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Full Length Research Paper

The effect of fungal competition on colonization of soybeans by moulds of the genus *Aspergillus*, *Trichothecium* and *Fusarium* and the ochratoxin A accumulation as related to temperature and moisture content

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The relationship between mould biomass and accumulation of ochratoxin A (OTA) on solid substrate (slightly cracked soybeans) at temperatures from 15 to 35°C and a water content in the substrate of 20 to 38% was been investigated. The experiments were carried out with the mould *Aspergillus ochraceus* NRRL 3174 in pure culture and in mixed culture, respectively; the latter with the moulds *Trichothecium roseum* ZMPBF 1226 and *Fusarium graminearum* ZMPBF 1244. The biomass growth during cultivation was monitored by measuring the analysis of chitin as glucosamine, and the concentration of OTA was measured by high performance liquid chromatography (HPLC) method using Hewlett-Packard instrument with fluorescence detector. It was established that the accumulation of the examined OTA primarily depend on the temperature of cultivation rather than on the growth of the biomass. The biomass of the mixed culture of *A. ochraceus*, *T. roseum* and *F. graminearum* after 35 days of cultivation reduced the amount of OTA by 48, 72 and 85%, respectively. The decrease of concentration of OTA is more pronounced in the substrate with a higher initial water content and at higher temperature of cultivation.

Key words: Moulds, mycotoxins, ochratoxin A, chitin, HPLC.

INTRODUCTION

Unavoidable, natural contaminants in foods may have either chemical or biological origin. Mycotoxins, toxic secondary metabolites of fungi, are of biological origin. Despite efforts to control fungal contamination, toxigenic fungi are ubiquitous in nature and occur regularly in worldwide food supplies due to mould infestation of susceptible agricultural products, such as cereal grains, nuts and fruits. The great toxicity of mycotoxins arose in a world-wide scale in 1960, when it was unambigously proved that the epizootic outbreaks of Turkey "X" disease had been caused by toxic groundnut meal, wherein occasionally fragments of mould hyphae had been detected. The mould was identified as *Aspergillus flavus* Link ex Fries, and the name for the new toxic principle was coined from the initials of the producing species "aflatoxin" (Blount, 1961). Thousands of mycotoxins exist, but only a few present significant food safety challenges. The natural fungal flora associated with foods is dominated by three genera – *Aspergillus*, *Fusarium*, and *Penicillium*, except for the *Fusarium* plant pathogens, which may include commensals as well as pathogens.

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When present in foods in sufficiently high levels, mycotoxins can have toxic effects that range from acute to chronic, and result in symptoms range from skin irritation to immunosuppression, birth defects, neurotoxicity and death (International Commission on Microbiological Specifications for Foods (ICMSF), 1996). Humans may be exposed to mycotoxins in two ways: by direct consumption of residue-containing milk or meat from animals which have ingested mycotoxin-contaminated feed. Human exposure is more likely in countries where fermented foods are an important part of the diet or where good sanitary standards are lacking (Duraković et al., 2011; Surekha et al., 2011). The ochratoxins encompass a group of closely related secondary metabolites of the fungal genera *Aspergillus* and *Penicillium*. The ochratoxins were first discovered in fungal cultures of *Aspergillus ochraceus* and were the first major group of fungal toxins to be characterized after the discovery of aflatoxins (van der Merwe et al., 1965). Subsequent studies revealed procedures of ochratoxin in 7 species of *Aspergillus* and 6 species of *Penicillium* (Harwing, 1974). According to the findings of numerous investigators, ochratoxins were found in nearly all mould-contaminated substrates (Ciegler et al., 1971). Of the toxins in this group, ochratoxin A (OTA) is the most toxic and the most abundantly produced, and is found widespread as a natural contaminant (van der Merwe et al., 1965; Harwing, 1974; Yazdani et al., 2010). Chemically, OTA is an amide formed from phenylalanine and a substituted chloroisocoumarin. The structures of ochratoxins A, B and C (OTA, OTB, and OTC) are presented in Figure 1.

Among several derivatives, OTA is the major and most toxic metabolite (Buchanan et al., 1981). It is structurally characterized as 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-R-methylisocoumarin linked with carboxy group to L-β-phenylalanine (Remiro et al., 2010). Both OTA and OTB (dechloroanalog of OTA) are found as natural contaminants in food, feed and their products (Scott et al., 1972). OTA has been listed as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC, 1999). It causes renal toxicity, nephropathy, and immunosuppression in several animal species, resulting in reduced performance parameters in animal production (Binder, 2007). OTA has also been detected in blood and other animal tissues and in milk, and has been implicated in the fatal human disease Balkan endemic nephropathy (Marquardt and Frolich, 1992). The fungal producers of ochratoxins are widely distributed in soil and decaying organic matter. Producing fungi have been associated with moldy feeds and have been isolated from numerous commodities (Scott et al., 1972; Krogh, 1978; Blažinkov et al., 2008; Duraković et al., 2008). The OTA was detected in cereals from the endemic nephropathy region of South-east Europe and in plant and animal products in several countries in different parts of the world. This information requires that OTA has to be given high priority for further investigations (Krogh, 1978; Binder, 2007).

Considering the fact that on natural substrates mixed mould cultures grow rather than pure ones, the problem of ochratoxin biosynthesis and accumulation during the growth of mixed mould cultures, as well as the possibility of their detoxication, becomes an especially significant one. Ochratoxin degradation by means of biological methods was the subject of numerous investigations (Mngadi et al., 2008; Yazdani et al., 2010). The ability of some fungal species to degrade ochratoxins was described by Varga et al. (2005), whereas Duraković et al., 2008 studied the influence of mould growth in mixed cultures on the biosynthesis of aflatoxins. Upon the results obtained, they inferred that aflatoxigenous moulds, growing in mixed cultures with aflatoxin-negative moulds, exhibit a lower capability of accumulation of aflatoxin in the culture media, than in growing in pure culture.

With respect to significance and because of the complexity of the problem of ochratoxin biosynthesis and biodegradation during mould growth in mixed cultures, in the present work we examined the conditions that may arise during the storage of soybeans. We were interested in finding out at which time and to what extent under chosen conditions of growth, a degradation of OTA takes place, when the mould *A. ochraceus* NRRL 3174 grows on soybeans in pure culture and in mixed culture, respectively, together with the moulds most frequently encountered as contaminants on soybean, but do not synthesizing ochratoxins themselves.

MATERIALS AND METHODS

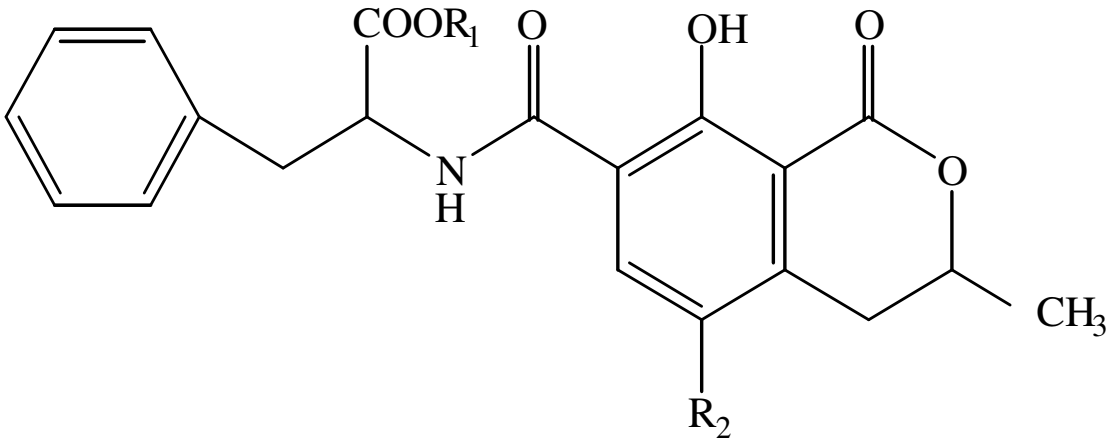
Microorganisms

The two moulds which most frequently occur on soybeans as natural contaminants were identified as *Trichothecium roseum* and *Fusarium graminearum*, and taken up into the Collection of Microorganisms of the Faculty of Food Technology and Biotechnology in Zagreb (Republic of Croatia), (ZMPBF) under serial Nos. 1226 and 1244. Isolation and identification of these fungal species were done on a medium low in nutrients as recommended by Gerlach (1981). Isolated moulds do not produce ochratoxins and chromatographically similar chemical compounds.

The mould *A. ochraceus* NRRL 3174, described as one of most potential ochratoxin producers, was used as a test organism. The mould was obtained from the USDA Fermentation Laboratory of the Northern Regional Research Center, Peoria, Illinois. The cultures were maintained on slants of Potato Dextrose Agar (PDA) stored at 4°C. Before each experiment, the organism was transferred to another PDA slant and incubated at 25°C for 7 days.

Isolation and determination of fungi

Twelve samples of completely commercially available soybeans were analyzed for determining fungal growth as a natural mycoflora. 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 for 7 and 14



The ochratoxins

	R_1	R_2
Ochratoxin A	**H**	**Cl**
Ochratoxin B	**H**	**H**
Ochratoxin C	**$-CH_2-CH_3$**	**Cl**

Figure 1. Structures of ochratoxins A, B, and C (Remiro et al., 2010).

days, respectively. The prevalent moulds that developed were then enumerated and sub-cultured on Sabouraud dextrose agar (Oxoid, New Hampshire, USA) for isolation of pure cultures. Pure cultures were identified using the stereoscopic microscope and fungal identification keys for *Aspergillus* (Klich and Pitt, 1988), *Fusarium* spp. (Nelson et al., 1983) and other common fungi (Beneke and Stevenson, 1987; Mngadi et al., 2008).

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 were 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 (ca 10^8 spores/ml) was carried out according to the method described by Lai et al. (1970). The inoculum was used at the rate of 1 ml/flask.

Substrate for mould growth and OTA accumulation

Slightly cracked soybeans were used as the medium for growth of investigated moulds and OTA accumulation. This cracking was to allow the mould spores to infect the kernel readily. Parameters of the cultivation were as follows: initial water content in the substrate: 20, 28 and 38%; initial number of conidia: 2×10^6 per gram of substrate; temperature of incubation: 15, 20, 28 and 35°C; cultivation time: 35 days.

The biosynthesis of OTA was performed with the mould *A. ochraceus* in pure culture, as well as with the three moulds *A. ochraceus*, *T. roseum,* and *F. graminearum*. In experiments with the pure culture of *A. ochraceus*, substrates were seeded with 2×10^6 spores per gram of each, whereas in experiments with the mixed culture the inoculation was carried out with 2×10^6 spores of each of investigated moulds per gram of the substrate. The cultivation was performed in a stationary culture with 50 g of substrate in 300 ml Erlenmeyer flasks.

Determination of biomass

At 7 days intervals during cultivation in the incubator, samples were taken for the determination of biomass content and for assay of OTA. The substrates were autoclaved at 121°C for 30 min before analysis to kill the spores and vegetative mycelia. The growth of the fungi was monitored by using the analysis of chitin measured as glucosamine, as a criterion (Donald and Mirocha, 1977; 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 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 hydrolyzed to glucosamine, deaminated to its corresponding aldehyde and measured spectrophotometrically. The chitin content is estimated from the standard curve of glucosamine-HCl read at 650 nm.

For determining 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).

Xiao-E et al., (2008) found the chitin content in healthy soybean seed to be 135 to 155 µg per gram of dry weight. The same authors determined the chitin content in the mycelium of *A. ochraceus* NRRL 3174 at 230 mg per gram of dry weight. At the same time, it was established that the mycelium of *T. roseum*

ZMPBF 1226 contained 260 mg of chitin per gram of dry weight, and mycelium of *F. graminearum* ZMPBF 1244 contained 185 mg chitin per gram of dry weight.

Detection and measuring of OTA

From all samples, OTA was extracted by shaking with 100 ml of chloroform in a laboratory shaker (50 cycles/min) for 1 h at room temperature. This procedure was repeated twice and the pooled extracts were dried over anhydrous sodium sulphate and concentrated in a rotary evaporator (50°C, 1.33 kPa) to a volume of approximately 5 ml. The extracts were purified by column chromatography using the method described by Nesheim (1980) and Abarca . (1994).

The detection and accumulation of OTA was determined by thin layer chromatography (TLC) and by high performance liquid chromatography (HPLC). Extracts were analyzed for OTA by TLC using silica gel-precoated plates (Merck, Darmstadt, F_{254}, 20 × 20 cm) and toluene / ethyl acetate / 90% formic acid (6:3:1 v/v/v) for development as described by Abarca . (1994), where after they were examined by comparing the Rf values of a known standard of OTA to the unknown samples under UV light. OTA primary standard to check the linearity was examined by Sigma-Aldrich, Germany. Working standard solution of 0.25 and 0.125 ng/ml of OTA were prepared by diluting standard solution with chloroform (1:1 v/v). Confirmation of TLC results was performed by HPLC analysis and was accomplished using a Hewlett-Packard Liquid Chromatography (pump and injection system), (Walborn, Germany) with JASCO FP-920 Fluorescence detector (Co. Ltd, Japan) and HP integrator 3395. The HPLC column was C_{18} Nova-Pak (4.6 x 250 mm) with particle size of 5 µM (Waters, Millipore, Milford, MA). Detection of OTA was carried out at $lambda_{ex}$ 330 nm and $lambda_{em}$ 460 nm from 21 to 25 min. The flow rate was 1 ml/min. for each mobile phase and the injected volume of working standard was 50 µL (AOAC International, 2005; Yazdani et al., 2010). OTA was quantified based on HPLC fluorometric response compared with that of an OTA standard (Abarca., 1994).

Determination of accumulation index

The accumulation index, which is the µg of OTA accumulated per g dry weight of mycelium per day, was calculated according to the formula (Marquardt and Frolich, 1992)

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

Mass spectroscopy

For the confirmation of structure, mass spectra of OTA were scanned, as well as mass spectra of the same toxin isolated from mould extracts. In this work, we used a “Shimadzu” EC-MS-QP 1000 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°C; scanning rate, 3 s/decade.

Figure 2 represents the mass spectra of the reference standard of OTA (upper spectrum), as well as of the same toxin isolated from soybeans contaminated with *A. ochraceus* NRRL 3174, *T. roseum* ZMPBF 1226 and *F. graminearum* ZMPBF 1244 (lower spectrum). The relative intensities (on the ordinate) were plotted versus the m/e ratio (on the abscissa). The spectra showed a weak molecular ion with m/e 403 (5%) as compared with the base peak m/e 57 (100%). The presence of chlorine in the molecule was evident from a number of ion pairs such as m/e 403 and 405, 239 and 241, and 213 and 125 and 127, having a ratio approximately 3:1 (chlorine isotopes 35 and 37). A dominant ion of mass 239 was apparently formed by a McLafferty rearrangement (McLafferty, 1973).

Statistical analysis

Repeatability and recovery were determined by spiking 50 g of soybeans with OTA standard solution at levels of 100, 500 and 1000 ng/g prior to addition of solvent and extraction. After 1 h at room temperature, OTA was extracted from spiked samples and quantified according to the protocol of Gilbert (2002). Mean recovery rates of duplicate experiments out of soybeans were between 81.8 and 95.6%, standard deviation (n = 5) between 6.5 and 16.3%, respectively (Table 1). There is no significant difference in the mean percent recoveries based on F-test for the analysis of variance of unequal sample size. The observed F-values at 0.1, 0.5 and 1.0 ppb are 0.26, 0.96 and 0.55, respectively, (F (0.95) = 3.35). Results of toxin investigated were not corrected for recovery.

RESULTS AND DISCUSSION

In experiments at 15°C, only slight growth of *A. ochraceus* was observed at this temperature and no accumulation of OTA was observed. Figures 3a to 5b represent the relation of mould biomass to the synthesized OTA during the growth of *A. ochraceus* in pure culture and the growth of *A. ochraceus*, *T. roseum* and *F. graminearum* in mixed culture at incubation temperature of 20, 28 and 35°C, respectively. According to Davis et al. (1972) and Escher et al. (1973), the minimal temperature for growth of moulds from these genera is 15 to 20°C and optimal temperature for growth of *A. ochraceus* and biosynthesis of ochratoxins is 25 to 27°C.

The initial water content in the substrate was 20, 28 and 38%, respectively. Lopez and Christensen (1967) stated 15.5% as the minimal water content for the growth of *A. ochraceus*. Diener and Davis (1968) and Hult et al. (1982) have demonstrated that a water content of 32 to 38% was optimal for the biosynthesis of ochratoxins on solid substrates. Out of three chosen initial water content in the substrate, the last one (38%) gave highest increments of biomass and best OTA 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. ochraceus* was 24.0 mg biomass (mycelium) dry weight/g substrate. The amount of OTA was low, about 15.49 µg OTA/g biomass (mycelium) dry weight. Maximum concentration was established to be 21 days after the inoculation (Figure 3a). In the mixed culture, the greatest amount of biomass was 29.0 mg biomass dry weight/g substrate and no accumulation of OTA in the first 7 days of cultivation. The accumulation of OTA was established after 14 days of cultivation and maximum accumulation was established after 21 days of cultivation (8.13 µg OTA/g biomass dry weight), (Figure 3b).

The obtained values were in good accordance with thefindings of Buchanan et al. (1981) and Duraković et al. (2008) who showed that the growth of toxigenous *Aspergillus* spp. on solid substrates at 20°C is very low. As expected, the temperature of 28°C was more favorable for both the growth of toxigenous mould and OTA accumulation. In experiments with the pure culture

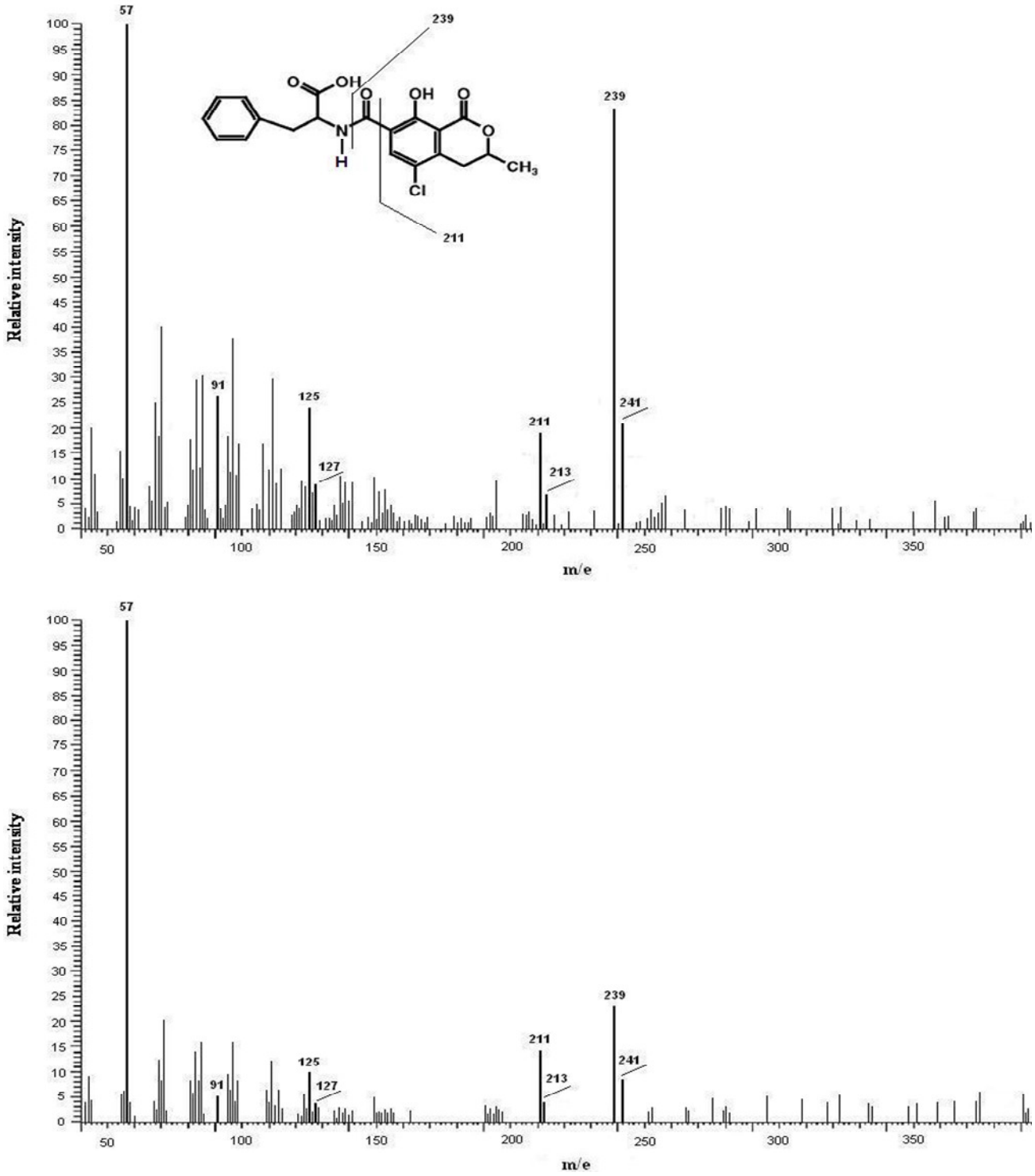


Figure 2. Mass spectra of 85 ng reference OTA; molecular weight = 403 (upper spectrum), and OTA isolated from soybeans contaminated with *A. ochraceus* NRRL 3174, *T. roseum* ZMPBF 1226 and *F. graminearum* ZMPBF 1244 at a measured level of 40 ng (lower spectrum). The formula is inserted, showing the fragments m/e 211 and 239, respectively. Fragments with intensity less than 2% were omitted.

of A. *ochraceus,* 41.50 mg mycelium 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 OTA was also established. At the time of maximum accumulation, it amounted to 53.70 µg OTA/g mycelium dry weight (Figure 4a). Under equal

Table 1. Percent OTA recoveries from spiked soybean samples.

Level spiked (ng/g)	Recoveries (%)	Average recoveries (%) ± SD
100	90 72 77 89 81	81.8 ± 7.7
500	91 89 87 84 101	90.4 ± 6.5
1000	70 100 94 99 115	95.6 ± 16.3

SD = standard deviation.

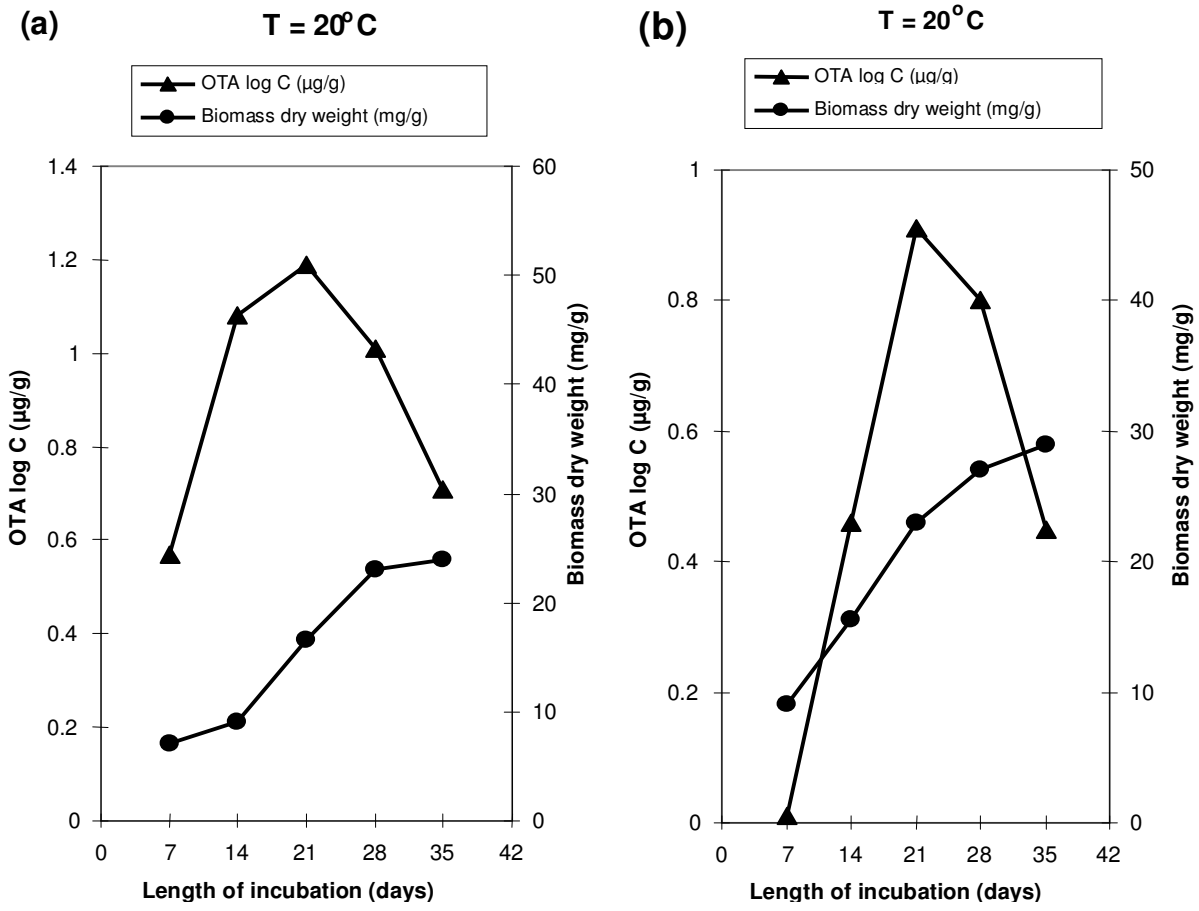


Figure 3. Ochratoxin A accumulation during the growth of *A. ochraceus* NRRL 3174 on soybeans in pure culture (a) and in mixed culture (b) at 20°C.

conditions of growth, the biomass content in the mixed culture was 50% higher (57.0 mg biomass dry weight/g of substrate) as compared with the pure culture, but the concentration of OTA was reduced. The highest values obtained after 21 days were 24.0 µg OTA/g biomass dry weight (Figure 4b). These values are in good accordance with the findings of Kuiper-Goodman and Scott (1989) who have stated 28 °C as the optimal temperature for growth of *A. ochraceus* NRRL 3174 and accumulation of OTA.

Furthermore, the temperature of 35°C was shown to promote of the growth of biomass, however, not of the OTA accumulation. The growth of *A. ochraceus* in pure culture at the above temperature resulted in somewhat greater amount of biomass (44.50 mg biomass dry weight/g of substrate) compared with the one at 28°C. The concentration of OTA was considerably lower (Figure 5a) 19.95 µg OTA/g biomass dry weight. In the mixed culture, there was an increase of biomass content to about 20% (49.50 mg mycelium dry weight/g of substrate) as compared with the growth at 28°C, and no synthesis of OTA was detected after first 7 days of cultivation. After 21 days of cultivation concentration of OTA was lower about 50% (9.55 µg OTA/g mycelium dry weight) in respect to values obtained at 28°C (Figure 5b). *A. ochraceus* is frequently found to be associated with

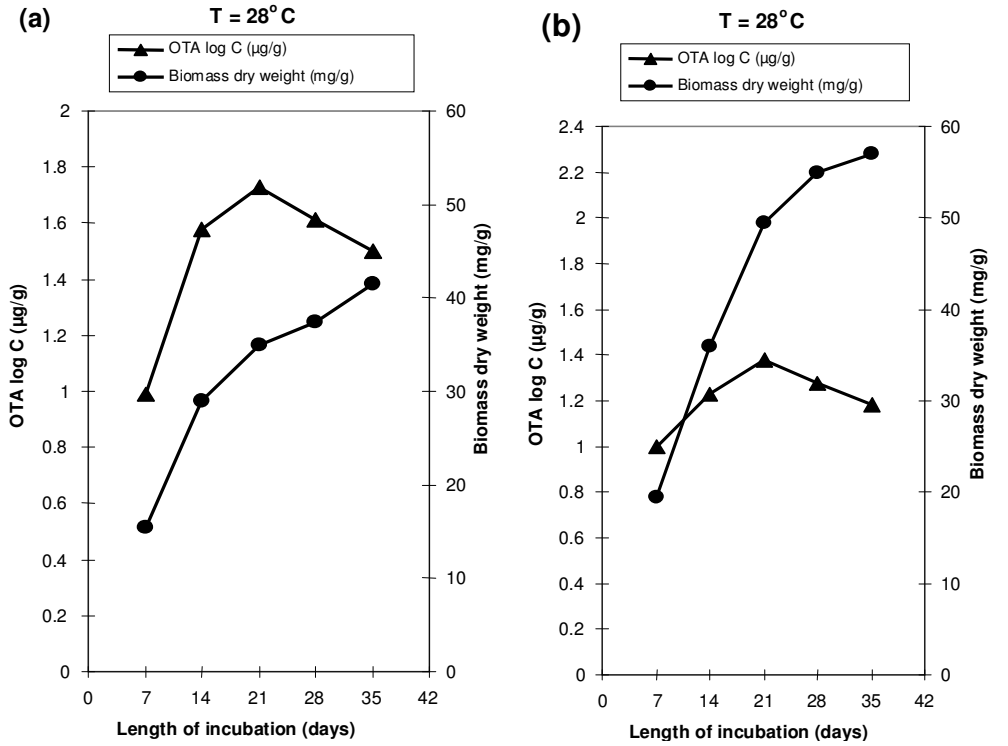


Figure 4. Ochratoxin A accumulation during the growth of *A. ochraceus* NRRL 3174 on soybeans in pure culture (a) and in mixed culture (b) at 28°C.

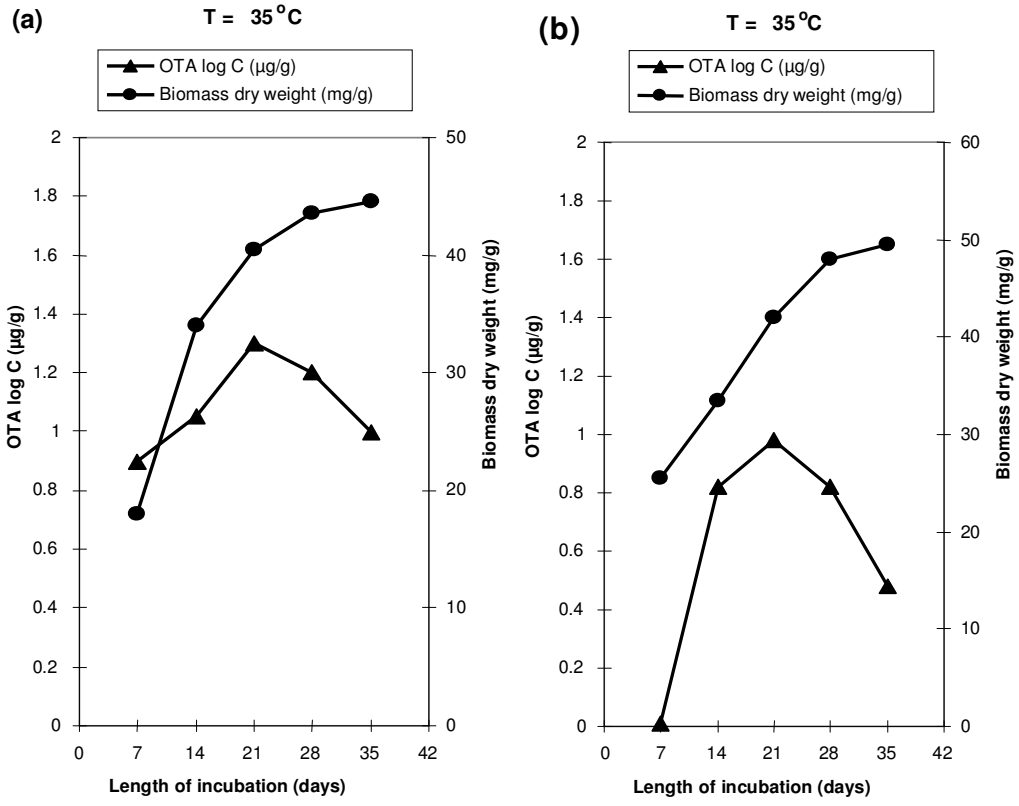


Figure 5. Ochratoxin A accumulation during the growth of *A. ochraceus* NRRL 3174 on soybeans in pure culture (a) and in mixed culture (b) at 35°C.

Table 2. Comparative representation of decrease of OTA accumulation during the growth of *A. ochraceus* NRRL 3174 in pure and in mixed culture with *T. roseum* ZMPBF 1226 and *F. graminearum* ZMPBF 1244 on soybeans with respect to incubation temperature and initial water content in the substrate.

Decrease of ochratoxin A accumulation after 35 days			
Temperature (°C)	**Pure culture (%)**	**Mixed culture[a] (%)**	**Initial water content (%)**
20	-66	-48	-
28	-41	-72	38
35	-50	-85	-
20	-36	Ø	
28	-24	-42	28
35	-32	-48	-
20	Ø	Ø	-
28	-15	Ø	20
35	Ø	Ø	-

[a] = in respect to highest values obtained with the pure culture of *A. ochraceous*; Ø = accumulation of OTA has not been proven.

numerous other microorganisms in stored grains and seed. Thus, the possibility arise that microbial competition between fungi for the substrate under favorable environmental conditions will restrict or reduce the amount of ochratoxin formed.

A. ochraceus or competing fungi might absorb or degrade ochratoxin following its formation in the substrate. Ashworth et al. (1965), Schroeder and Ashworth (1965) and Duraković et al. (2008) 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. Magan and Aldred (2007), Duraković et al. (2008) and Mngadi et al. (2008) noted that a *Penicillium* sp. reduced aflatoxin and ochratoxin yields when grown in cultural competition with *A. flavus* and *A. ochraceus*. Whether this was accomplished by competition for available substrate or by destruction of mycotoxin, was not demonstrated. Regarding OTA, several reports describe OTA degrading activities of microbial flora (Galtier and Alvinerie, 1976; Hult et al., 1976; Xiao et al., 1991; Madhyastha et al., 1992). Degradation of OTA was observed in the milk due to the action of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* species (Škrinjar et al., 1996), while two other bacteria *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994) and *Phenylobacterium immobile* (West and Lingens, 1983) could also convert OTA to the much less toxic ochratoxin α (OTα) in liquid cultures.

The ability of some fungal species to degrade mycotoxins was the subject of numerous investigations. Thus, Duraković et al. (2008) studied the influence of moulds growth in mixed culture on the biosynthesis of aflatoxin. 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. Furthermore, recent reports describe that the OTA is degrading activities of some *Aspergillus*, *Rhizopus,* and *Pleurotus* isolates and their enzymes (Stander et al., 2000; Varga et al., 2005; Varga and Kozakiewicz, 2006). We observed that OTA is also efficiently detoxified by moulds *Trichothecium roseum* and *Fusarium graminearum* isolated from moldy soybeans. Some authors have suggested that mycotoxin accumulation can be stimulated by fungal competition as a competing strategy (Birzele et al., 2000). Different studies, however, have not been able to confirm this point (Reid et al., 1999), or support this hypothesis (Surekha et al., 2011).

Table 2 comparatively shows the decrease of OTA concentration in the substrate after 35 days of growth of *A. ochraceus* in pure and in mixed culture with the two mentioned moulds at all parameters of cultivation. The values were calculated based on the highest amounts of OTA accumulated. Hence, it appears that at the same cultivation temperature, a more considerable decrease of OTA concentration occurs in the substrate with a higher initial water content. As shown in Figure 6a to c, the amount of OTA produced by mixed cultures is much less than in the pure culture of *A. ochraceus*. Since *T. roseum* and *F. graminearum* were isolated as a naturally occurring mycoflora on soybeans, it is reasonable to expect that such mixed mycoflora should be found in fields and storehouses. Although these moulds under equal conditions did not produce either OTA or chromategraphically similar compounds, little is known about other possible metabolites that might cause stimulation or decrease in OTA accumulation.

The results reveal that depending on parameters of

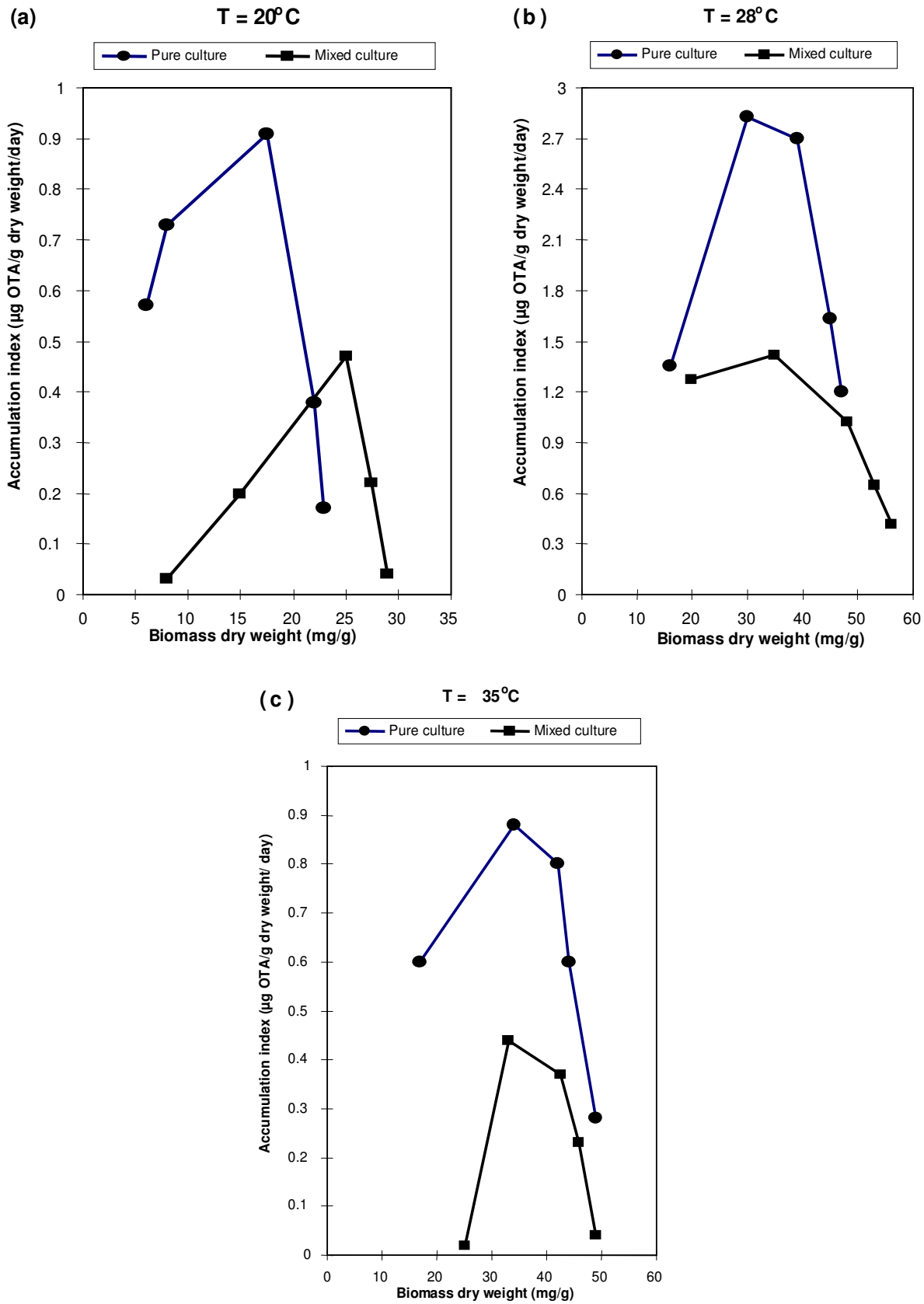


Figure 6. Ochratoxin A accumulation index curves of *A. ochraceus* NRRL 3174 in pure and in mixed culture at: (a) 20°C; (b) 28°C; (c) 35°C during 35 days of cultivation.

cultivation growth, the biomass of *A. ochraceus* NRRL 3174 growing in pure culture reduces the concentration of OTA by 66, 41, and 50% (Figures 3a to 5b). These findings suggest the ability of the toxigenous mould to partly metabolize OTA in a certain period of growth, and/or modify this toxin into compounds with differing chemical characteristics. Under equal conditions of cultivation, the mixed culture biomass decreases the concentration of OTA by 48, 72 and 85% in respect to temperature of cultivation and a water content in the substrate (Figures 3a to 5b). We observed that OTA accumulation was also efficiently reduced by isolates of *T. roseum* and *F. graminearum* isolated from moldy soybeans.

The data illustrations represent values standing in good accordance with the findings of Varga et al. (2005) and Duraković et al. (2008), who have shown that the simultaneous growth of toxigenous and non-toxigenous moulds in mixed culture resulted in a decrease of mycotoxin concentration of up to as much as 85%. In the further studies, we concentrated on investigations of OTA degrading activities of *Trichothecium* and *Fusarium* isolates.

Conclusions

The determination of mould biomass on solid substrates by the “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 ochratoxin synthesized. Thus, for example at 35°C, more mycelium grew than at 28°C, but the amount of the produced ochratoxin A was considerably smaller. It was shown that more OTA was accumulated in the substrate on which only *A. ochraceus* NRRL 3174 was cultivated, than if, under equal conditions, the same mould was grown in mixed culture with two other ochratoxin-negative moulds. The biomass of the mixed culture of *A. ochraceus*, *T. roseum* and *F. graminearum* was more capable of eliminating OTA from the substrate than the biomass of the same strain of *A. ochraceus* in pure culture. Therefore, the natural mycoflora may act as an additional control factor, as long as they do not produce any other mycotoxins.

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Abbreviations:

OTA, Ochratoxin **A; NRRL,** Northern Regional Research Laboratory, Peoria, Illinois (USA); ZMPBF, Collection of Microorganisms of the Faculty of Food Technology and Biotechnology in Zagreb (Republic of Croatia); HPLC, high performance liquid chromatography; **OTB,** ochratoxin **B; OTC,** ochratoxin **C; PDA,** potato dextrose agar; **TLC,** thin layer chromatography**; OTα**, ochratoxin α.

REFERENCES

Abarca ML (1994). Ochratoxin A Production by Strains of *Aspergillus niger* var. *niger*. Appl. Environ. Microbiol. 60(7): 2650-2652.

AOAC International, Association of Official Analytical Chemists (2005). Official Methods of Analysis of AOAC International. In: Horwitz W (Ed.), 18th edition, Arlington, Va.

Ashworth LJ, Schroeder HW, Langley BC (1965). Aflatoxins: Environmental factors governing occurrence in Spanish peanuts. Science, 148(3674): 1228-1229.

Beneke ES, Stevenson KE (1987). Classification of food and beverage fungi. In: Beuchat, L.R. (Ed.), Food Beverage Mycology, 2nd edition. Van Nostrand Reinhold, New York, pp. 1-50.

Binder EM (2007). Managing the risk of mycotoxins in modern feed production. Anim. Feed Sci. Technol. 133: 149-166.

Birzele B, Prange A, Kramer J (2000). Deoxynivalenol and ochratoxin A in German wheat and changes of level in relation of storage parameters. Food Addit. Contamin. 17: 1027-1035.

Blažinkov M, Sikora S, Mačešić D, Uher D, Duraković L (2008). The effect of rhizobial inoculation and liming on alfalfa production in Croatia. Cereal Res. Commun. 36(Part 1, Supplement 5): 343-346.

Blount WP (1961). Turkey “X” disease. J. Brit. Turkey Fed. 9: 52-77.

Buchanan RL, Tice G, Marino D (1981). Caffeine inhibition of ochratoxin A production. J. Food Sci. 47: 319-321.

Davis ND, Searcy JW, Diener UL (1972). Medium scale production and purification of ochratoxin A, a metabolite of *Aspergillus ochraceus*. Appl. Microbiol. 26: 27-30.

Diener UL, Davis ND (1968). Limiting temperature and relative humidity for growth and production of aflatoxin and free fatty acids by *Aspergillus flavus* in sterile peanuts. J. Am. Oil Chem. Soc. 44: 259-263.

Donald W, Mirocha CJ (1977). Chitin as a measure of fungal growth in stored corn and soybean seed. Cereal Chem. 64: 466-474.

Duraković S, Duraković L, Vahčić N, Skelin A, Duraković Z (2008). Affect of moulds growth in mixed cultures on production of aflatoxins on maize hybrid. Cereal Res. Commun. 36(35): 1615-1618.

Duraković L, Blažinkov M, Skelin A, Sikora S, Delaš F, Mrkonjić-Fuka M, Huić-Babić K, Redžepović S (2011). Temperature-dose relationships with aflatoxin M_1 in milk on the brine shrimp (*Artemia salina*) larvae. Mljekarstvo, 61(2): 145-153.

Escher FE, Koehler PE, Ayres JC (1973). Production of ochratoxin A and B on country cured hams. Appl. Microbiol. 26: 27-30.

Galtier P, Alvinerie M (1976). In vitro transformation of ochratoxin A by animal microbial flora. Ann. Rech. Vet. 7: 91-98.

Gerlach W (1981). The present concept of *Fusarium* classification. In: Nelson PE, Toussoun TA, Cook RJ (Eds.). *Fusarium* Diseases, Biology, and Taxonomy. Pennsylvania State University Press, University Park, pp. 413-426.

Gilbert J (2002). Validation of analytical methods for determining mycotoxins in foodstuffs. TrAC, Trends Anal. Chem. 21: 468-486.

Harwing J (1974). Ochratoxins and related metabolites. In: Purchase, I.F.H. (Ed.), Mycotoxins, Vol. III. Elsevier Scientific Publishing Company, Amsterdam, Oxford, New York, pp. 345-367.

Hult K, Teiling A, Gatenbeck S (1976). Degradation of ochratoxin A by a ruminant. Appl. Environ. Microbiol. 32: 443-444.

Hult K, Pleština R, Habazin-Novak V, Radić B, Ceović S (1982). Ochratoxin A in human blood and Balkan endemic nephropathy. Arch. Toxicol. 51: 313-321.

Hwang CA, Draughon FA (1994). Degradation of ochratoxin A by *Acinetobacter calcoaceticus*. J. Food Prot. 57: 410-414.

IARC, International Agency for Research on Cancer (1999). Overall evaluations of carcinogenicity to humans. IARC Monographs, I: 1-36.

ICMSF, International Commission on Microbiological Specifications for

Foods (1996). Toxigenic fungi: *Aspergillus*. In: Roberts TA, Baird-Parker AC, Tompkin, RB (Eds.). Microorganisms in foods: Microbiological specifications of food pathogens. International Commission on Microbiological Specifications for Foods. Blackie Acad. Professional, Chapman Hall, London, UK, pp. 347-381.
Klich MA, Pitt JL (1988). A laboratory guide to common *Aspergillus* species and their teleomorphs. CSIRO Publishers, New South Wales, pp. 65-96.
Krogh P (1978). Causal associations of mycotoxic nephropathy. Acta Pathol. Microbiol. Scand., Sect. A, Suppl. 269: 1-28.
Kuiper-Goodman T, Scott PM (1989). Review: Risk assessment of the mycotoxin ochratoxin A. Biomed. Environ. Sci. 2: 179-248.
Lai M, Semeniuk G, Hesseltine CW (1970). Conditions for production of ochratoxin A by *Aspergillus* species in synthetic medium. Appl. Microbiol. 19: 542-544.
Lopez LC, Christensen CM (1967). Effect of moisture content on growth and sporulation of *Aspergillus flavus*. University of Minnesota Press, Minneapolis.
Madhyastha MS, Marquardt RR, Frohlich AA (1992). Hydrolysis of ochratoxin A by the microbial activity of digesta in the gastrointestinal tract of rats. Arch. Environ. Contamin. Toxicol. 23: 408-472.
Magan N, Aldred D (2007). Environmental and fungal interactions: maintaining a competitive edge. In: Van West P, Avery S, Stratford M (Eds.). Stress in yeast and filamentous fungi. Elsevier Ltd., Amsterdam, pp. 19-35.
Marquardt RR, Frolich AA (1992). A review of recent advances in understanding ochratoxicosis. J. Anim. Sci. (Champaign, IL, U.S.) 70(12): 3968-3988.
McLafferty FW (1973). Mass spectra of ochratoxins. In: Benjamin WA (Ed.). Interpretation of mass spectra, 2nd ed. Marcel Dekker, New York, pp. 56-63.
Mngadi PT, Govinden R, Odhav B (2008). Co-occurring mycotoxins in animal feeds. Afr. J. Biotechnol. 7(13): 2239-2243.
Nelson PE, Toussoun TA, Marasas WFO (1983). *Fusarium* species. An Illustrated Manual for Identification. The Pennsylvania State University Press, 3-49: 118-121.
Nesheim S (1980). Ochratoxins. In: Stoloff L (Ed.). Mycotoxins Methodology. Association of Official Analytical Chemists, 13th ed. Washington, D.C., pp. 16-18.
Reid LM, Nicol RW, Ouellet T, Savard M, Young JC, Stewart DW, Schaafsma AW (1999). Interaction of *Fusarium graminearum* and *Fusarium moniliforme* in maize cars: disease progress, fungal biomass, and mycotoxin accumulation. Phytopathology, 89: 1028-1037.
Remiro R, Ibànez-Vea M, Gonzàlez-Penas E, Lizarraga E (2010). Validation of a liquid chromatography method for the simultaneous quantification of ochratoxin A and its analogues in red wines. J. Chromatogr. A. 1217: 8249-8256.
Schroeder HW, Ashworth LJ (1965). Aflatoxins in Spanish peanuts in relation to pod and kernel condition. Phytopathology, 55: 464-465.
Scott PM, Van Walbeek W, Kennedy B, Anyeti D (1972). Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. J. Agric. Food Chem. 20: 1103-1109.
Stander M, Bornscheuer T, Henke E, Steyn PS (2000). Screening of commercial hydrolases for the degradation of ochratoxin A. J. Agric. Food Chem. 48: 5736-5739.
Surekha M, Saint K, Reddy VK, Reddy AR, Reddy SM (2011). Fungal succession in stored rice (*Oryza sativa* Linn.) fodder and mycotoxin production. Afr. J. Biotechnol. 10(4): 550-555.
Škrinjar M, Rašić JL, Stojičić V (1996). Lowering ochratoxin A level in milk by yoghurt bacteria and bifidobacteria. Folia Microbiol. Prague, Czech Repub. 41: 26-28.
Van der Merwe KJ, Steyn PS, Fourie L, Scott DB, Theron JJ (1965). Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. Nature, 205: 1112-1113.
Varga J, Pèterig Z, Tàbori K, Tèren J, Vàgvölgyi C (2005). Degradation of ochratoxin A and other mycotoxins by *Rhizopus* isolates. Int. J. Food Microbiol. 99: 321-328.
Varga J, Kozakiewicz Z (2006). Ochratoxin A in grapes and grape-derived products. Trends Food Sci. Technol. 17: 72-81.
Wegst W, Lingens F (1983). Bacterial degradation of ochratoxin A. FEMS Microbiol. Lett. 17: 341-344.
Xiao H, Marquardt RR, Frohlich AA, Philips GD, Vitti TG (1991). Effect of hay and a grain diet on the rate of hydrolysis of ochratoxin A in the rumen sheep. J. Anim. Sci. Champaign, IL, U.S. 69: 3706-3714.
Xiao-E C, Xu-Bo F, Wen-Sui X (2008). Strain improvement and optimization of the media composition of chitosanase-producing fungus *Aspergillus* sp. Afr. J. Biotechnol. 7(14): 2501-2508. CJ 22-326.
Yazdani D, Abidin-Zainal MA, Tan YH, Kamaruzaman S (2010). Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. Afr. J. Biotechnol. 9(45): 7654-7659.