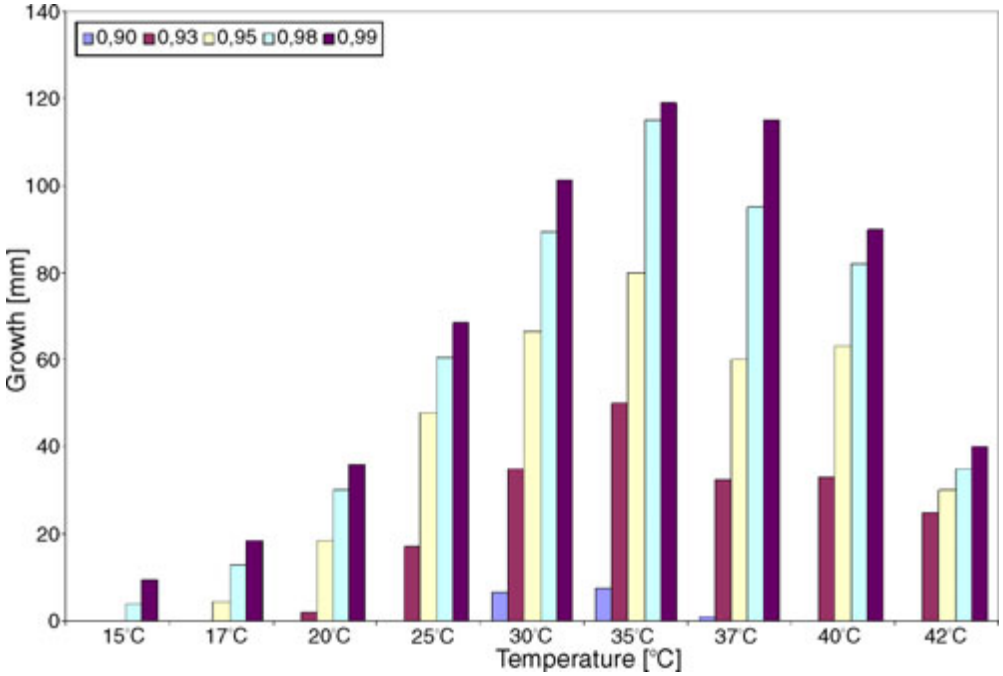

Fig. 1 Growth profile of Aspergillus parasiticus BFE96p at various combinations of temperature × aw. Growth was determined by measuring the diameter of the colony

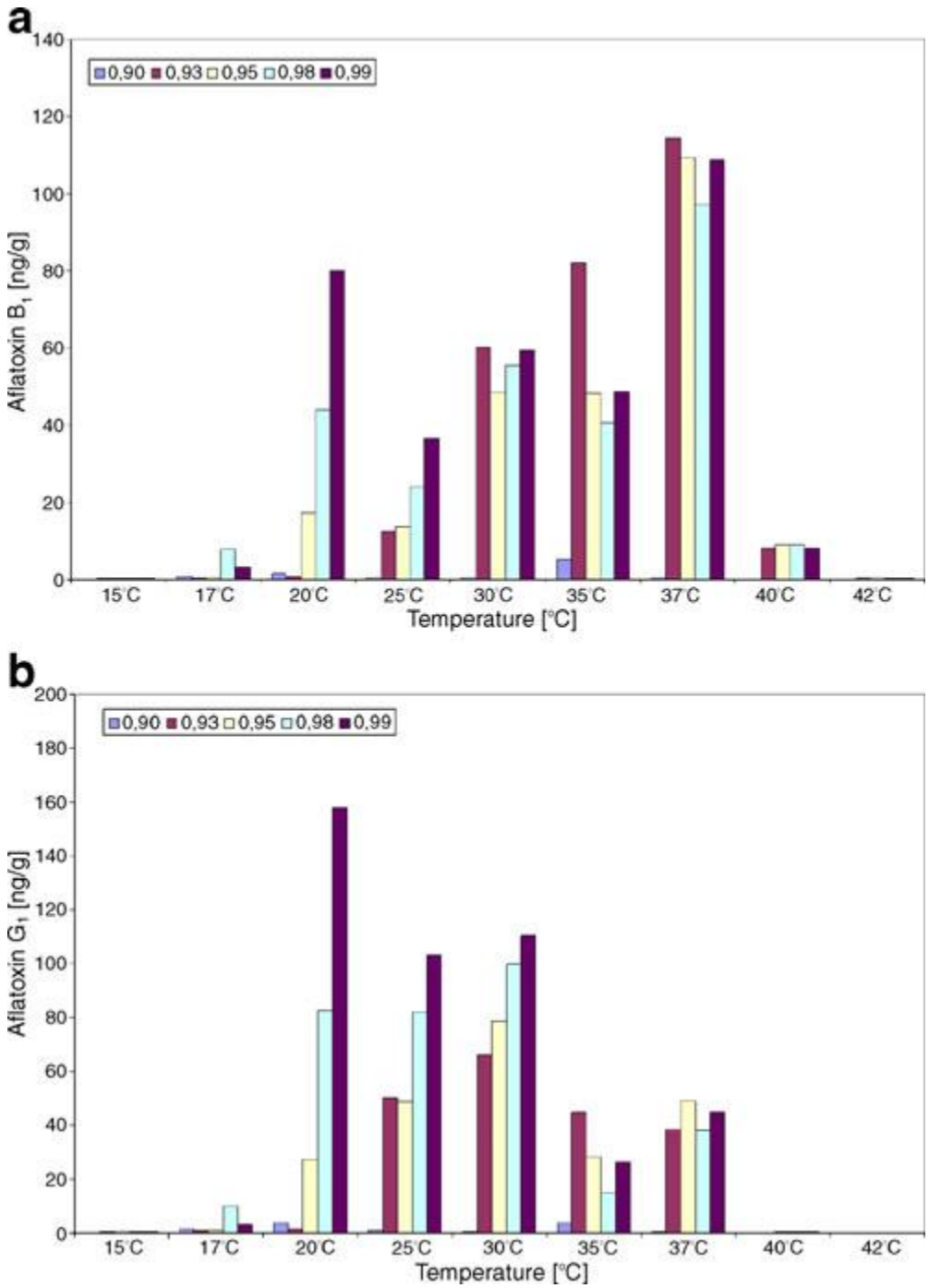

Fig. 2 Production profile of aflatoxin B1 (a) and aflatoxin G1 (b) in relation to various combinations of temperature × aw. The aflatoxin produced was quantified by HPLC-MS

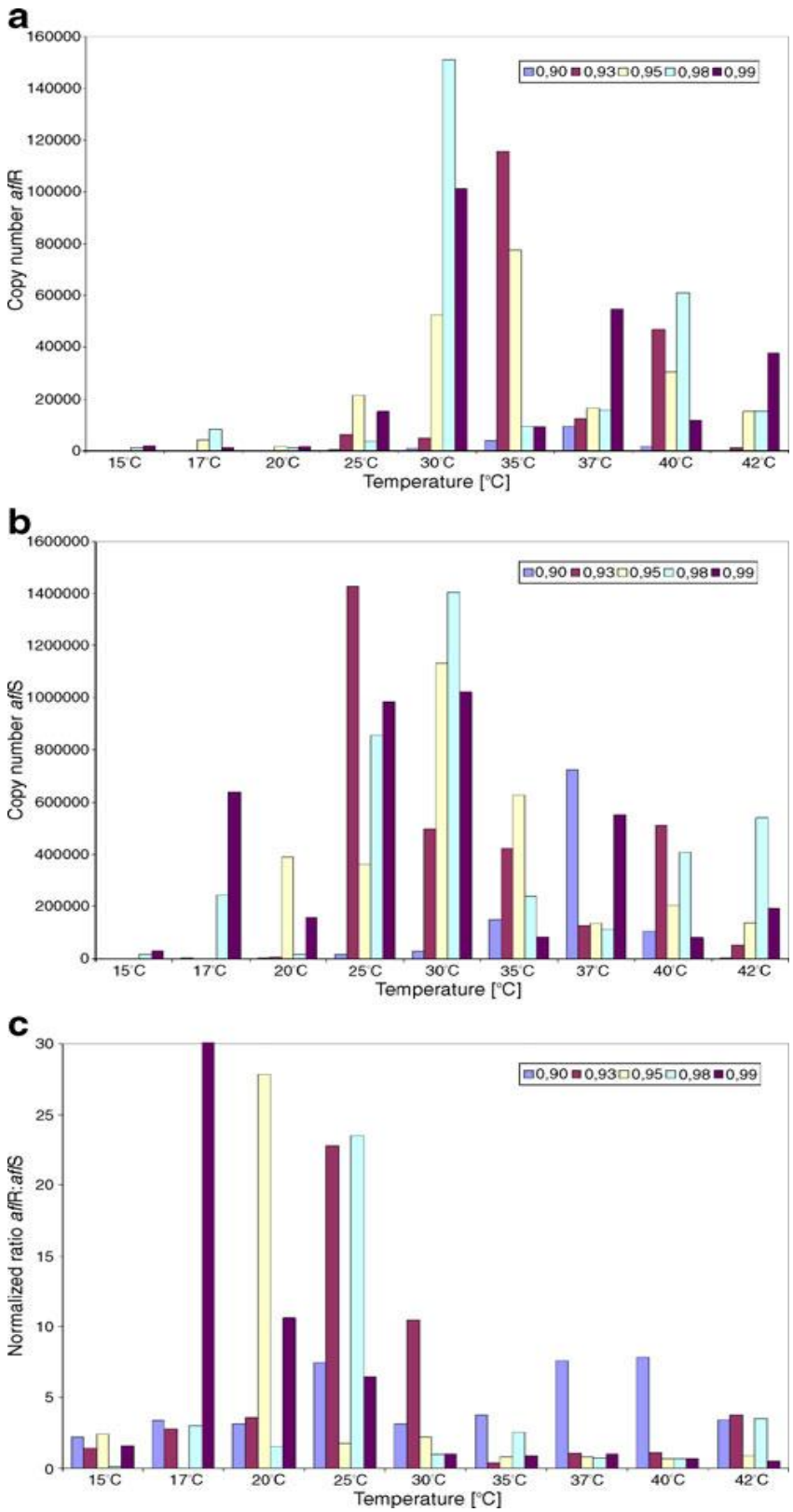

Fig. 3 Expression profile of aflR (a) and aflS (b) determined by Real-Time PCR at various combinations of temperature × aw. The ratio of the normalised expression values (aflS:aflR) is shown in (c)