**ORIGINAL SCIENTIFIC PAPER**

# The Effect of Experimental Fungal Emulation on Colonization of Soybeans by Moulds of the Genus *Aspergillus* and *Fusarium* and on Aflatoxin $B_1$ Accumulation in Relation to Temperature and Moisture Content *in vitro*

**Lejla Duraković[1*], Alma Tudić[2], Ksenija Markov[1], Domagoj Čvek[1], Frane Delaš[1]**

[1]*Faculty of Food Technology and Biotechnology, Department of Biochemical Engineering, Laboratory for General Microbiology and Food Microbiology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia*
[2]*Croatian Waters, Sector of Development and Water Economy, Vukovarska 220, 10000 Zagreb, Croatia*

***Summary***

*One strain of Aspergillus flavus ATCC 26949 and one strain of Fusarium graminearum ZMPBF 1244 were used to investigate the effect of growth in mixed culture on aflatoxin $B_1$ ($AFB_1$) accumulation in respect to time and temperature of cultivation in vitro. The experiments were carried out with aflatoxigenous mould A. flavus ATCC 26949 in pure culture and in mixed culture, respectively, the latter with the common mould F. graminearum ZMPBF 1244. F. graminearum does not produce aflatoxins and chromatographycally similar compounds. The amount of biomass was estimated by measuring the chitin content; the aflatoxin accumulation by means of UV light and ELISA method. It was established that the accumulation of $AFB_1$ primarily depend on the temperature of cultivation, rather than on growth of the mycelium. The biomass of the mixed culture of A. flavus and F. graminearum after 35 days of cultivation reduces the amount of $AFB_1$ by 55 to 76% in respect to highest values obtained in experiments with the pure culture of A. flavus. Decrease of this toxin amount is more pronounced in the substrate at the higher water content and at the higher temperature of cultivation. As a general rule, cultures grown in mixed culture under investigate temperature conditions produced less amount of $AFB_1$ than A. flavus grown in pure culture.*

***Keywords:*** *moulds, mycotoxins, mixed cultures, chitin, ELISA*

***Sažetak***

*Jedan soj Aspergillus flavus ATCC 26949 i jedan soj Fusarium graminearum ZMPBF 1244 upotrijebljeni su u istraživanju utjecaja rasta u mješovitoj kulturi na nakupljanje aflatoksina $B_1$ ($AFB_1$) u odnosu na vrijeme i temperaturu uzgoja in vitro. Istraživanja su provedena pomoću aflatoksikogene plijesni A. flavus ATCC 26949 u čistoj i u mješovitoj kulturi, odnosno kasnije zajedno s plijesni F. graminearum ZMPBF 1244. Plijesan F. graminearum ne proizvodi aflatoksine i slične spojeve. Količina biomase plijesni određena je mjerenjem količine hitina; nakupljanje aflatoksina pomoću UV svjetla i ELISA postupka. Dokazano je da nakupljanje $AFB_1$ prvenstveno ovisi o temperaturi uzgoja plijesni, više nego o rastu micelija. Biomasa mješovite kulture vrsta A. flavus i F. graminearum nakon 35-og dana uzgoja pokazala je smanjenje tvorbe $AFB_1$ za 55 do 76% u odnosu na najviše vrijednosti dobivene u pokusima s čistom kulturom A. flavus. Smanjenje količine tog toksina bilo je više izraženo u supstratu koji je sadržavao veću količinu vode i pohranjen pri višoj temperaturi. U pravilu, ako je pri uzgoju plijesni porasla mješovita kultura pri istraživanim temperaturnim uvjetima, te su kulture proizvele manju količinu $AFB_1$ u odnosu na količinu koju je proizvela plijesan A. flavus porasla u čistoj kulturi.*

***Ključne riječi:*** *plijesni, mikotoksini, mješovite kulture, hitin, ELISA*

## Introduction

The important beneficial role of certain filamentous fungi in the production of cheeses, antibiotics, enzymes, Single Cell Proteins and numerous other products has been well documented (Duraković and Duraković, 2000), and techtnical aspects of some of these processes are considered elsewhere. However, it must not be overlooked that specific filamentous fungi have an even longer history for their detrimental influences on man, animals and plants. The biotechnological hazards of microorganisms, including fungi, had been already established (Seargeant and Evans, 1979). In man and animals fungi can cause infections or mycoses, promote alergic responses in sensitive subjects or poison with toxic metabolites (Emmons et al., 1977). Toxicity of fungi to man and animals can arise by mycetismus (mushroom poisoning) or by mycotoxicoses.

Large number of turkey poults and duckling died on British farms in 1960 as a result of consuming groundnut meal imported from Brazil. The dead of birds was caused by the liver damage resulting from toxic metabolic products of the mould *Aspergillus flavus* (named aflatoxins), which contaminated the Brazilian groundnut (Blount, 1961). The microflora of seeds, cereal grains, and oilseeds consists of a large and varied population of fungi, bacteria and actinomycetes (Semeniuk, 1954). The fungi are the most common cause of post-harvest deterioration in these field crops. They render unfit for human consumption and animal perhaps as much as 1% world's supply of grain and oilseed (Johnson, 1948). The over characteristics of fungal spoilage in these commodities are very similar. Spoil-

Corresponding author: lejla.durakovic@pbf.hr

age fungi attack food and feed crops after harvest whenever environmental conditions became favorable for their proliferation. Moisture content of the seed or grain, its viability and physical state, ambient temperature, length of storage, and the activity of stored product insects and mites are the main factors that determine both the initiation and extent of mould growth. Storage fungi are the dominant type of mould associated with stored seeds. They are usually only superficially present on seeds at harvest, but some invasion of these microorganisms into dead plant material such as blossom ends, leaf fragments, or straw, etc. does occur. These fungi principally include species *Aspergillus* and *Penicillium*. They are primarily responsible for post-harvest spoilage, and are active in stored grain with a moisture content in the range of 13.3 to 18% (Lopez and Christensen, 1967).

In addition to the long-recognized role of spoilage moulds in destroying stored crops, it is only recently that their tremendous capacity for toxin production has come to be recognized. This toxigenic capacity has stimulated a great deal of research on moulds associated with many stored crops. The capacity of fungi to produce toxic metabolites has been known since the turn of the century. As early as 1913, Alsberg and Black of the U.S. Department of Agriculture conjectured that the products of mould growth might be involved in diseases. Certainly the first described form of a mycotoxicosis, ergotism, has been known to man for much of this recorded history.

The term mycotoxin was adopted in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100000 turkey poults died. This mysterious turkey "X" disease was linked to a peanut meal contaminated with secondary metabolites from *Aspergillus flavus* (Bennett and Klich, 2003). Mycotoxins are, in general, low molecular weight, non-antigenic fungal metabolites, capable of eliciting a toxic response in man and animals (Kuiper-Goodman, 1998). Most antibiotics are in practice mycotoxins, the difference being one of degree rather than kind. Mycotoxins may further be considered as important environmental pollutants synthesized typically, but not always, on grains, nuts and other plant materials. Aquisition by the host is by ingestion, inhalation or contact and quite small amounts of the compounds can represent significan health hazard (WHO, 1979; Hayes, 1980).

The most abundant moulds that produce these toxins and contaminate human foods and animal feeds through fungal growth prior and during harvest or during (improper) storage are *Aspergillus*, *Penicillium* and *Fusarium* (Bhatnagar et al., 2004). On the basis of adverse effects on human and animal health and widespread contamination aflatoxins, deoxynivalenol, ochratoxin A, fumonisins and zearalenone are considered as the most important mycotoxins on a worldwide scale (Miller, 1995). Aflatoxins are produced by several species of *Aspergillus* (*A. bombycis*, *A. flavus*, *A. nomius*, *A. ochraceorostus*, *A. parasiticus* and *A. pseudotamarii*), *Emericella astellata* and *Peteromyces alliaceus*, classified within the section *Flavi* of subgenus *Circumdati* (Samson et al., 2006; Varga and Samson, 2008). Aflatoxins are not produced by other fungi (Samson et al., 2006). Due to their toxicity including carcinogenic activity, aflatoxins affect not only the health to humans and animals but also the economics of agriculture and food (Hwang et al., 2004). On the basis of adverse effects on human and animal health and widespread contamination aflatoxins are considered as the most important mycotoxins on a worldwide scale (Miller, 1995; Lee et al., 2003). Chemically, aflatoxins are difuranocoumarin derivatives produced *via* polyketide pathway (Klich and Pitt, 1988). Of these, the most important, due to its toxicity and occurrence, is aflatoxin $B_1$ ($AFB_1$).

## Materials and methods

### Isolation and determination of fungi

Twelve samples of completely commercially available soybeans were analyzed for determining fungal growth as a natural microflora. Sub-samples of each twelve samples were extracted and analyzed in triplicate. Aliquots of 1.0 g of each sample were placed on chloramphenicol agar and incubated at 25 °C during 7 and 14 days, respectively. The prevalent of grown moulds were enumerated and sub-cultured on Sabouraud dextrose agar (Oxoid, New Hampshire, USA) to obtain pure culture. Pure cultures were identified using the stereoscopic microscope and fungal identification keys for *Aspergillus* (Klich and Pitt, 1988), *Fusarium* sp. (Nelson et al., 1983) and other common fungi (Mngadi et al., 2008).

The two moulds which most frequently occur on soybeans as natural contaminants were identified as *Fusarium graminearum* and *Aspergillus ochraceus*. *F. graminearum* was taken up into the Collection of Microorganisms of the Faculty of Food Technology and Biotechnology in Zagreb, Croatia (ZMPBF) under serial no. 1244. Isolation and identification of this fungal species should be done on a medium low in nutrients as recommended by Gerlach (1981).

Isolated mould does not produce aflatoxins and chromatographically similar compounds. The mould *A. flavus* ATCC 26949, described as one of most potent aflatoxin producers was used as a test organism. The mould was obtained from the American Type Culture Collection (ATCC), Washington, D.C., USA. The cultures were maintained on slants of Potato Dextrose Agar (PDA) and stored at 4 °C. Before each experiment, the organism was transferred to another PDA slant and incubated at 25 °C during 7 days.

### Preparation of inoculum

To obtain the inoculum, the moulds were subcultured on PDA slants from the stock cultures. The slants were incubated for 7 days at 25 °C until they were well sporulated. The spore material from the slants was suspended in 5 mL sterile water solution of Triton X-100 and transferred for surface culture to Roux bottles containing PDA slant (100 mL, 4%), which were subsequently incubated for 10 days at 25 °C. The harvesting of the spore suspension (cca 5 x $10^8$ spores/mL) was carried out according to the method described by Lai et al. (1970) and Duraković et al. (2010). This inoculum was used at the rate of 1 mL/flask.

### Substrate for mould growth and $AFB_1$ accumulation

The basal substrate used in this study was soybeans. Soybeans (*Glycine max*) have been grown in the Far East since early times and have become of supreme importance as a source of oil and protein through the world during the 20th century

(Keinan et al., 2002). Soya meal is a main protein source in animal nutrition (Divi et al., 1997); other soy products, flour, textured products, tofu, fermented products among other, are used as feedstuffs. Such food are consumed in particular by health conscious people (Keinan et al., 2002), which represent a significant part of vegetarian diets and frequently are used also for infant food formulas (Divi et al., 1997).

Fifty grams of soybeans were slightly cracked, that is, the surface were slightly abraded, but kernels were left whole. This cracking was to allow the mould conidia to infect kernels readily. The beans were cracked before sterilization by autoclaving.

Parameters of the cultivation were as follows:

- initial water content in the substrate: 20, 28 and 38%;
- initial numbedr of conidia: 1 x $10^7$ per gram of substrate;
- temperature of cultivation: 15, 20, 25 and 35 °C;
- cultivation time: 35 days.

The biosynthesis of $AFB_1$ was performed with the mould *A. flavus* in pure culture, as well as with mixed culture of moulds *A. flavus* and *F. graminearum*. In experiments with the pure culture of *A. flavus*, substrates were seeded with 1 x $10^7$ conidia per gram of each mould, whereas in experiments with the mixed culture the inoculation was carried out with 1 x $10^7$ conidia of each of investigated moulds per gram of substrate. The cultivation was performed in a stationary culture with 50 grams of substrate in 300 mL Erlenmeyer flasks. The water content was adjusted by adding appropriate amount of distilled water to the samples and following the liquid and solid phase to equilibrate on a laboratory shaker for 30 minutes.

## Determination of biomass

The growth of the fungi was monitored by measuring the chitin content in the substrate as described by Donald and Mirocha (1977) and Xiao-E et al. (2008). Chitin, a polymer of N-acetyl-D-glucosamine, is a constituent of the cell walls of most fungi and can be used as a measure of total fungal growth, since little or no chitin-like materials occur in sound cereal grains. In the analytical method devises, the polymer is not measured directly but rather is hydrolised to glucosamine, deaminated to its corresponding aldehyde and measured spectrophotometrically. The chitin content is estimated from the standard calibration curve of glucosamine-HCl read at 650 nm. For determining of biomass dry weight six different amounts of mould moist mycelium were weighed during its growth on Sabouraud agar at 25 °C. The samples were dried at 60 °C during two hours, and then at 105 °C to constant mass. On the basis of the data obtained, the calibration curve was made, from which was, according to the chitin content, directly determined amount of biomass dry weight (Donald and Mirocha, 1977).

Duraković (2007) and Xiao-E et al. (2008) stated that after 28 days and under comparable environmental conditions, chitin content in mycelium dry weight of mould *A. flavus* ATCC 26949 was 230 mg per gram of dry weight. At the same time, it was established that the mycelium of *Fusarium graminearum* ZMPBF 1244 contained 185 mg of chitin per gram of dry weight. Duraković (2007) found the chitin content in healthy soybean seed to be 135 to 155 µg per gram of dry weight.

## Aflatoxin assay

Aflatoxin $B_1$ was extracted from samples with chloroform as described by AOAC International (2005). The chloroform was evaporated under nitrogen gas, the samples dissolved in methanol and filtered through 0.25 µm organic solvent filter (Millipore Co., Bedford, Massachusets, USA). To confirm the identity of $AFB_1$ Thin Layer Chromatography (TLC) was used. Silica gel precoated plates (250 µm thick; Merck, Darmstadt, Germany) were activated at 115 °C before the use. TLC plates were developed in chloroform : acetone : petroleum ether (33 : 6 : 1), (modified Duraković, 2007), where after they were examined by comparing $R_f$ values of a known standards to the unknown samples under UV light at 365 nm. Aflatoxin primary standards to check the linearity were provided by Carl Roth (Karlsruhe, Germany). Six mililiter of methanol solution with the following specification: 250 ng/mL and 125 ng/mL of $AFB_1$. Working standard solutions of 0.25 and 0.125 ng/mL of $AFB_1$ were prepared by diluting the primary standard solution with methanol - acetonitrile (1 : 1 v / v). Quantification of $AFB_1$ was assayed by ELISA, according to the standard calibration curve (Cavaliere et al., 2004).

## Test principle

The basis of test is antigen-antibody reaction. The wells in the microtiter plates are coated with capture antibodies directed against anti-aflatoxin antibodies. Standards of the sample solutions, aflatoxin-enzyme conjugate and antiaflatoxin antibodies are added. Free and enzyme conjugated aflatoxin compete for the aflatoxin antibody binding sites (competitive enzyme immunoassay). At the same time, the aflatoxin-antibodies are also bound by immobilized capture antibodies. Any unbound enzyme conjugate is then removed in washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made spectrophotometrically at 450 nm (optional reference wavelength $\leq$ 600 nm). The absorption is inverse proportional to the aflatoxin concentration in the sample.

## Determination of production index

The production index, which is the µg of $AFB_1$ produced per gram dry weight of mycelium per day, was calculated according to the formula (Duraković et al., 2012):

$$\text{Production index (µg / g dry weight / day)} = \frac{AFB_1 \text{ at Mycelial Dry Weight}}{\text{Days of Incubation}}$$

## Statistical analysis

$AFB_1$ recoveries from the soybean samples were performed using the method recommended by AOAC International (2005).

Repeatability and recovery were determined by spiking 50 g of soybeans with toxin standard solution at the level of

***Table 1.*** *Percent $AFB_1$ recoveries from spiked samples of soybeans by ELISA*

| Amount added (ng/g) | Mean amount recovered (ng/g) | Mean recoveries, (%) ± SD | CV[a] |
|---|---|---|---|
| 50 | 48.9 | 97.8 ± 5.8 | 10.8 |
| 100 | 97.2 | 97.2 ± 9.2 | 11.0 |
| 200 | 202.8 | 98.6 ± 6.2 | 11.4 |
| 300 | 305.7 | 98.1 ± 7.1 | 8.9 |
| 400 | 410.6 | 97.4 ± 9.9 | 11.2 |
| 500 | 514.6 | 97.3 ± 9.6 | 11.9 |
| **Average** | | | |
| 310 | 263.3 | 97.7 ± 7.9 | 10.9 |

[a]**CV** = Coefficient of variation

50 to 500 ng/g prior to the addition of solvent and extraction. After 1 h, $AFB_1$ was extracted from spiked samples and quantitated according to the protocol of Cahill et al. (1999). Recovery rates of duplicate experiments were between 97.2 and 98.6; standard deviation (n = 5) was between 5.8 and 9.9, respectively (Table 1).

There is no significant difference in the mean percent recoveries. Results of toxin investigated were not corrected for recovery.

## Results and discussion

The quality of stored soybeans has generally been determined by qualitative determination of the number of kinds of fungi in the beans after surface disinfection. Aflatoxin assay usually takes 5 to 7 days to obtain results. This method is time-honored and usually dependable but is not rapid enough for the grain industry. With this in mind, a chemical method was devises which takes about 4 to 5 hours for analysis. It is based on the chemical determination of chitin, a constituent of fungal cell walls. It has an advantage in that it will reflect total mycelium (viable and nonviable) based on chitin content. The method used in this study was based on the method of chitin detection in fungus-infected host tissue devises by Donald and Mirocha (1977), Duraković (2007) and Xiao-E et al. (2008). This method may be useful to the grain industry in rapidly determining the quality of grain.

The influence of investigated moulds growth in pure and in mixed cultures at different environmental conditions and $AFB_1$ accumulation are indicated in Table 2 and Figures 1a to 4c. Mould growth and $AFB_1$ accumulation were observed for 35 days at temperatures of 15, 20, 25 and 35 °C after inoculation of *A. flavus* and *A. flavus* and *F. graminearum*, respectively. According to previous findings (Gourama and Bullerman, 1995; Wicklow et al., 1998; Weinderbörner, 2001) the optimal temperature for the biosynthesis of $AFB_1$ is 28 to 32 °C.

In experiments at 15 °C, only slight growth of *A. flavus* was observed at this temperature and no accumulation of $AFB_1$ was observed. Figures 1a to 4c represent the relation of mould biomass to the synthesized $AFB_1$ during the growth of *A. flavus* in pure culture and the growth of *A. flavus* and *F. graminearum* in mixed culture at incubation temperature of 20, 25 and 35 °C, respectively. According to previous findings (Gourama and Bullerman, 1995; Wicklow et al., 1998; Weinderbörner, 2001) the minimal temperature for growth of moulds from these genera is 15 to 20 °C and optimal temperature for growth of *A. flavus* and biosynthesis of $AFB_1$ is 25 to 27 °C and 28 to 32 °C, respectively.

The initial water contents in the substrates were 20, 28 and 38%, respectively. Lopez and Christensen (1967) stated 15.5% as the minimal water content for the growth of *A. flavus*. Diener and Davis (1968), Hult et al. (1982), and Duraković et al. (2008) have demonstrated that a water content of 32 to 38% was optimal for the biosynthesis of $AFB_1$ on solid substrates. Out of three chosen initial water contents in the substrate, the last one (38%) gave highest increments of biomass and best $AFB_1$ accumulation. The results cited below all refer to this water content.

The greatest amount of biomass obtained at 20 °C during the growth of pure mould culture of *A. flavus* was 18.0 mg biomass dry weight/g of substrate. The amount of $AFB_1$ is low, about 10.96 µg $AFB_1$/g biomass dry weight. Maximum con-

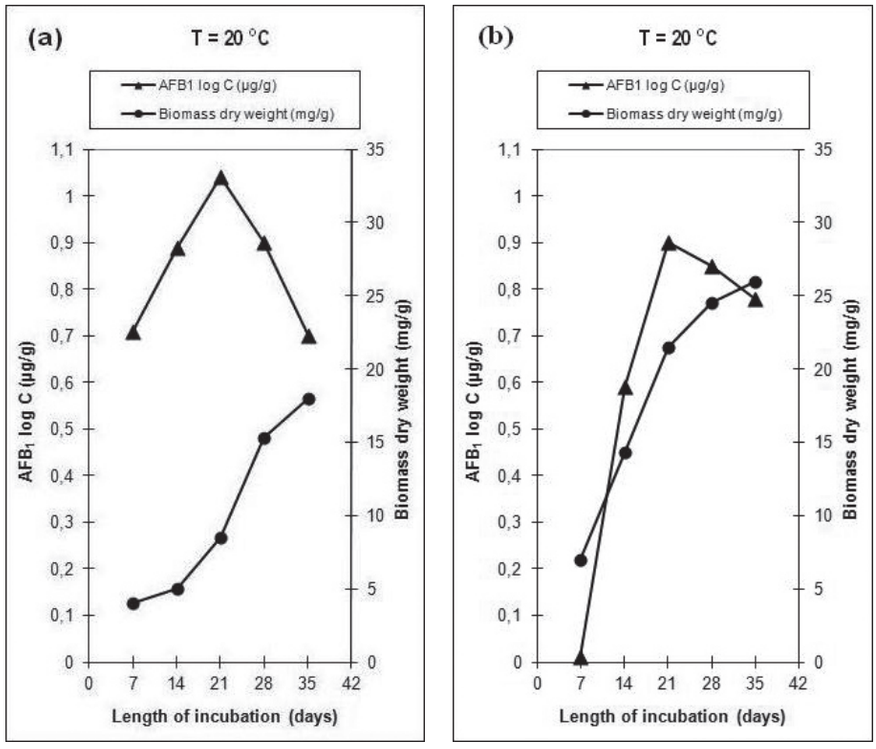


***Figure 1.*** *Aflatoxin $B_1$ accumulation during the growth of A. flavus ATCC 26949 on soybeans in pure culture (a) and in mixed culture (b) at 20 °C.*

centration was established to be 21 days after the inoculation (Figure 1a).

In the mixed culture, the greatest amount of biomass was 26.0 mg biomass dry weight/g of substrate and no accumulation of $AFB_1$ was established after 14 days of cultivation, and maximum accumulation was established after 21 days of cultivation (7.94 µg $AFB_1$/g biomass dry weight (Figure 1b).

The obtained values are in good accordance with the findings of Buchanan et al. (1981), Duraković et al. (2008), and Yazdani et al. (2010) who showed that the growth of toxigenous *Aspergillus* spp. on solid substrates at 20 °C is very low.

As expected, the temperature of 25 °C was more favorable for both the growth of toxigenous mould and $AFB_1$ accumulation. In experiments with the pure culture of *A. flavus*, 34.0 mg biomass dry weight/g of substrate was detected, which is almost twice the amount obtained with the same mould culture at 20 °C, and a higher accumulation of $AFB_1$ was also established. At the time of maximum accumulation, it amounted to: 38.01 µg $AFB_1$/g mycelium dry weight (Figure 2a).

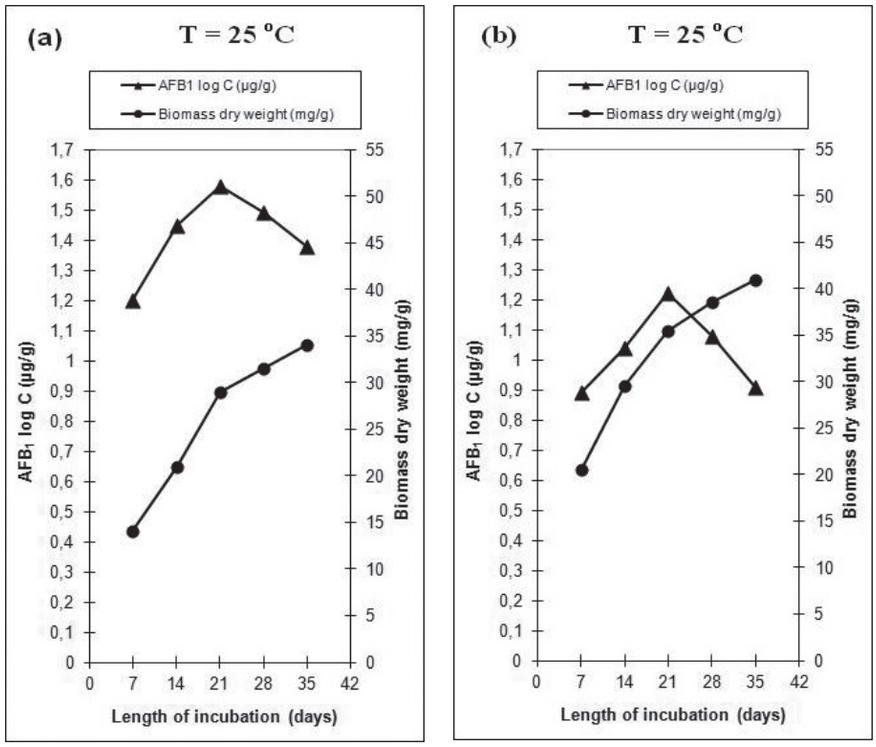


***Figure 2.*** *Aflatoxin $B_1$ accumulation during the growth of A. flavus ATCC 26949 on soybeans in pure culture (a) and in mixed culture (b) at 25 °C*

Under equal conditions of growth, the biomass content in the mixed culture was 45% higher (41.0 mg biomass dry weight/g of substrate) as compared with the pure culture, and the concentration of $AFB_1$ was lessened. The highest values obtained after 21 days were 16.59 µg $AFB_1$/g biomass dry weight (Figure 2b).

The values are in good accordance with the findings of Cho and Kang (2000) and Duraković et al. (2008, 2010) who have stated 25 to 28 °C as the optimal temperature for growth of *A. flavus* ATCC 26949 and accumulation of $AFB_1$.

The temperature of 35 °C was shown to be promotive of the growth of biomass, however not of the $AFB_1$ accumulation. Growing of *A. flavus* in pure culture at the above temperature resulted in somewhat greater amount of biomass (38.0 mg biomass dry weight/g of substrate) compared with the one at 25 °C. The accumulation of $AFB_1$ was considerably lower (Figure 3a) 11.22 µg $AFB_1$/g biomass dry weight.

In the mixed culture, there was an increase of biomass content to about 15% (46.0 mg mycelium dry weight/g of substrate) as compared with the growth at 25 °C and no synthesis of $AFB_1$ could have been detected after first 14 days of cultivation. After 21 days of cultivation, concentration of $AFB_1$ was lower about 30% (11.22

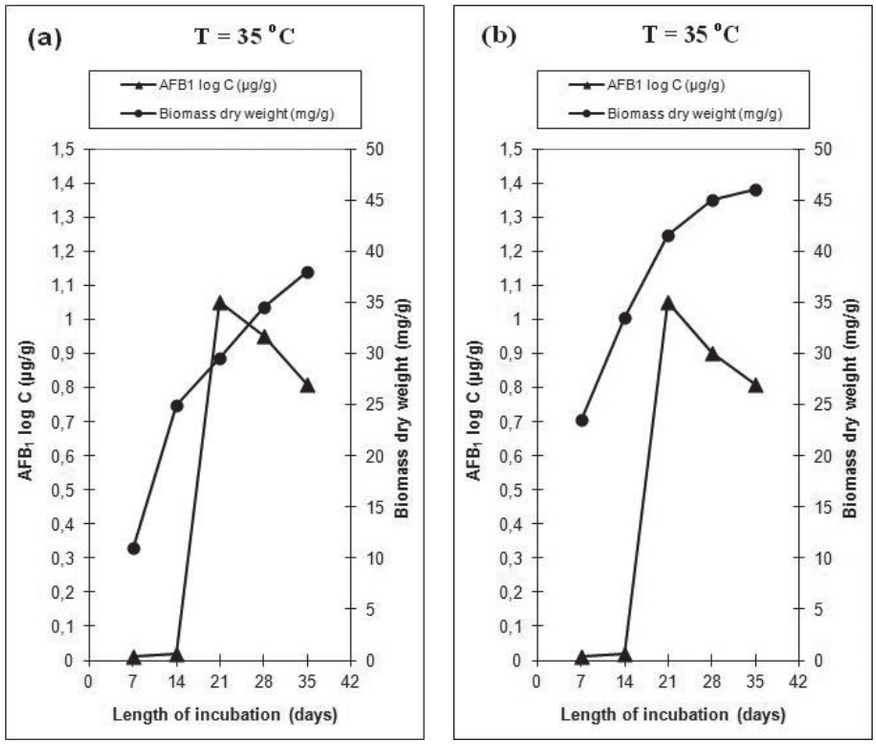


***Figure 3.*** *Aflatoxin $B_1$ accumulation during the growth of A. flavus ATCC 26949 on soybeans in pure culture (a) and in mixed culture (b) at 35 °C*

µg $AFB_1$/g mycelium dry weight) in respect to values obtained at 25 °C (Figure 3b).

Figures 4a to 4c represent the influence of temperature incubation on production index during the growth of investigated moulds in pure and in mixed cultures. The results represented in Figures 4a to 4c reveal that by increasing of temperature, production index was significantly decreased.

Soya, which is the one of major crop, is used as food and fodder after harvest, almost through all the year. High levels of relative humidity during cultivation and maturation, as well as insufficient drying during harvesting and storage of grain can led to fungal activity and contamination with mycotoxin production, which reduces the value of food and feed. Rapid drying allows few organisms to grow, but with slow drying, many saprophytic fungi are capable to colonize fodder. Type of storage fungi to be colonized will vary with the storage conditions and the amount of initial water content of fodder. Association of mycotoxigenic fungi and contamination of soybeans with different mycotoxins has been reported from different parts of the world (Keinan et al., 2002; Mngadi et al., 2008; Yazdani et al., 2010).

Mould *A. flavus* is frequently associated with numerous other microorganisms in stored grains and seed. Thus, the possibility arises that microbial competition between fungi for the substrate under favorable environmental conditions will restrict or reduce the amount of aflatoxin formed. *A. flavus* or competing fungi might absorb or degrade aflatoxin following its formation in the substrate. Schmidt and Esser (1985) concluded that microbial competition or microbial breakdown might be responsible for smaller amounts of aflatoxin in the kernels of parasite damaged pods than in kernels from broken pods. Ashworth et al. (1965) demonstrated that several fungi could break down aflatoxin in peanut and in an aflatoxin-containing liquid medium. They also found that *A. niger* and *Rhizoctonia solani* limited the development of *A. flavus* and aflatoxin accumulation in the substrate. Wildman et al. (1967), Duraković (2007), and Duraković et al. (2008, 2010) noted that a *Penicillium* sp. reduced aflatoxin yields when grown in cultural competition with *A. flavus*. Whether this was accomplished by competition for available substrate or by destruction of aflatoxin was not demonstrated.

Some authors have suggested that mycotoxin accumulation can be stimulated by fungal competition strategy (Birzele et al., 2000). Different studies, however, have not been able to confirm this point (Reid et al., 2011). One of the limiting factors of fungal growth on solid substrates is also available solid surface. In mixed cultures the species with higher growth rate will overgrow the substrate, thus preventing the growth of other species (Duraković, 2007; Duraković et al., 2008).

Table 2 comparatively shows the decrease of $AFB_1$ concentration in the substrate after 35 days of growth of *A. flavus* in pure and in mixed culture with the mentioned mould at all parameters of cultivation. The values were calculated on the basis of the highest amounts of $AFB_1$ accumulated. Hence, it appears at the same cultivation temperature, a more considerable decrease of $AFB_1$ concentration occurs in the substrate with a higher initial water content.

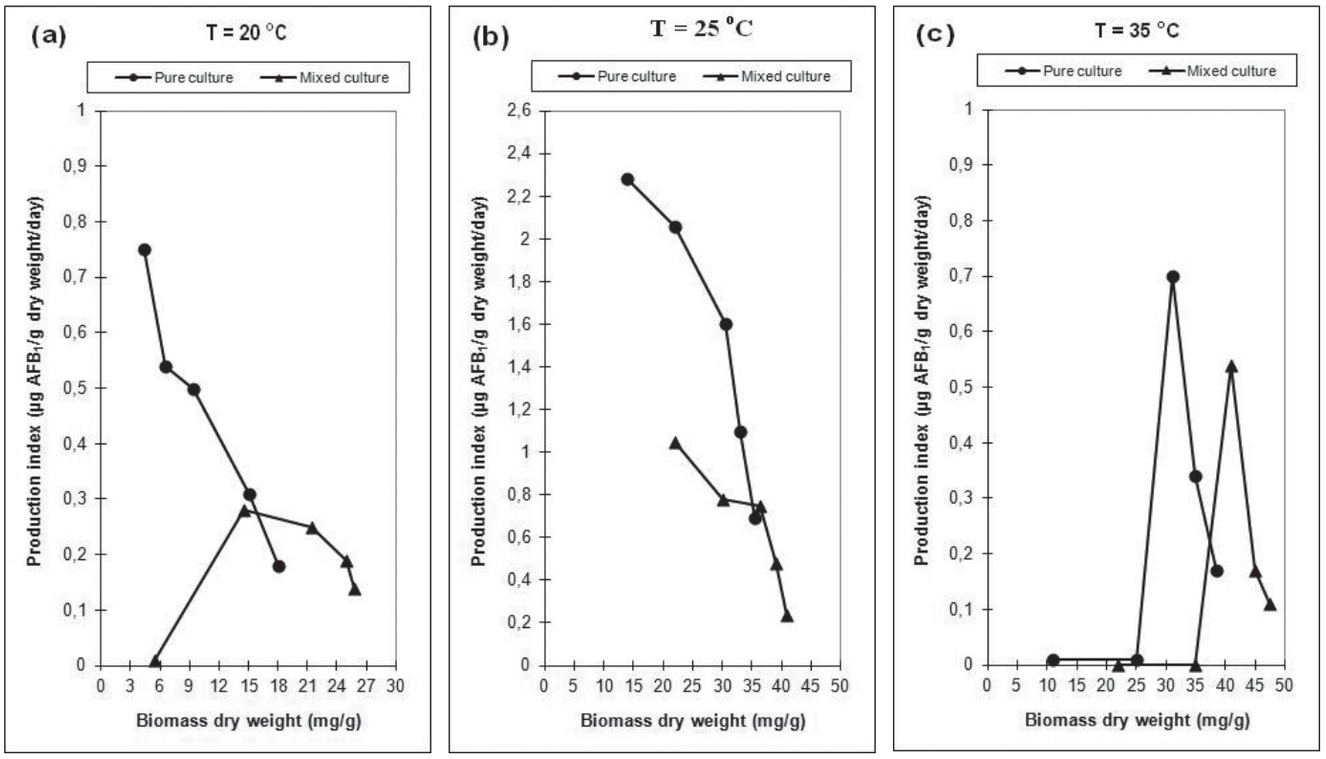


***Figure 4.*** *Aflatoxin $B_1$ production index curves of A. flavus ATCC 26949 in pure and mixed culture at: (a) 20 °C; (b) 25 °C; (c) 35 °C during 35 days of cultivation.*

***Table 2.*** *Comparative representation of decrease of $AFB_1$ accumulation during the growth of A. flavus ATCC 26949 in pure and in mixed culture with F. graminearum ZMPBF 1244 on soybeans with respect to incubation temperature and initial water content in the substrate.*

| Decrease of aflatoxin $B_1$ accumulation after 35 days | | | |
|---|---|---|---|
| **Temperature (°C)** | **Pure culture (%)** | **Mixed culture[a] (%)** | **Initial water content (%)** |
| 20 | - 44 | - 62 | 38 |
| 25 | - 35 | - 76 | |
| 35 | - 53 | - 55 | |
| 20 | - 24 | Ø | 28 |
| 25 | - 32 | - 40 | |
| 35 | - 25 | - 45 | |
| 20 | Ø | Ø | 20 |
| 25 | - 12 | Ø | |
| 35 | Ø | Ø | |

[a] = In respect to highest values obtained with the pure culture of *A. flavus*; Ø = accumulation of $AFB_1$ has not been proven.

## Conclusions

The determination of mould biomass on solid substrates by "Chitin method" is a comparatively rapid procedure and can be successfully applied for evaluating the extent of fungal contamination on cereals.

A rank growth of the toxigenous mould was found to be in no relation with the amount of the aflatoxin $B_1$ synthesized. Thus, e.g. at 35 °C, more mycelium grew than at 25 °C, but the amount of the produced $AFB_1$ was considerably smaller. It was shown that more $AFB_1$ was accumulated in the substrate on which only *A. flavus* ATCC 26949 was cultivated, than if, under equal conditions, the same mould was grown in mixed culture with aflatoxin-negative mould.

The biomass of the mixed culture was more capable of eliminating $AFB_1$ from the substrate than the biomass of the same strain of *A. flavus* in pure culture.

As a conclusion, it has been shown that natural mycoflora may act as an additional control factor, as long as they do not produce any other mycotoxins.

According to obtained results we can suggest that increase of temperature and decrease of moisture can posditively inhibitory influence on $AFB_1$ production.

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