ORIGINAL SCIENTIFIC PAPER

Aflatoxin Accumulation During the Growth of Mould Aspergillus flavus ATCC 26949 on Corn in Pure and Mixed Culture as Related to Various Temperature and Moisture Content

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Summary

The relationship between mould biomass and the biosynthesis of aflatoxin B_1 and G_1 on solid substrates (whole and crushed maize grain) at temperatures from 15-40°C and a water content in the substrate of 20-38% has been investigated. The experiments have been carried out with the mould Aspergillus flavus ATCC 26949 in pure culture and in mixed culture respectively, the latter with the mould Trichothecium roseum ZMPBF 1226. The biomass growth during cultivation was measured by the chitin content, and the concentration of aflatoxins was determined by means of a ELISA. It has been established that the biosynthesis of the examined aflatoxins and their ratio primarily depend on the temperature of cultivation, rather than on the growth of the mycelium. The biomass of the mixed culture of A. flavus and T. roseum after 35 days of cultivation reduces the amount of aflatoxin B_1 by 19-40%, and the amount of aflatoxin G_1 by 30-45%. The decrease of concentration of both toxins is more pronounced in the substrate with a higher initial water content and at a higher temperature of cultivation.

Keywords: moulds, mixed cultures, mycotoxins, ELISA

Sažetak

Istražen je kvantitativni odnos biomase plijesni i aflatoksina B_1 i G_1 na čvrstim supstratima (cijelo i lomljeno zrno kukuruza) pri temperaturama od 15-40°C i sadržaju vode u supstratu od 20-38%. Pokusi su provedeni pomoću plijesni Aspergillus flavus ATCC 26949 u čistoj kulturi i mješovitoj kulturi s plijesni Trichothecium roseum ZMPBF 1226. Tijekom uzgoja praćen je rast biomase određivanjem sadržaja hitina, a koncentracija aflatoksina je određivana imunoenzimnim postupkom (ELISA). Utvrđeno je da sinteza istraživanih toksina i njihov međusobni odnos nisu izravno ovisni o rastu biomase, nego ponajprije ovise o temperaturi na kojoj se plijesni uzgajaju. Biomasa mješovite kulture A. flavus i T. roseum nakon 35 dana uzgoja, smanjuje tvorbu aflatoksina B_1 za 19-40%, a aflatoksina G_1 za 30-45%. Smanjenje količine oba toksina izrazitije je u supstratu s većom početnom količinom vode i pri višoj temperaturi uzgoja.

Ključne riječi: plijesni, mješovite kulture, mikotoksini, ELISA

Introduction

The problem of mould metabolites, known collectively as mycotoxins, arose on a world-wide scale in 1960. when it was unambiguously proved that numerous cases of Turkey "X"-disease had been caused by groundnut meal, wherein mould fragments were occasionally found (Blount, 1961.). The mould was identified as *Aspergillus flavus* Link ex Fries, and the subsequently found toxic principle was named aflatoxin, in accordance with its origin. The study of aflatoxins received a further stimulation when it was established that apart from their toxic potency they exhibit carcinogenic effects (Lancaster *et al.*, 1961.b).

According to the findings of numerous investigators, aflatoxins were found in nearly all mold-contaminated substrates (Ciegler *et al.*, 1971.; Šutić and Stojanović, 1973.; Martinez, 1979.; Pantović and Adamović, 1980.; Yamazaka, 1980.; Chelkowski *et al.*, 1981.; International Agency for Research on Cancer (IARC), 1993.a.; International Commission on Microbiological Specifications for Foods (ICMSF), 1996.).

Considering the fact, that on natural substrates mixed mould cultures grow rather than pure ones, the problem of aflatoxin biosynthesis during the growth of mixed mould cultures, as well as the possibility of their detoxification, becomes an especially significant one (Park, 1993.; Duraković *et al.*, 2007.; 2008.).

The control of fungi is a key problem in many areas of applied microbiology and new antifungal agents are sought constantly (Varga and Toth, 2005.). The extensive antimicrobial activity of dehydroacetic acid (DHA) and its newly synthesized analogues has only recently been recognized. Some investigators found that analogues of DHA had activity against moulds from genus *Aspergillus* including *A. parasiticus, A. flavus and A. ochraceus* (Duraković *et al.*, 1989.; 1991.; 1993.; 1994.).

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Aflatoxin degradation by means of biological methods was the subject of numerous investigations. Ciegler *et al.* (1966.) and Alberts *et al.* (2006.) studied the microbial detoxification of aflatoxin B_1. Out of some 1000 species of microorganisms (bacteria, yeasts, moulds, actinomycetes and algae), only the bacterium *Flavobacterium aurantiacum* NRRL B-184 was capable of eliminating aflatoxin from the substrate.

The ability of some fungal species to degrade aflatoxin was described by Mann and Rehm (1975.; 1977.), whereas Massimango *et al.* (1979.) and Ginterova *et al.* (1980.) studied the influence of mould growth in mixed culture on the biosynthesis of aflatoxin B_1. Upon the results obtained, they inferred that aflatoxicogenous moulds, growing in mixed culture with aflatoxin-negative moulds, exhibit a lower capability of accumulating aflatoxin in the culture media, than if growing in pure culture (Duraković *et al.*, 2007.; 2008.).

With respect to the significance, and because of the complexity of the problem of aflatoxin biosynthesis and biodegradation during mould growth in mixed cultures, in the present work were examined the conditions which may arise during the storage of maize.

Therefore we were interested to find out if, at which time, and to what extent, under chosen conditions of growth, a degradation of aflatoxin B_1 and G_1 takes place, when the mould *A. flavus* ATCC 26949 grows on maize in pure culture and in mixed culture respectively, together with mould most frequently encountered as contaminant on maize, but not synthesizing aflatoxins themselves.

Materials and methods

The mould which most frequently occur on maize as natural contaminant was identified as *Trichothecium roseum* and taken up into the Collection of Microorganisms of the Faculty of Food Technology and Biotechnology of Zagreb (ZMPBF) under the serial No. 1226. The mould *A. flavus* ATCC 26949, described as one of the most potent aflatoxin producers, was used as a test microorganism.

The biosynthesis of aflatoxins B_1 and G_1 was performed with the mould *A. flavus* in pure culture, as well as with the two moulds, *A. flavus* and *T. roseum* ZMPBF 1226 in mixed culture.

The investigation of biosynthesis of aflatoxin B_1 and G_1 consisted of the following: Cultivation of each of the investigated moulds in pure culture for the sake of production of the inoculum; cultivation of *A. flavus* on whole and crushed maize grain in pure and mixed culture; determination of biomass in pure and mixed culture; extraction and purification of the extracts by column chromatography; qualitative analysis of aflatoxin by thin-layer chromatography and quantitative assay of aflatoxins by ELISA.

The parameters of biosynthesis were as follows: substrate: whole and crushed maize grain (ZG SK 502A hybrid); initial water content in the substrate: 20%, 28%, and 38% respectively; initial spore number in the substrate: 1.5 x 10^6 per gram; incubation temperature: 15^{0}C, 20^{0}C, 30^{0}C and 40^{0}C respectively; cultivation time: 35 days.

The obtain the inoculum, the moulds were cultivated in pure culture on a sporulation medium – potato dextrose slant agar. After 7 days incubation at 28^{0}C the spores were suspended in aliquots of 5 mL of sterile water solution of Triton X-100. Whole and crushed maize grains respectively were used as the medium for aflatoxin biosynthesis. The cultivation was performed in stationary culture with 50 g of substrate in each 500 mL – Erlenmayer flask.

In experiments with the pure culture of *A. flavus*, substrates were seeded with 1.5 x 10^6 spores per gram of each, whereas in experiments with the mixed culture the inoculation was carried out with 1.5 x 10^6 spores of each of the investigated moulds per gram of the substrate.

At 7 – days intervals during cultivation in the incubator, samples were taken for the determination of biomass content and for the assay of aflatoxin B_1 and G_1.

The biomass content in such solid substrates was determined by the "Chitin method", as described by Donald and Mirocha (1977.). The essential feature of this method is the basic hydrolysis of chitin in the samples to chitosan. After a series of chemical reactions, chitosan was separated from the substrate by centrifugation at 6.000 x g determination was performed by spectrophotometer at 650 nm according to the standard calibration curve for N-acetylglucosamine. Duraković (1981.) found the chitin content in healthy maize grain (the hybrid ZG SK 502A was used) to be 105-120 µg per gram of dry weight. The same author determined the chitin content in the mycelium of *A. flavus* ATCC 26949 at 185 mg per gram of dry weight. At the same time it was established that the mycelium of *T. roseum* ZMPBF 1226 contained 260 mg chitin per gram of dry weight.

The identification of aflatoxin B_1 and G_1 by thin – layer chromatography was performed with the solvent system chloroform : acetone (9 : 1, v/v), recommended by Hesseltine (1986.), and with the solvent system chloroform : acetone : petroleum ether (33 : 6 : 1 , v/v/v), (modified; Duraković, 1981.).

After preparative thin – layer chromatography (Silicagel F_{254} ; layer thickness 2 mm), the aflatoxins were assayed quantitatively by ELISA, according to the standard calibration curves (Cavaliere *et al.*, 2004.).

For the confirmation of structure, mass spectra of aflatoxins B_1 and G_1 were scanned, as well as mass spectra of the same toxins from mould extracts. In this work we used a "SHIMADZU" EC-MS-QP1000 mass spectrometer, and the scanning conditions were as follows: energy of ionization: 70 eV; ion source current: 1 mA; trap current: 100 µA; ion source temperature: 200^{0}C; scanning rate: 3 s/decade.

Results and discussion

Figures 1 and 2 represent the relation of biomass to the synthesized aflatoxins B_1 and G_1 during the growth of *A. flavus* in pure culture and the growth of *A. flavus* and *T. roseum* in mixed culture, at incubation temperatures of 15^{0}C, 20^{0}C, 30^{0}C, and 40^{0}C respectively. According to Sorensen *et al.* (1967.), and Shindler *et al.* (1967.), the optimal temperature for the biosynthesis of aflatoxin B_1 is 28 – 32^{0}C, whereas for aflatoxin G1 it is 24 – 28^{0}C.

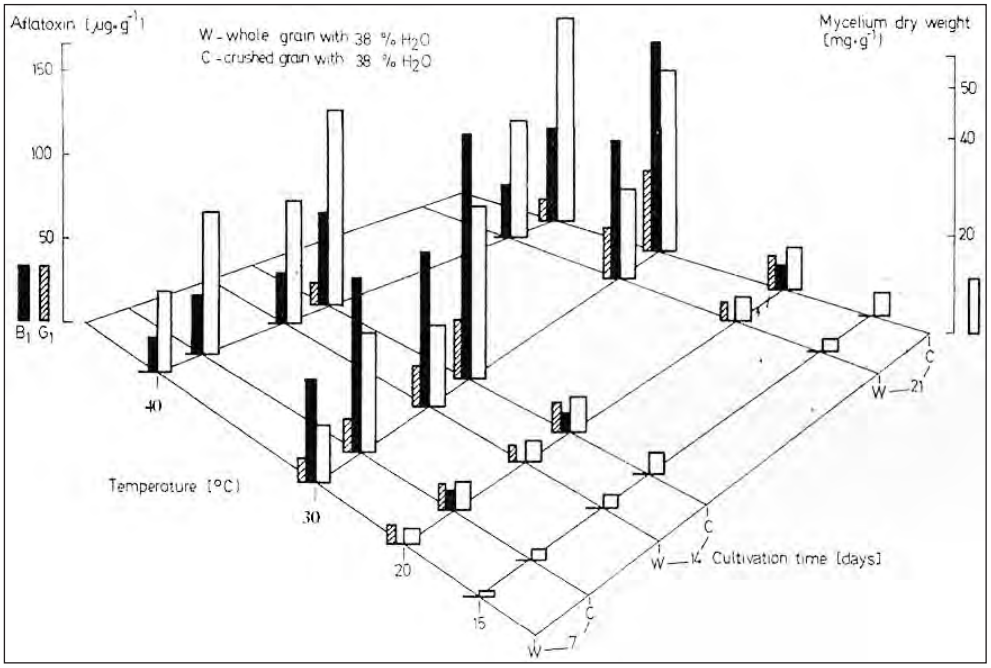

Figure 1. *Comparison of mould biomass from pure culture of A. flavus ATCC 26949 with synthesized aflatoxins B_1 and G_1, with respect to time, cultivation temperature and substrate.*

The initial water content in the substrate was 20%, 28% and 38%. Lopez and Christensen (1967.) stated 15.5% as the minimal water content for the growth of *A. flavus*. Diener and Davis (1968.) and Duraković *et al.* (1989.; 2008.) have demonstrated that a water content of 32 – 38% was optimal for the biosynthesis of aflatoxins on solid substrates.

Out of the three chosen initial water contents in the substrate, the last one (38%) gave highest increments of biomass and best aflatoxin production.

At all cultivation parameters crushed maize grain proved a better substrate for both the growth of biomass and the synthesis of aflatoxins. The results cited below all refer to this substrate.

At 15^{0}C no synthesis of aflatoxins, neither B_1 nor G_1, was evidenced, although a small increment of biomass was observed (Figures 1 and 2).

The greatest amount of biomass obtained at 20^{0}C during growth of the pure mould culture was 6.80 mg/g substrate. In the mixed culture it amount to 14.20 mg/g substrate. The synthesis of aflatoxins B_1 and G_1 was established only in the pure culture of *A. flavus*, and the greatest amounts obtained were (Figure 1.): 18.65 µg B_1/g mycelium dry weight and 19.30 µg G1/mycelium dry weight.

As expected, the temperature of 30^{0}C was more favourable for both the growth of the toxicogenous mould and the aflatoxin synthesis. In experiments with pure culture of *A. flavus* on crushed maize grain, 35.40 mg mycelium/g substrate was detected, which is almost twice the amount obtained with the same mould on whole maize grain. On this substrate a higher production of both investigated toxins was also established.

At the time of maximum accumulation it amounted to: 139.80 µg B_1/g mycelium dry weight and 47.25 µg G_1/g mycelium dry weight (Figure 1.).

Under equal conditions of growth, the biomass content of the mixed mould culture was slightly higher (36.90 mg/g substrate), but the concentration of both aflatoxins was lessened. The highest values obtained were (Figure 2.): 89.70 µg B_1/g mycelium dry weight and 30.50 µg G_1/g mycelium dry weight.

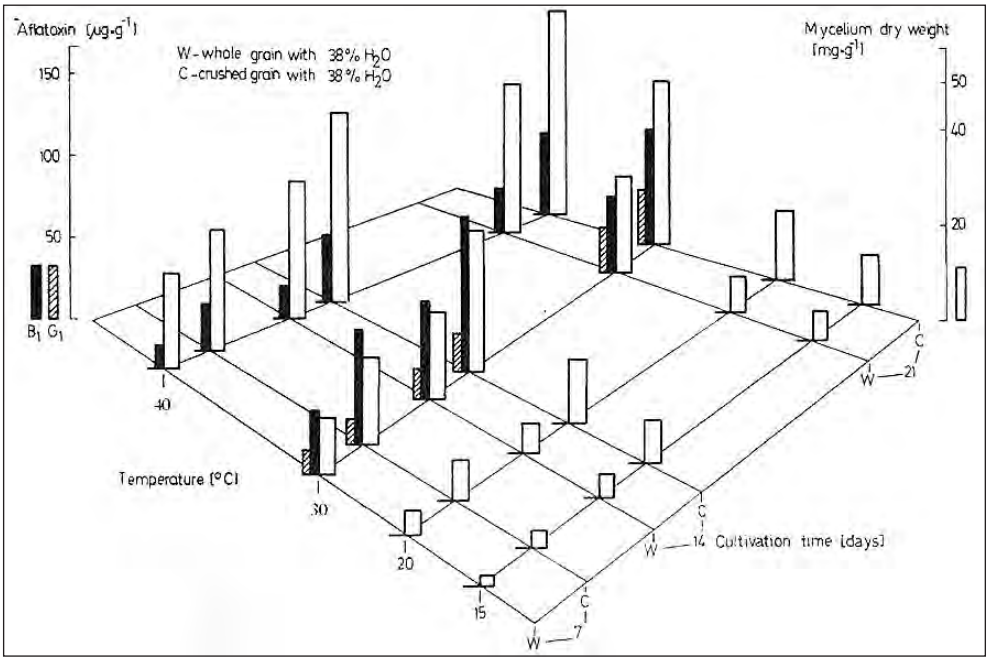

Figure 2. *Comparison of mould biomass from mixed culture of A. flavus ATCC 26949 and T. roseum ZMPBF 1226 with synthesized aflatoxins B_1 and G_1, with respect to time, cultivation temperature and substrate.*

The temperature of 40^{0}C was shown to be more promotive of the growth of biomass, however not of the aflatoxin synthesis. Growing *A. flavus* in pure culture at the above temperature resulted in a somewhat greater amount of biomass (44.20 mg/g substrate), compared with the one at 30^{0}C.

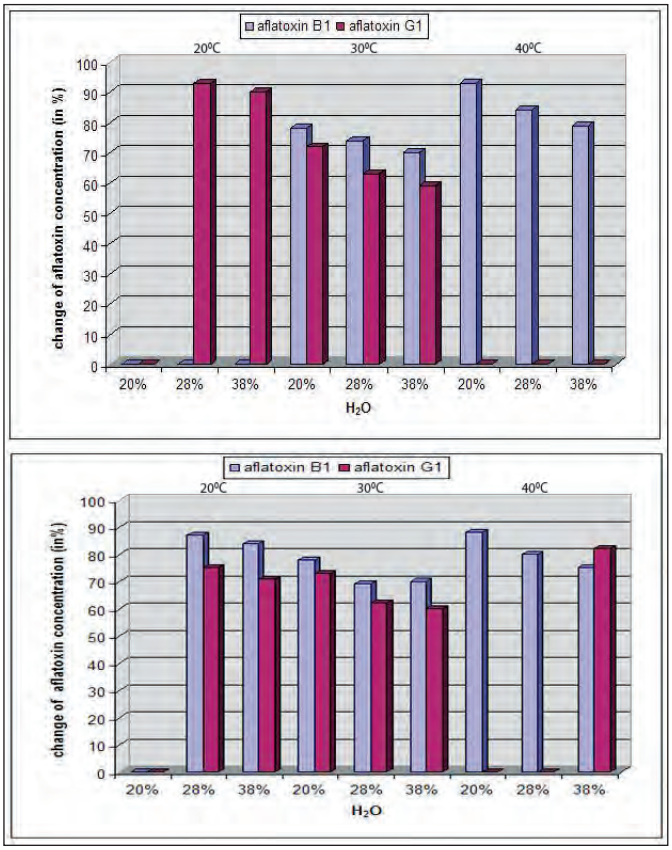

Figure 3. *Comparative representation of concentration change of aflatoxins B_1 and G_1, synthesized during growth of the mould A. flavus ATCC 26949 in pure culture on whole maize grain (above) and crushed maize grain (below), with respect to incubation temperature and initial water content in the substrate. (Measured after 35 days).*

The concentration of aflatoxins was considerably lower (Figure 1.): 49.40 µg B_1/g mycelium dry weight and 10.70 µg G_1/g mycelium dry weight.

In the mixed culture there was an increase of biomass content of about 20% (43.90 mg/g substrate), as compared with the growth at 30^{0}C, and no synthesis of aflatoxin G_1 could have been detected. The highest concentration of aflatoxin B_1 was 51.60 µg/g mycelium dry weight, i.e. about 15% lower than in pure culture of *A. flavus* (Figure 2.).

Comparing the amounts synthesized at various cultivation temperatures it appears that not only the amount of the two toxins, but also their ratio depend on the temperature. E.g., at 20^{0}C a greater amount of aflatoxin G_1 is synthesized (ratio B_1 : G_1 = 1 : 1.5). At 30^{0}C the amount of B_1 exceeds by far that of G_1 (ratio B_1 : G_1 = 4 : 1), whereas at 40^{0}C this becomes even more pronounced (B_1 : G_1 = 4.5 : 1).

Figures 3. and 4. comparatively show the decrease of concentration of the two toxins after 35 days of cultivation of the investigated moulds in pure and in mixed culture respectively. The results reveal that, depending on parameters of growth, the biomass of *A. flavus* ATCC 26949 growing in pure culture reduces the concentration of aflatoxin B_1 by 10 – 31%, and that of G_1 by 6 – 33%. These findings suggest of the ability of the toxicogenous mould to partly metabolize aflatoxins in a certain period of growth, and/or to modify them into compounds with differing chemical characteristics.

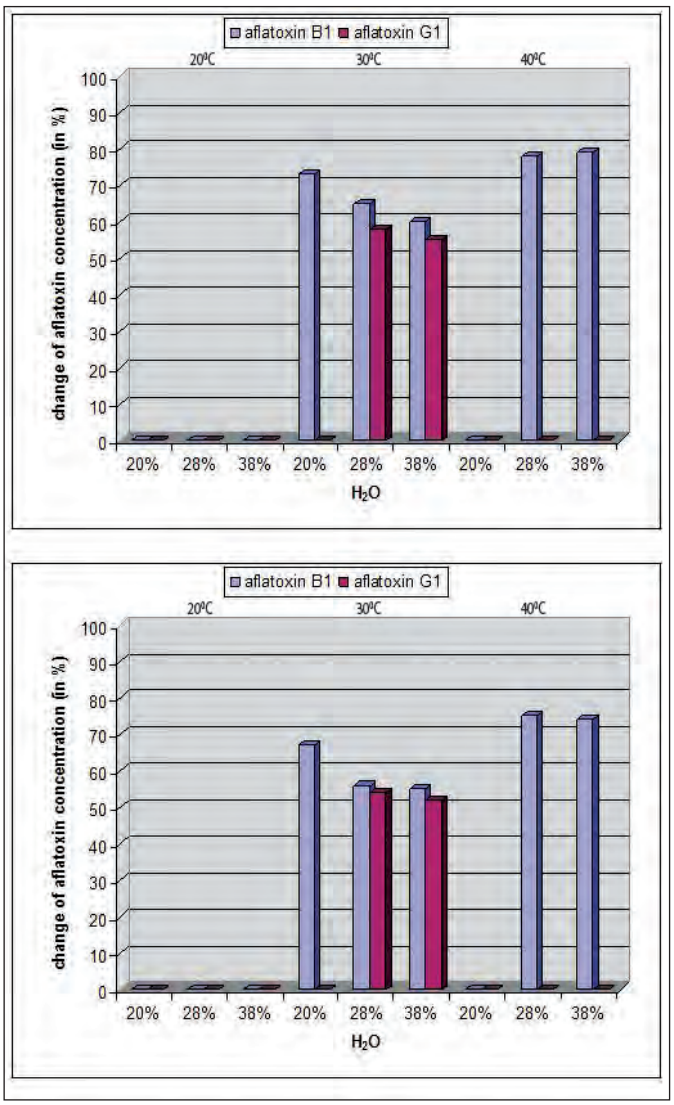

Figure 4. *Comparative representation of concentration change of aflatoxins B_1 and G_1, synthesized during growth of the moulds A. flavus ATCC 26949 and T. roseum ZMPBF 1226 in mixed culture on whole maize grain (above) and crushed maize grain (below), with respect to incubation temperature and initial water content in the substrate. (measured after 35 days).*

Under equal conditions of cultivation the mixed culture biomass decreases the concentration of aflatoxin B_1 by 25 – 43%, and that of G_1 by 32 – 49%. The data on illustrations represent values, standing in good accordance with the findings of Masimango *et al.* (1979.), Ginterova *et al.* (1980.) and Duraković *et al.* (1989.; 2008.) who have shown that the simultaneous growth of toxicogenous and nontoxicogenous moulds in mixed culture resulted in a decrease of aflatoxin concentration of up to as much as 75%.

Figure 5. represents mass spectra of reference standard of aflatoxin B_1, as well as of the same toxin isolated from the substrate. The relative intensities (on the ordinate) are plotted versus the m/e – ratio (on the abscissa).

Characteristic molecular peeks are seen: at m/e – ratio 312, 284 and 256 for aflatoxin B_1.

Higher peaks were recorded with aflatoxin standard, because its concentration was substantially higher than the concentration of aflatoxin in mould extract.

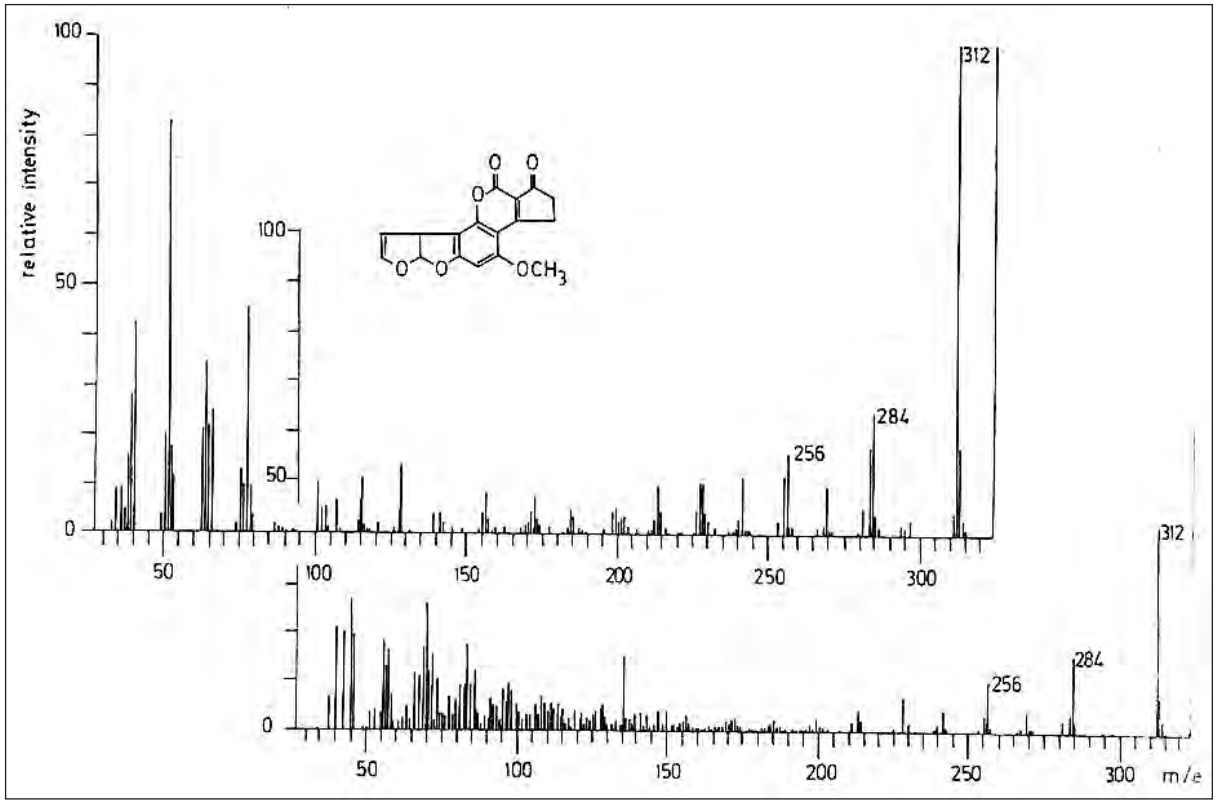

Figure 5. *Presentation of mass spectrum of aflatoxin B_1 standard which represents approximately 70 ng (spectrum above). The spectrum shows 3 main ions which represent M, (M-CO), and (M-2CO) which are seen at m/e 312, 284 and 256. At figure 5, the spectrum below was given from AFB1 isolated from the sample extract which represents 50 g of maize grain defined by ELISA, that contains approximately 40 ng AFB_1/g. The spectrum represents 3 main characteristics of AFB_1 ions. Ion masses larger than 320 were not detected.*

Tables 1. and 2. show the decrease of concentration of both aflatoxins in the substrate after 35 days of growth of the moulds in pure and mixed culture at all chosen parameters of cultivation. The values were calculated on the basis of the highest amounts of aflatoxins synthesized. Hence it appears that at the same cultivation temperature a more considerable decrease of concentration of both investigated toxins occurs in the substrate with a higher initial water content.

A relatively unfrequent occurence of aflatoxins on cereals in our climate is probably due to the fact that elevated temperatures enhance the synthesis of these toxins.

Considering that the mean summer temperature in continental parts of Croatia ranges from 20 to 24^{0}C, it is unlikely that during the growth of cereals in the field a substantial growth of the aflatoxin – producing fungus and the toxin biosynthesis itself would take place. However, during storage of cereals without sufficient ventilation of the stored commodities, significant differences in temperature and water content due to the process of respiration can arise. An uneven distribution of water in the goods can locally lead to a rank growth of mould, albeit the mean water content is considered to be within allowed limits.

The respiration of the growing mould increases the humidity of adjacent grains, thus stimulating further growth, regardless of the mean water content in the whole heap of grains.

Table 1. *Decrease of concentration of aflatoxins B_1 and G_1 during growth of the mould A. flavus ATCC 26949 in pure culture after 35 days of cultivation with respect to incubation temperature, initial water content in the substrate, and the substrate itself.*

Decrease of aflatoxin concentration after 35 days (per cent)					
Temperature (^{o}C)	B_1		G_1		Initial water content
	Whole grain	Crushed grain	Whole grain	Crushed grain	
20	Ø	-15	-9	-25	38%
30	-23	-24	-30	-32	
40	-14	-18	Ø	-15	
20	Ø	-8	-6	-20	28%
30	-17	-20	-27	-28	
40	-12	-16	Ø	Ø	
20	Ø	Ø	Ø	Ø	20%
30	-13	-17	-18	-24	
40	-7	-10	Ø	Ø	

Ø – synthesis of has not been aflatoxin proved

Results obtained in the course of our investigations match well with the findings of other investigators studying the problems of growth of toxicogenous moulds and the biosynthesis of aflatoxins (Sorensen *et al.*, 1967.; Shindler *et al.*, 1967.; Lopez and Christensen, 1967.; Ainsworth *et al.*, 1965.; Diener and Davis, 1968.; Masimango *et al.*, 1979.; Ginterova *et al.*, 1980.; Duraković *et al.*, 1989.; 2008.).

Table 2. *Decrease of concentration of aflatoxins B_1 and G_1 during growth of the moulds A. flavus ATCC 26949 and T. roseum in the mixed culture after 35 days of cultivation, with respect to incubation temperature, initial water content in the substrate, and the substrate itself.*

Decrease of aflatoxin concentration after 35 days (per cent)					
Temperature (°C)	B_1		G_1		Initial water content
	Whole grain	Crushed grain	Whole grain	Crushed grain	
20	Ø	Ø	Ø	Ø	38%
30	-40	-45	-42	-45	
40	-21	-23	Ø	Ø	
20	Ø	Ø	Ø	Ø	28%
30	-29	-40	-30	-42	
40	-19	-19	Ø	Ø	
20	Ø	Ø	Ø	Ø	20%
30	-22	-24	Ø	Ø	
40	Ø	Ø	Ø	Ø	

Ø – synthesis of has not been aflatoxin proved

Conclusions

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

By the use of the solvent system chloroform : acetone : pethroleum ether (33 : 6 : 1, v/v/v), obtained in our experiments, a better resolution of aflatoxins B_1 and G_1 by thin – layer chromatography was achieved than with the commonly used system chloroform : acetone (9 : 1, v/v).

A rank growth of the toxicogenous mould was found to be in no relation with the amount of the aflatoxins synthesized. Thus e.g., at 30°C more mycelium grew than at 20°C, but the amount of the produced acflatoxins was considerably smaller.

It was shown that more aflatoxins B_1 and G_1 were 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 two other aflatoxin – negative moulds.

The biomass of the mixed culture of *A. flavus* and *T. roseum* was more capable of eliminating aflatoxins B1 and G1 from the substrate than the biomass of the same strain of *A. flavus* in pure culture.

Acknowledgements

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