Arh. hig. rada toksikol., 37 (1986) 3—17.

Original Scientific Paper
UDC 615.918:582.282

THE RELATIONSHIP BETWEEN *TRICHOTHECIUM ROSEUM* AND *ASPERGILLUS PARASITICUS* AND THE PRODUCTION OF AFLATOXINS

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(Received for publication August 21, 1985)

The relationship between mould biomass and the biosynthesis of aflatoxins B_1 and G_1 on solid substrates (whole and crushed maize grain) at temperatures from 15—35 °C and a water content in the substrate of 20—38% has been investigated. The experiments have been carried out with the aflatoxigenous mould *Aspergillus parasiticus* NRRL 2999 in pure culture and in mixed culture respectively, the latter with the mould *Trichothecium roseum* ZMFT 1226. Mould *T. roseum* does not produce aflatoxins and chromatographically similar compounds.

The amount of biomass was estimated by measuring the chitin content, and the aflatoxin concentration by means of a fluorodensitometer. 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. parasiticus* and *T. roseum* after seven weeks of cultivation reduces the amount of aflatoxin B_1 by 20—38%, and the amount of aflatoxin G_1 by 30—40%. The decrease of concentration of both toxins is more pronounced in the substrate with a higher initial water content and a higher temperature of cultivation.

Aflatoxins constitute a family of biologically potent metabolites produced by certain strains of the closely related fungal species *Aspergillus flavus* and *A. parasiticus*. Aflatoxins were originally discovered as the etiological agents in a poultry pathology called »turkey X disease« (1). Subsequently, turkey X disease was recognized as one species form of a larger toxigenic syndrome in vertebrates now collectively called »aflatoxicoses« (2).

In addition to acute and chronic toxicity, aflatoxins display carcinogenic, mutagenic and teratogenic activity in a wide range of animal species (3—5).

According to the results of numerous investigators, aflatoxins were found in nearly all kinds of mould-infected natural substrates (6—10).

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.

Aflatoxin degradation by means of biological methods was the subject of numerous investigations. Thus *Ciegler and co-workers* (11) 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* (12, 13), whereas *Masimango and co-workers* (14) and *Ginterova and co-workers* (15) studied the influence of mould growth in mixed culture on the biosynthesis of aflatoxin B_1. Upon the results obtained, they inferred that aflatoxigenous 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.

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 we 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 aflatoxins B_1 and G_1 takes place, when the mould *A. parasiticus* NRRL 2999 grows on maize in pure culture and in mixed culture, together with mould most frequently encountered as contaminant on maize, but not synthesizing aflatoxins themselves.

MATERIAL AND METHODS

Microorganisms

The two moulds which most frequently occur on maize as natural contaminants were identified as *Trichothecium roseum* and *Fusarium sp.*, and taken up into the Collection of Microorganisms of the Technological Faculty of Zagreb (ZMTF) under the serial Nos. 1226 and 1215. The mould *A. parasiticus* NRRL 2999, 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. parasiticus* in pure culture, as well as with the two moulds, *A. parasiticus* and *T. roseum* ZMTF 1226 in mixed culture.

Cultivation of inoculum

Potato-dextrose agar slants, as the medium for sporulation, were inoculated with pure mould culture. After incubation at 28 °C for seven days, the conidia were harvested with an inoculation loop into a sterile 5 ppm — solution of Triton X-100 in distilled water. The suspension thus obtained was homogenized in a tissue grinder. Then it was diluted to 5 x 10^7 spores/mL. Each 50 g of substrate could be inoculated with 1 mL of the diluted suspension.

Determination of mould biomass

Whole and crushed maize grains were used as the medium for aflatoxin biosynthesis. The cultivation was performed in stationary culture with 50 g of substrate in 500 mL — Erlenmeyer flasks.

Parameters of cultivation were the following:
— Initial water content in the substrate: 20%, 28%, 38%;
— Initial number of conidia: 1 x 10^6 per gram of substrate
— Temperature of incubation: 15 °C, 20 °C, 28 °C and 35 °C
— Cultivation time: 49 days

In experiments with the pure culture of *A. parasiticus*, substrates were seeded with 1x10^6 spores per gram of each, whereas in experiments with the mixed culture the inoculation was carried out with 1 x 10^6 spores of each of the investigated moulds per gram of substrate.

The water content was adjusted by adding appropriate amounts of distilled water to the samples and allowing the liquid and solid phase to equilibrate on a laboratory shaker for 30 minutes.

Every seven days during the cultivation period two flasks were taken out of the incubator as samples for the determination of biomass and aflatoxins.

The growth of the fungus was monitored by measuring chitin in the substrate, as described by *Donald and Mirocha* (16). The values thus obtained are expressed in terms of biomass dry weight.

Detection and measurement of aflatoxins

Aflatoxins were extracted from each sample with 150 mL of chloroform for two hours with agitation. The extracts were vacuum-filtered and the filtrates evaporated in a flash-evaporator at 50 °C, reducing their volume to about 5 mL. Subsequently, the extracts were purified by column chromatography (17).

The aflatoxins were identified by thin-layer chromatography on silica gel-precoated plates. Good separation was achieved with the sol-

vent system chloroform-acetone-petroleum ether (33:6:1, v/v/v) (18). The spots were visualized at 366 nm.

The aflatoxins were measured with »Camag« — fluorodensitometer. As a further confirmation of identity of aflatoxins B_1 and G_1 isolated from the extracts and the laboratory standards, mass spectra of both substances were made with Kratos MS 25, Kratos DS 50S mass spectrograph.

RESULTS

Figures 1 and 2 represent the relation of biomass to the synthesized aflatoxins B_1 and G_1 during the growth of *A. parasiticus* in pure culture and the growth of *A. parasiticus* and *T. roseum* in mixed culture, at incubation temperatures of 15 °C, 20 °C, 28 °C, and 35 °C respectively.

Figures 3 and 4 represent the concentration change of aflatoxins B_1 and G_1 after 49 days of cultivation, depending on the amount of biomass, the initial water content in the substrate and cultivation temperature.

Figures 5 and 6 represent mass spectra of reference standards of aflatoxins B_1 and G_1, as well as of the same toxins isolated from the substrate.

Tables 1 and 2 show the decrease of concentration of both aflatoxins in the substrate after 49 days of growth of the moulds in pure and in mixed culture at all chosen parameters of cultivation.

DISCUSSION

At 15 °C there was no evidence of the synthesis of aflatoxins, either B_1 or G_1, although a small increment of biomass was osberved (Figs. 1—2). According to *Sorensen and co-workers* (19), and *Shindler and co-workers* (20), the optimal temperature for the biosynthesis of aflatoxin B_1 is 28—32 °C, whereas for aflatoxin G_1 it is 24—28 °C.

The initial water content in the substrate was 20%, 28% and 38%. *Lopez and Christensen* (21) stated 15.5% as the minimal water content for the growth of *A. flavus*. *Diener and Davis* (22) demonstrated that a water content of 32—35% 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.

The greatest amount of biomass obtained at 20 °C during growth of the pure mould culture was 7.7 mg/g substrate. In the mixed culture it amounted to 16.0 mg/g substrate. The synthesis of aflatoxins B_1 and G_1

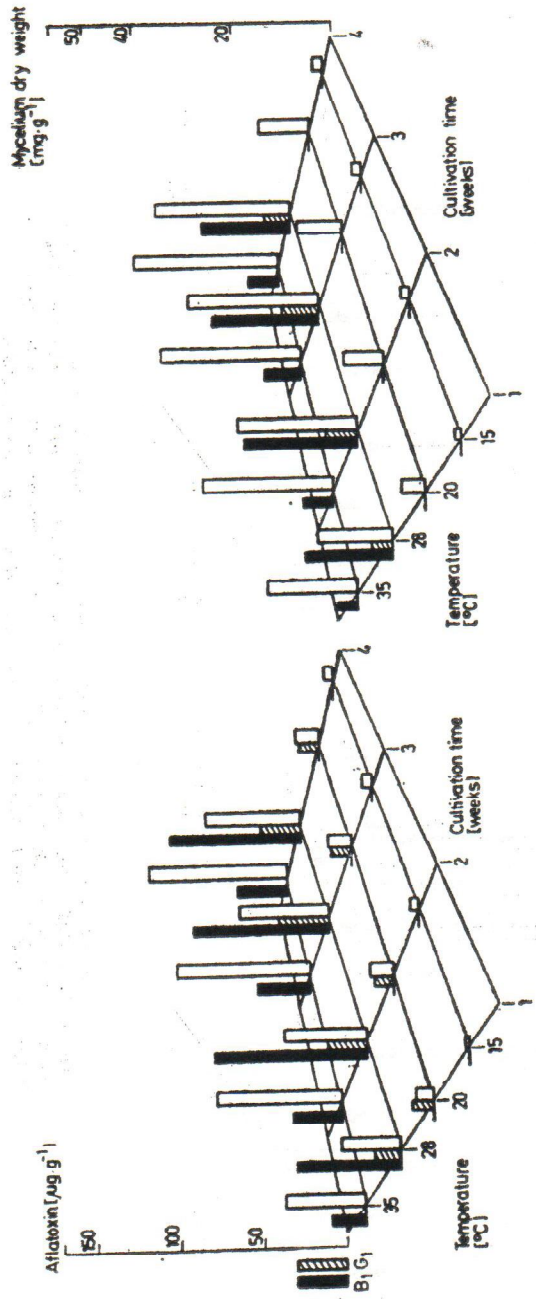

Fig. 1. *Comparison of mould biomass from pure culture of A. parasiticus NRRL 2999 (left) and mixed culture of A. parasiticus and T. roseum ZMTF 1226 (right) on whole grain with 38% water content with synthesized aflatoxins B_1 and G_1 with respect to time and cultivation temperature.*

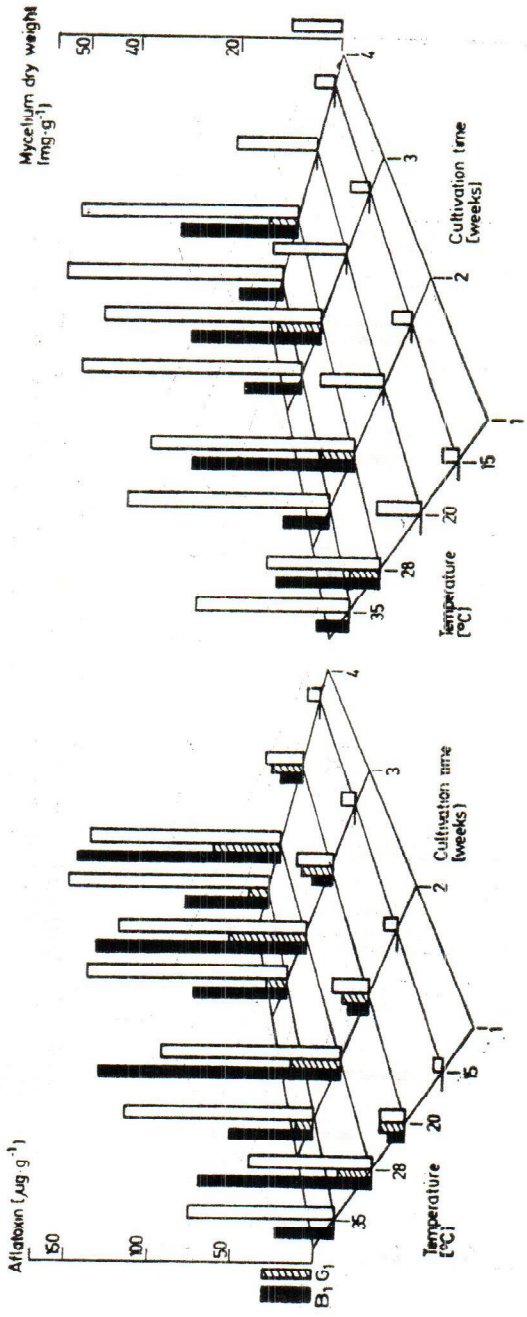

Fig. 2. *Comparison of mould biomass from pure culture of A. parasiticus NRRL 2999 (left) and mixed culture of A. parasiticus and T. roseum ZMTF 1226 (right) on crushed grain with 38% water content with synthesized aflatoxins B_1 and G_1 with respect to time and cultivation temperature.*

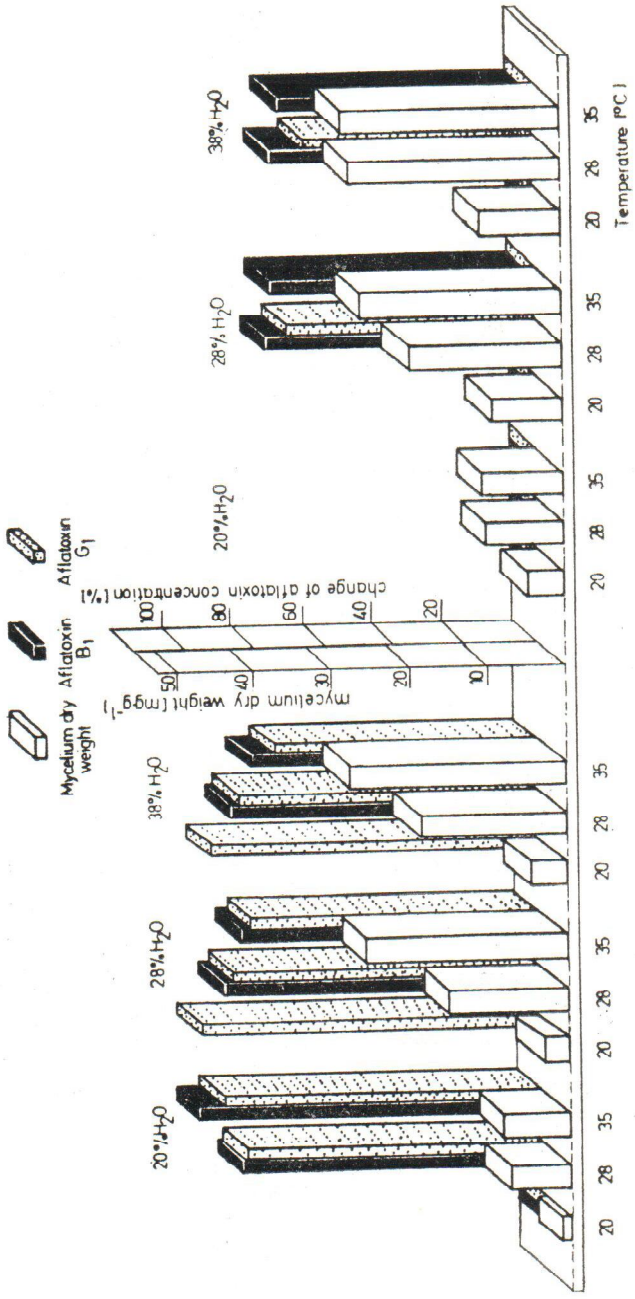

Fig. 3. *Comparative representation of concentration change of aflatoxins B_1 and G_1, synthesized during growth of the mould A. parasiticus NRRL 2999 in pure culture (left) and in mixed culture of the moulds A. parasiticus and T. roseum ZMTF 1226 (right) on whole maize grain, with respect to mould biomass, incubation temperature and initial water content in the substrate. (Measured after seven weeks).*

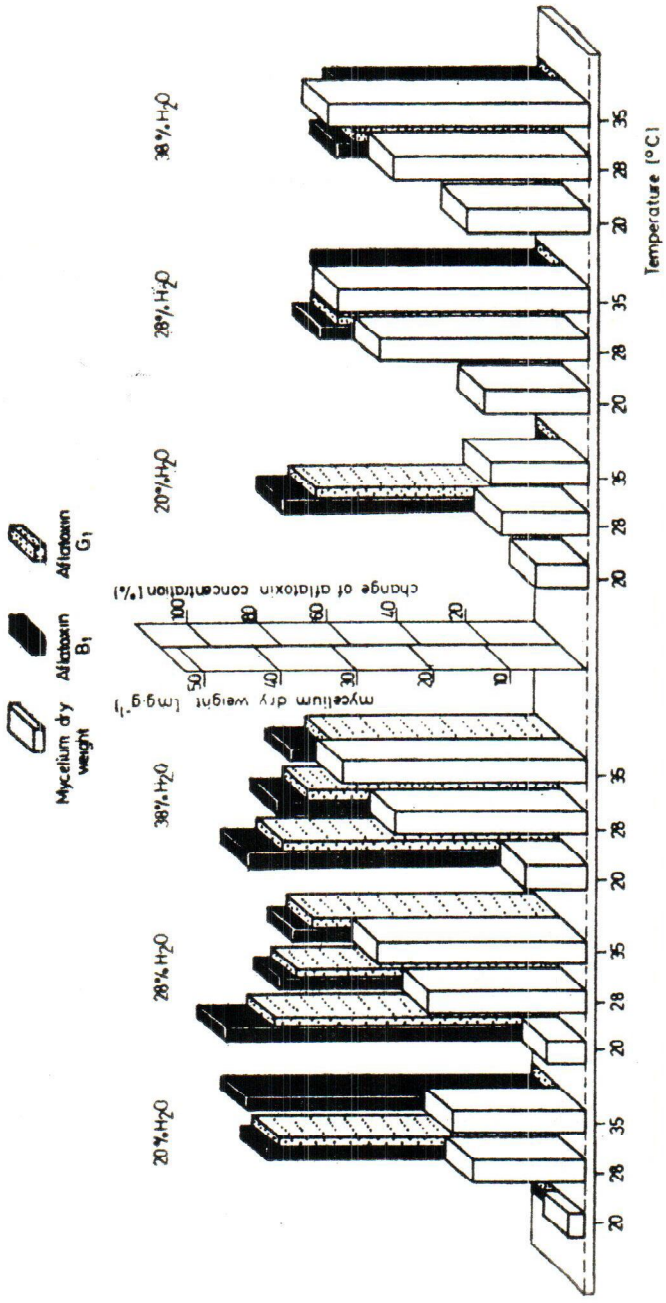

Fig. 4. *Comparative representation of concentration change of aflatoxins B_1 and G_1 synthesized during growth of the mould A. parasiticus NRRL 2999 in pure culture (left) and in mixed culture of the moulds A. parasiticus and T. roseum ZMTF 1226 (right) on crushed maize grain, with respect to mould biomass, incubation temperature and initial water content in the substrate. (Measured after seven weeks).*

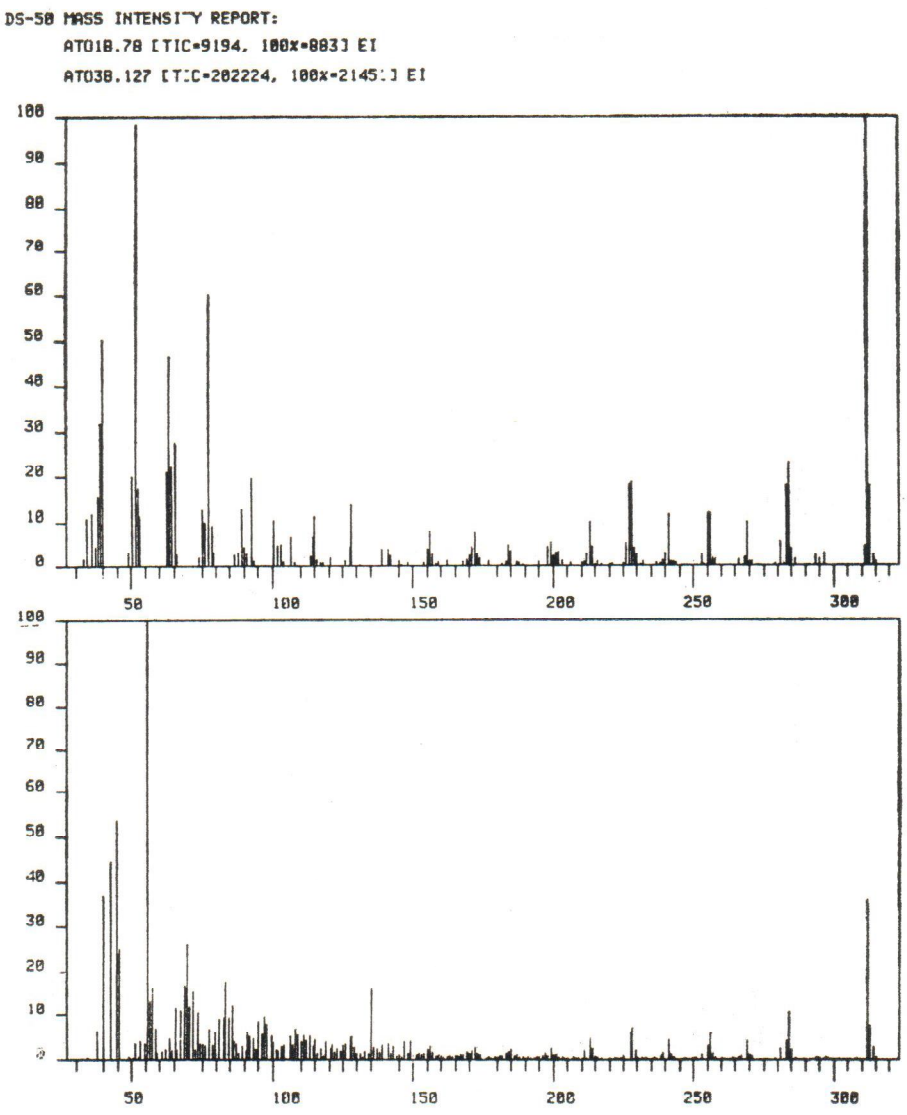

Fig. 5. *Mass spectra of aflatoxin B_1 — standard (above) and aflatoxin B_1 obtained through biosynthesis (below).*

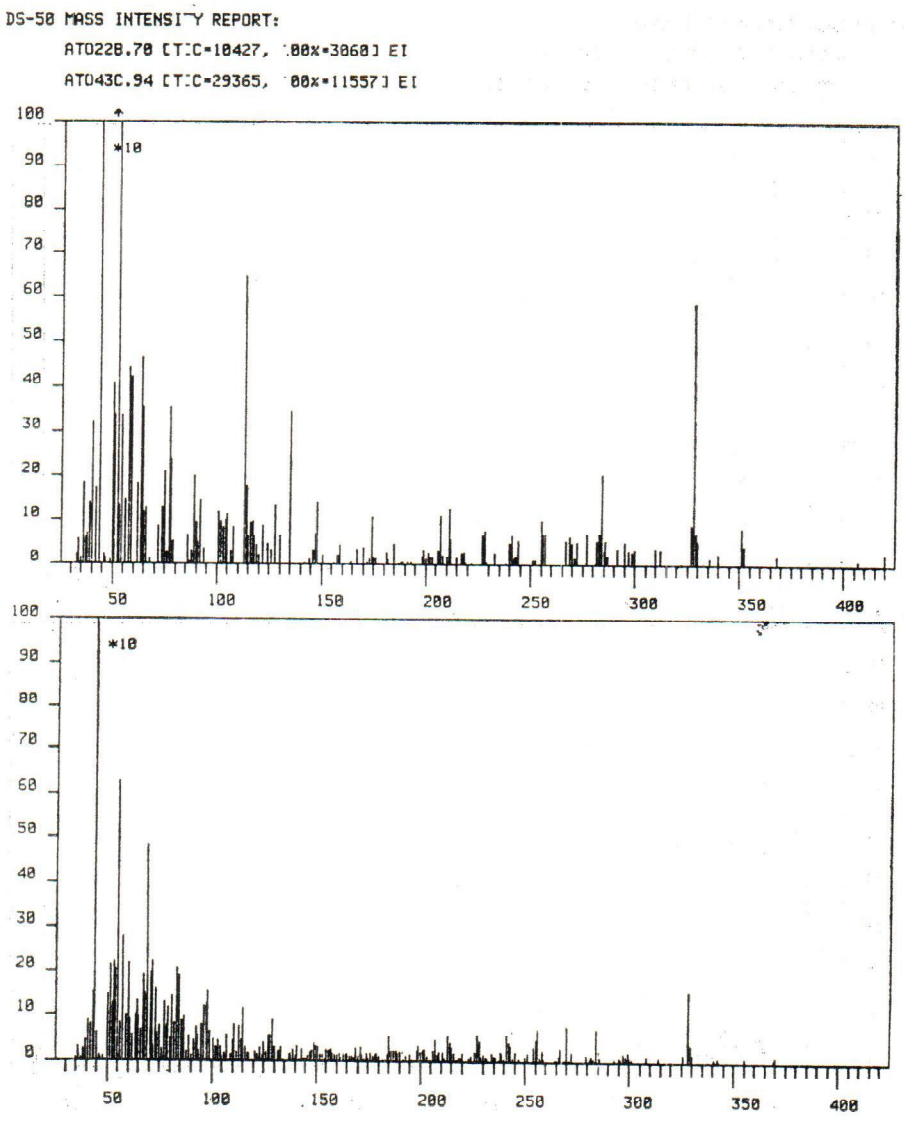

Fig. 6. *Mass spectra of aflatoxin G_1 — standard (above) and aflatoxin G_1 obtained through biosynthesis (below).*

Table 1.

Decrease of concentration of aflatoxins B_1 and G_1 during growth of the mould A. parasiticus NRRL 2999 in pure culture after seven weeks of cultivation with respect to incubation temperature, initial water content in the substrate, and the substrate itself.

Temperature (°C)	Decrease of aflatoxin concentration after seven weeks (per cent)				Initial water content
	B_1		G_1		
	Whole grain	Crushed grain	Whole grain	Crushed grain	
20	0	0	0	0	
28	—15	—16	—17	—20	20%
35	— 5	—10	—10	0	
20	0	— 5	— 5	—15	
28	—11	—15	—13	—25	28%
35	—17	—25	—19	—29	
20	0	—10	— 8	—20	
28	—13	—20	—15	—28	38%
35	—20	—23	—25	—35	

0 — synthesis of aflatoxin has not been proved

Table 2.

Decrease of concentration of aflatoxins B_1 and G_1 during growth of the moulds A. parasiticus NRRL 2999 and T. roseum ZMTF 1226 in the mixed culture after seven weeks of cultivation, with respect to incubation temperature, initial water content in the substrate, and the substrate itself.

Temperature (°C)	Decrease of aflatoxin concentration after seven weeks (per cent)				Initial water content
	B_1		G_1		
	Whole grain	Crushed grain	Whole grain	Crushed grain	
20	0	0	0	0	
28	0	20	0	30	20%
35	0	0	0	0	
20	0	0	0	0	
28	25	30	30	36	28%
35	27	35	0	0	
20	0	0	0	0	
28	25	35	35	40	38%
35	28	38	0	0	

0 — synthesis of aflatoxin has not been proved

was established only in the pure culture of *A. parasiticus*, and the greatest amounts obtained were 12.0 μg B_1/g mycelium dry wt. and 20.0 μg G_1/g mycelium dry wt. (Fig. 2).

As expected, the temperature of 28 °C was more favourable for both the growth of the toxigenous mould and the aflatoxin synthesis. In experiments with pure culture of *A. parasiticus* on crushed maize grain, 35.5 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: 147.0 μg B_1/g mycelium dry wt. and 47.0 μg G_1/g mycelium dry wt. (Fig. 2).

Under equal conditions of growth, the biomass content of the mixed mould culture was slightly higher (44.5 mg/g substrate), but the concentration of both aflatoxins was lessened. The highest values obtained were 98.5 μg B_1/g mycelium dry wt. and 26.5 μg G_1/g mycelium dry wt. (Fig. 2).

The temperature of 35 °C was shown to promote the growth of biomass, but not of the aflatoxin synthesis. Growing *A. parasiticus* in pure culture at the above temperature resulted in a somewhat greater amount of biomass (42.0 mg/g substrate), compared with growth at 28 °C. The concentration of aflatoxins was considerably lower: 54.0 μg B_1/g mycelium dry wt. and 12.5 μg G_1/g mycelium dry wt. (Fig. 2).

In the mixed culture there was an increase of biomass content of about 10% (46.0 mg/g substrate), as compared with growth at 28 °C, and no synthesis of aflatoxin G_1 could be detected. The highest concentration of aflatoxin B_1 was 35.0 μg/g mycelium dry wt., i.e. about 35 % lower than in the pure culture of *A. parasiticus* (Fig. 2).

Comparing the amounts synthesized at various cultivation temperatures it appears that not only the amount of the two toxins, but also their ratios depend on the temperature, e. g. at 20 °C a greater amount of aflatoxin G_1 is synthesized (ratio $B_1 : G_1 = 1 : 1.5$). At 28 °C the amount of B_1 exceeds by far that of G_1 (ratio $B_1 : G_1 = 3 : 1$), whereas at 35 °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 seven weeks 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. parasiticus* NRRL 2999 growing in pure culture reduces the concentration of aflatoxin B_1 by 5 — 25%, and that of G_1 by 5 — 35%. These findings suggest the ability of the toxigenous mould to partly metabolize aflatoxins in a certain period of growth, and/or to modify them into compounds with differing chemical characteristics.

Under equal conditions of cultivation the mixed culture biomass decreases the concentration of aflatoxin B_1 by 20 — 38%, and that of G_1

by 30—40%. The data on figures represent values, standing in good accordance with findings of *Masimango and co-workers* (14) and *Gintero-va and co-workers* (15), who have shown that the simultaneous growth of toxicogenous and non-toxicogenous moulds in mixed culture resulted in a decrease of aflatoxin concentration of up to as much as 75%.

Figures 5 and 6 represent mass spectra of reference standards of aflatoxins B_1 and G_1, as well as of the same toxins isolated from the substrates. The relative intensities (on the ordinate) are plotted versus the m/e — ratio (on the abscisa).

Characteristic molecular peaks are seen: at m/e — ratio 312 for aflatoxin B_1; at m/e — ratio 328 for aflatoxin G_1.

Higher peaks were recorded with aflatoxin standards, because their concentration was substantially higher than the concentration of aflatoxins in mould extracts.

Tables 1 and 2 show the decrease of concentration of both aflatoxins in the substrate after seven weeks 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 aflatoxin synthesized. Hence it appears that at the same cultivation temperature a significant decrease of concentration of both investigated toxins occurs in the substrate with a higher initial water content.

The relatively infrequent occurrence 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 the continental parts of Croatia ranges from 20 to 24 °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, although 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.

CONCLUSION

The application of the chitin-method for the determination of mould biomass on maize showed that it was a suitable procedure for rapid and precise estimation of the extent of mould contamination of this commodity.

The mere growth of an aflatoxicogenous fungus on maize should not be taken as proof that aflatoxins are present in the substrate.

These toxins are elaborated under strictly defined conditions. Water content and temperature appear to be the most important parameters.

It was shown that more aflatoxins B_1 and G_1 were accumulated in the substrate on which only *A. parasiticus NRRL 2999* was cultivated, than if, under equal conditions, the same mould had been grown in mixed culture with the mould not synthesizing aflatoxin.

The biomass of the mixed culture of *A. parasiticus* and *T. roseum* was more capable of eliminating aflatoxins B_1 and G_1 from the substrate than the biomass of the same strain of *A. parasiticus* in pure culture.

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Duraković, S. et al.: The relationship between *Trichothecium roseum* and *Aspergillus parasiticus* and the Production of Aflatoxins. Arh. hig. rada toksikol., Vol. 37 (1986) No. 1, pp. 3—17.

Sažetak

ODNOS IZMEĐU PLIJESNI *TRICHOTHECIUM ROSEUM* I *ASPERGILLUS PARASITICUS* I BIOSINTEZA AFLATOKSINA

Istražen je kvantitativan odnos biomase plijesni i aflatoksina B_1 i G_1 na čvrstim supstratima (cijelo i lomljeno zrno kukuruza) pri temperaturi od 15—35 °C i sadržaju vode u supstratu od 20—38%. Pokusi su provedeni s pomoću aflatoksikogene plijesni *Aspergillus parasiticus* NRRL 2999 u čistoj kulturi i u mješovitoj kulturi s plijesni *Trichothecium roseum* ZMTF 1226. U preliminarnim istraživanjima dokazano je da plijesan *T. roseum* ZMTF 1226 ne sintetizira aflatoksine, niti kromatografski slične spojeve.

Rast biomase praćen je mjerenjem sadržaja hitina, a koncentracija aflatoksina određivana je tankoslojnom kromatografijom pomoću fluorodenzitometra. Utvrđeno je da sinteza istraživanih toksina i njihov međusobni odnos prvenstveno ovise, ne o količini biomase plijesni, nego o temperaturi uzgoja. Biomasa mješovite kulture plijesni *A. parasiticus* i *T. roseum* reducira, nakon sedam tjedana uzgoja, količinu aflatoksina B_1 za 20—38%, a aflatoksina G_1 za 30—40%. Smanjenje količine oba istraživana toksina izrazitije je u supsratu s većom početnom količinom vode i pri višoj temperaturi uzgoja.

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Primljeno 21. VIII 1985.