

INFLUENCE OF CHOSEN MICROBES AND SOME CHEMICAL SUBSTANCES ON THE PRODUCTION OF AFLATOXINS

*Iveta Brožková, Petra Šmahová, Jarmila Vytrásová, Petra Moťková,
Marcela Pejchalová, David Šilha*

ABSTRACT

Aflatoxins are produced as secondary metabolites by *A. flavus*, *A. parasiticus*, *A. nomius* and *A. tamarii*. The aflatoxin biosynthetic pathway involves several enzymatic steps and genes (*apa-2*, *ver-1*) that appear to be regulated by the *aflR* gene in these fungi. The aim of this work was the detection of aflatoxins by the HPLC method and the ascertainment of factors influencing their production. *A. parasiticus* CCM F-108, *A. parasiticus* CCF 141, *A. parasiticus* CCF 3137 and two isolates *A. flavus* were used. These toxigenic isolates were recovered from spice (strain 1) and wraps (strain 2). The gene for the production of aflatoxin B1 for each species of fungi was detected using an optimized PCR method. *Rhodotorula* spp.*, *Lactococcus lactis* subsp. *lactis* CCM 1881, *Flavobacterium* spp. and fungal strain *Pythium oligandrum** were tested for inhibition of aflatoxins production and fungal growth. Having used the HPLC detection, various preservatives (propionic acid, citric acid, potassium sorbate) were tested from the viewpoint of their influence on the growth of aflatoxigenic fungi followed by the production of aflatoxins. The growth of *A. flavus* and *A. parasiticus* and aflatoxin production in Potato Dextrose Agar supplemented with propionic acid (1000-2000-3000 mg/kg), citric acid (2000-3000-4000 mg/kg) and potassium sorbate (500-800-1000 mg/kg) was tested by Agar Dilution Method. After 72 h of incubation was evaluated growth of fungi, all samples were frozen for later extraction and aflatoxins quantification by HPLC. Effect of peptone and sucrose additions were studied in yeast extract (2%) supplemented with peptone (5-10-15%) or sucrose (15%). Growth inhibition of *Aspergillus* by *Pythium oligandrum* was tested on wood surface. As shown, the highest inhibition effect on the aflatoxins production was obtained when propionic acid was applied in concentrations since 1000 mg/kg. A total inhibition of the fungi growth and aflatoxins production was observed in all samples containing peptone in the concentration range tested. Significant limitation of the growth and production of aflatoxins was also observed in the presence of other microorganisms such like *Pythium oligandrum* and *Rhodotorula* spp.

Keywords: aflatoxin; preservatives; *Pythium oligandrum*; *Rhodotorula*; inhibition; *Aspergillus flavus*; *A. parasiticus*

INTRODUCTION

Microscopic filamentous fungi present a worldwide problem. Fungal physiology refers to the nutrition, metabolism, growth and reproduction of fungal cells. It also generally relates to interaction of fungi with their biotic and abiotic environment, including cellular response to stress. The dynamics of fungal activities are central to the efficiency of forestry and agricultural operations, in all three forms: mutualistic symbionts, pathogens and saprophytes as they mobilize nutrients and affect the physical-chemical environment (Gqaleni et al., 1997; Chourasia, 1993; Paster, 1999).

Fungi are also responsible for the organic pollutants detoxification and for the bioremediation of heavy metals in the environment.

The fungal negative effect on humans, animals and plants is the main reason for monitoring the contamination of food and feed (Gunterus et al. 2007, Huang et al. 2009). Some fungi colonize plant seeds and thereby cause loss of stored harvests and reduce seed germination rates, they can also produce mycotoxins which have mutagenic properties. Some fungi are pathogens of plants or animals

(and even other fungi). Fungi can also cause diseases in humans, many of them as so-called opportunistic pathogens that attack people whose immune systems are weakened.

Aspergillus species compete with *Fusarium* and *Penicillium* species for the dominance among the world's fungal flora (Nesci et al., 2003).

Aflatoxins are secondary metabolites produced by *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* and the newly described *Aspergillus tamarii* and have the highest toxicity among mycotoxins (hepatotoxicogenic, hepatocarcinogenic and immunosuppressive effects). Due to their toxicity including the carcinogenic activity, aflatoxins affect not only the health of humans and animals but also the economics of agriculture and food. For the production of aflatoxins it is particularly important where their biosynthetic pathway stops.

Aflatoxinogenic fungi are important contaminants of certain foods and animal feeds because of their ability to produce aflatoxins. When these fungi invade and grow in commodities such as peanuts, corn and cottonseed,

the resulting contamination with aflatoxins often makes the commodities unfit for consumption.

Historically, identification of filamentous fungal (mould) species has been based on morphological characteristics, both macroscopic and microscopic. These methods may often be time-consuming and inaccurate, which necessitates the development of identification protocols that are rapid, sensitive, and precise. The polymerase chain reaction (PCR) has shown a great promise in its ability to identify and quantify individual organisms from a mixed culture environment; however, the cost effectiveness of single organism PCR reactions is quickly becoming an issue (Nigam and Singh, 2000; Bennett and Papa, 1988; Weidenbörner, 2001).

The aim of this work was the determination of aflatoxins by the HPLC method and the ascertainment of factors influencing their production.

MATERIAL AND METHODS

Bacterial strains, growth medium and cultural conditions

Six fungal, two bacterial and one yeast strains were used: *A. parasiticus* CCM F-108, *A. parasiticus* CCF 141, *A. parasiticus* CCF 3137 and two isolates *A. flavus*. These toxigenic isolates were recovered from spice (strain 1) and wraps (strain 2). *Rhodotorula* spp.*, *Lactococcus lactis* subsp. *lactis* CCM 1881, *Flavobacterium* spp. and fungal strain *Pythium oligandrum** were tested for inhibition of aflatoxins production and fungal growth. Strains of CCM were provided by Czech Collection of Microorganisms (Brno, Czech Republic). Strains of CCF were provided by Department of Botany (Faculty of Science, Charles University, Praha, Czech Republic), the others (*) were provided by Department of Biological and Biochemical Sciences (Faculty of Chemical technology, University of Pardubice, Pardubice, Czech Republic). A suspension of bacterial and yeast or fungal strain was prepared from freshly grown colonies on MRS for *Lactococcus lactis*, Malt agar for yeast 24-48 h incubation at optimum temperature. Concentration of bacterial and yeast cells was adjusted to 10^8 cfu/mL using 0.5 McFarland turbidity scale. Suspensions of fungal spores were prepared from 7-days cultures grown on Malt agar's slant at 24 °C. The density of spores was adjusted to 10^6 spores/mL using Bürker counting cell. All the nutrient media used throughout the study were purchased from HiMedia, India. The growth of *A. flavus* and *A. parasiticus* and aflatoxin production was tested by Agar Dilution Method. Control Petri dishes were kept without bacteria and yeast in similar way. After 7 days of incubation was evaluated growth of fungi, all samples were frozen for later extraction and aflatoxins quantification by HPLC (Malir et al., 2006).

The growth of *A. flavus* and *A. parasiticus* and aflatoxin production in Potato Dextrose Agar (PDA, HIMEDIA, India) supplemented with propionic acid (1000-2000-3000 mg/kg), citric acid (2000-3000-4000 mg/kg) and potassium sorbate (500-800-1000 mg/kg) was tested by Agar Dilution Method. After 72 h of incubation was evaluated growth of fungi, all samples were frozen for later extraction and aflatoxins quantification by HPLC (Malir et al., 2006).

Effect of peptone and sucrose additions were also studied. Spore concentrations ranging 10^6 spores/mL were inoculated onto 10 mL of yeast extract (2%) supplemented with peptone (5-10-15%) or sucrose (15%). Each sample was examined in duplicate. Negative control (without peptone or sucrose) was also included.

After incubation (25 °C, 72 h) was evaluated growth of fungi on Malt agar, production of aflatoxins was quantification by HPLC (Malir et al., 2006).

Growth inhibition of *Aspergillus* by *Pythium oligandrum* was tested on wood surface (5x5 cm).

Extraction of aflatoxins

The sample was agitated with 50 mL of a mixture of 60% methanol with 0,8 g NaCl for 3 min at room temperature. 5 mL of solution was diluted by 10 mL deionized water. Extraction of aflatoxins was performed by Afla B affinity column (VICAM, USA). After filtration of the substrate was added 1mL to developer (8 mL deionized water, 1mL trifluoroacetic acid, 1 mL acetic acid). Then the amount of aflatoxins were detected by HPLC.

Conditions for determination of aflatoxins by HPLC

The HPLC system has been consisted of a column (OmniSpher 5 C18 (3x150, 5 µm), Varian), a precolumn (ChromSep Guard ColumnC18 (10x2,5 µm), Varian). A 100 µl sample was injected to HPLC system. Microfiltered methanol-acetonitrile-water (1:1:4 v/v) was used as isocratic mobile phase with a flow rate of 0,5 mL/min at room temperature. Aflatoxins detection was by fluorescence with excitation and emission wavelengths of 364 nm and 456 nm, respectively.

RESULTS AND DISCUSSION

Production of aflatoxins in potato dextrose broth

The optimized PCR reaction selectivity was tested on collection cultures and it was proven that both PCR methods are highly specific for the determination of aflatoxinogenic strains of *A. flavus* and *A. parasiticus* isolated from food and feed. When verifying the PCR selectivity to detect the regulatory gene important for the aflatoxin B₁'s production, a positive reaction was observed also in the non-aflatoxinogenic strain of *A. versicolor* CCM F-585. The aflatoxin B₁'s biosynthetic production pathway renders various intermediate products, one of which is versicolorin A, the final metabolite of the *A. versicolor* CCM F-585 fungus (Zachová et al., 2003). The production of aflatoxins was demonstrated only by *Aspergillus parasiticus* CCF 141, *Aspergillus parasiticus* CCF 3137 and *Aspergillus flavus* CCM-F 108 (Table 1).

Strain of *Aspergillus parasiticus* CCF 141 produced aflatoxin B₁ (6.6 ng/mL), aflatoxin B₂ (1.0 ng/mL), aflatoxin G₁ (6.9 ng/mL) and aflatoxin G₂ (1.0 ng/mL) (Table 1). Production of aflatoxin B₁ corresponds to the data in the literature on the production of aflatoxin B₁ found in *Aspergillus parasiticus* FVB 1981 isolated from peanut sauce, which were incubated in YES broth at 28 °C for 7 days, 10.8 ng/mL (Abarca et al., 1988). Strain of *Aspergillus parasiticus* CCF 3137 produced 18.7 ng/mL aflatoxin B₁, 0.8 ng/mL aflatoxin B₂, 6.5 ng/mL aflatoxin G₁ and 0.3 ng/mL aflatoxin G₂ (Table 1). By contrast, Tsai et al. (1984) states in YES broth at 25 °C for 72 hours difference in the production of aflatoxin B₁ *Aspergillus*

parasiticus NRRL 2999, which was only 8.2 ng/mL (Tsai et al., 1984). Strain of *Aspergillus flavus* CCM F-108 produced by aflatoxin B1, in the amount of 331.5 ng/mL and aflatoxin B2 in the amount of 2.44 ng/mL (Table 1). Authors Kady and Maraghy (1994) indicate comparable production of aflatoxin B1 to the *Aspergillus flavus* isolated from meat products cultivated in potatoes broth for 10 days at 28 °C, and that was 310 ng/mL. Production of aflatoxin B2, however, did not demonstrated in their experiment.

The production of aflatoxins by strains *A. flavus* from the samples of sunflower, barleycorn, spice *Steak afpa*, *Tandoori*, spices for chicken was not detected. This is consistent with authors Razzaghi-Abyaneh et al. (2006). Aflatoxins were produced only by isolates from wraps and spice (Table 2). Strains producing aflatoxins were used for further experiments.

Fungal growth inhibition and the production of aflatoxins by a conserving substance

Growth of selected strains of fungi was monitored in the presence of the preservative on the soil potato agar at 25 °C for 72 hours. Agar Dilution Method was used to determine the inhibition of mold growth. Size of colony was compared with the size of colonies on the medium without preservatives. Citric acid inhibited at least growth of *Aspergillus flavus* CCM F-108 and the most growth of *Aspergillus parasiticus* CCF 3137 (Table 3). Propionic acid inhibited growth of at least *Aspergillus parasiticus* CCF 3137 and the most inhibited *Aspergillus flavus* CCM F-108, whose growth was completely suppressed. Growth of *Aspergillus parasiticus* CCF-141 was inhibited completely also (Table 3). Suppression of growth of *Aspergillus flavus* detected by the agar diluted method on Sabouraud agar at 21°C for 5 days provides Čonková et al. (1993), namely even lower concentrations of propionic acid than was used for our experiment (Table 3). Potassium sorbate inhibited the growth of most *Aspergillus*

flavus CCM F-108 and a minimum growth of *Aspergillus parasiticus* CCF 3137. This strain was isolated from environment of the rainforest in Malaysia, and is therefore probably more resistant than the strains of *Aspergillus parasiticus* CCF 141 and *Aspergillus flavus* CCM F-108, obtained from the Czech Republic. Significant inhibition of growth of *Aspergillus flavus* CCM F-108 is already at a concentration of potassium sorbate and 500 mg/kg (Table 3). The concentration of potassium sorbate (higher than 1500 mg/kg) is needed for pronounced inhibition of growth for *Aspergillus parasiticus*, so there, as confirmed the authors Liewen and Marth (1984) in their study. Propionic acid inhibits growth of *Aspergillus* the most of preservatives (Table 3).

Aflatoxins production in the presence of preservatives

Conserving substance factors were tested in potato dextrose broth (30 °C, 72 h). All the preservatives inhibited the production of aflatoxins. When aflatoxins were created, aflatoxins were produced in quantities less than the detection limit determination. *A. parasiticus* CCM F-108 and *Aspergillus parasiticus* CCF 141 were the most sensitive to preservatives. Same results are featured by Chourasia (1993).

Similar to our findings also Čonková et al. (1993) feature the fungicidal efficiency of propionic acid resulting from the experiment at 88.9%. In their study, Tsai et al. (1984) determined the production of aflatoxins by strain *Aspergillus parasiticus* NRRL 2999 in the presence of 0.2 - 0.4% propionic acid.

The largest production of aflatoxins was determined in the presence of citric acid. Production of aflatoxin B1 and G1 decreased with increasing concentration of citric acid (Figure 1, Table 4 and Table 5).

In the presence of potassium sorbate were *Aspergillus parasiticus* CCF 3137 produced only aflatoxins B1 and G1, at a concentration of 500 mg/kg (B1 0,29 ng/mL, G1 0,036 ng/mL).

Table 1 Amount of aflatoxins in potato dextrose broth (25 °C, 72 hours).

Collection cultures	AFB1 (ng/mL)	AFB2 (ng/mL)	AFG1 (ng/mL)	AFG2 (ng/mL)
<i>A. parasiticus</i> CCF 141	6.6	1	6.9	1
<i>A. parasiticus</i> CCF 3137	18.7	0.8	6.5	0.3
<i>A. parasiticus</i> CCM-F 108	331.5	2.44	<0.025	<0.030
<i>A. flavus</i> CCM-F 585	<0.025	<0.035	<0.025	<0.030
<i>A. tamarii</i> CCF 3206	<0.025	<0.035	<0.025	<0.030
<i>A. parasiticus</i> CCM-F 550	<0.025	<0.035	<0.025	<0.030
<i>A. flavus</i> var <i>columnaris</i>	<0.025	<0.035	<0.025	<0.030
<i>A. flavus</i> CCM-F 171	<0.025	<0.035	<0.025	<0.030
<i>A. flavus</i> CCM-F 449	<0.025	<0.035	<0.025	<0.030

Table 2 Amount of aflatoxins in potato dextrose broth (25 °C, 72 hours).

Strains of <i>A. flavus</i> isolated from samples	AFB1 ng/mL	AFB2 ng/mL	AFG1 ng/mL	AFG2 ng/mL
Wraps (strain 2)	210	4	<0,025	<0,030
Spice (strain 1)	650	15	<0,025	<0,030

Table 3 Averages of fungal colonies on potato dextrose agar (25 °C after 72h).

Conserving substance	<i>A. parasiticus</i> CCM-F 108 (mm)	<i>A. parasiticus</i> CCM-F 141 (mm)	<i>A. parasiticus</i> CCF 3137 (mm)
	Without conserving substance	30 ±0.4	38 ±1.4
Citric acid 2000 mg/kg	29 ±0.7	27 ±1.4	27 ±0.7
Citric acid 3000 mg/kg	25 ±0.7	23 ±0.7	22 ±0.4
Citric acid 4000 mg/kg	21 ±0.4	20 ±1.8	18 ±1.4
Propionic acid 1000 mg/kg	Without growth	28 ±1.4	32 ±1.4
Propionic acid 2000 mg/kg	Without growth	Without growth	10 ±1.4
Propionic acid 3000 mg/kg	Without growth	Without growth	7 ±1.4
Potassium sorbate 500 mg/kg	18 ±0.4	36 ±1.1	45 ±2.1
Potassium sorbate 800 mg/kg	15 ±0.7	33 ±1.4	39 ±1.4
Potassium sorbate 1000 mg/kg	11 ±0.7	30 ±2.5	35 ±1.4

Information on the aflatoxin production inhibition by organic acids and potassium sorbate are rather sparse, some data can be found in **Tsai et al. (1984)**.

The reduction of aflatoxins by peptone and sucrose

The aflatoxin pathway gene expression analysis was carried out in inducing (YES medium) and non-inducing (YEP medium) conditions (**Sweeney et al., 2000; Scherm and Palomba, 2004; Scherm et al., 2005**). In our experiment, peptone and sucrose inhibited most of the aflatoxin production in all of the concentrations.

The growth of aflatoxigenic fungi was not particularly influenced by the presence of peptone and sucrose.

Fungal growth inhibition and aflatoxins production by *Pythium oligandrum*

***Pythium oligandrum* on wood block**

The growth of neither *Aspergillus* nor *Pythium oligandrum* was visible on wood block after the incubation period, therefore swabbing by sterile swab was carried out. Swab cotton was then shaken out to physiological solution and the surface of the potato dextrose agar was inoculated with 0.1 mL of the homogenate and incubation was allowed for 72 h at 25 °C.

After the incubation period, the *Pythium oligandrum* growth was quite prominent, while the growth of *A. flavus* was strongly inhibited.

Without the presence of *Pythium oligandrum* spores of *A. flavus* survive on wooden plate.

***Pythium oligandrum* in liquid medium**

This section of the experiment proved a major growth inhibition of fungi, in some strains up to four orders; hence also the aflatoxins production was reduced by *Pythium oligandrum*.

Pythium oligandrum Dresch., has been identified as one of the most numerous species observed in agricultural soils. In certain areas, it has proven its ability to induce plant disease suppression (**Floch et al., 2009**). Inoculation with *P. oligandrum* or its elicitors can sensitive plants and make them respond more quickly and efficiently to pathogen attacks and trigger systemic resistance. Additionally, *P. oligandrum* has been reported to promote plant growth according to a recent study this phenomenon is mediated by tryptamine, an auxin produced by *P. oligandrum*. Interactions between host fungi and *P. oligandrum* involve a complex set of events (**Rey et al., 2005**). In our study also showed that *P. oligandrum* not only inhibited of growth of *Aspergillus*, but inhibited production of aflatoxins as well.

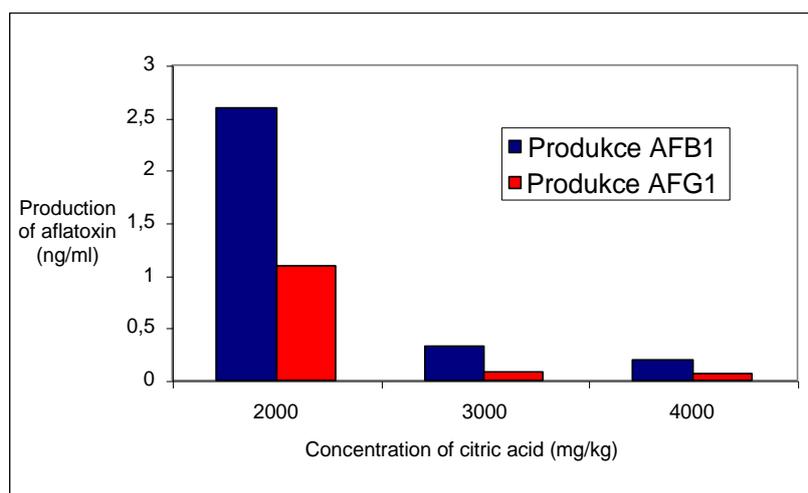


Figure 1 The dependence of the aflatoxin production on the citric acid concentration in *Aspergillus parasiticus* CCF 3137 (potato dextrose broth, 25 °C, 72 h).

Table 4 Aflatoxin production by *A. parasiticus* CCF 3137 in potato dextrose broth with preservatives (25 °C, 72 hours).

<i>Aspergillus parasiticus</i>		AFB1	AFB2	AFG1	AFG2
CCF 3137		ng/ml	ng/ml	ng/ml	ng/ml
Propionic acid	1000 mg/kg	0.3	0.04	0.1	<0.030
	2000 mg/kg	0.2	0.03	<0.025	<0.030
	3000 mg/kg	0.2	0.03	<0.025	<0.030
Citric acid	2000 mg/kg	2.6	0.045	1.1	<0.030
	3000 mg/kg	0.33	<0.035	0.08	<0.030
	4000 mg/kg	0.2	<0.035	0.07	<0.030
Potassium sorbate	500 mg/kg	0.29	<0.035	0.036	<0.030
	800 mg/kg	<0.025	<0.035	<0.025	<0.030
	1000 mg/kg	<0.025	<0.035	<0.025	<0.030

Fungal growth inhibition and aflatoxins production using different microorganisms

Fungal growth inhibition using different microorganisms

The size of fungal colony was measured on a medium for selected microorganisms (Malt, MRS, BHI) (Table 6). After incubation, an analysis of aflatoxins was carried out using HPLC.

After microaerophile cultivation on MRS at 30 °C, *Aspergillus parasiticus* CCF 141 and *Aspergillus parasiticus* CCF 3137 occurred for the production of aflatoxins, which was probably caused by low pH. The fungi were stressed and resisted the production of aflatoxins in this medium, as well as his work indicates Nesci et al. (2003). Author tested the effect of pH on

aflatoxin production of *Aspergillus parasiticus* RCT 1920 and *Aspergillus parasiticus* RCD 106 on corn MALT agar at 25 °C for 5 days.

Ehrlich et al. (2005) also examined the production of aflatoxins B1 and G1 in the presence of different acidic pH. Found that gene expression for the production of aflatoxin B1 with decreasing pH increases and is the largest production of aflatoxin B1, production of aflatoxin G1 is stopped.

In our case, stress of the *A. parasiticus* probably contributed microaerophilic environment of cultivation and production of nisin by *Lactococcus*. *Lactococcus* and *Rhodotorula* inhibited growth all strains of *A. parasiticus* (Figure 2 and Figure 3).

Table 5 Aflatoxin production by *A. flavus* from spices for chicken (Strain 1) in potato dextrose broth with preservatives (25 °C, 72 hours).

<i>A. flavus</i> Strain 1		AFB1	AFB2	AFG1	AFG2
		ng/ml	ng/ml	ng/ml	ng/ml
Propionic acid	1000 mg/kg	<0.025	<0.035	<0.025	<0.030
	2000 mg/kg	<0.025	<0.035	<0.025	<0.030
	3000 mg/kg	<0.025	<0.035	<0.025	<0.030
Citric acid	2000 mg/kg	2.76	0.074	<0.025	<0.030
	3000 mg/kg	<0.025	<0.035	<0.025	<0.030
	4000 mg/kg	<0.025	<0.035	<0.025	<0.030
Potassium sorbate	500 mg/kg	0.068	<0.035	<0.025	<0.030
	800 mg/kg	0.035	<0.035	<0.025	<0.030
	1000 mg/kg	0.029	<0.035	<0.025	<0.030

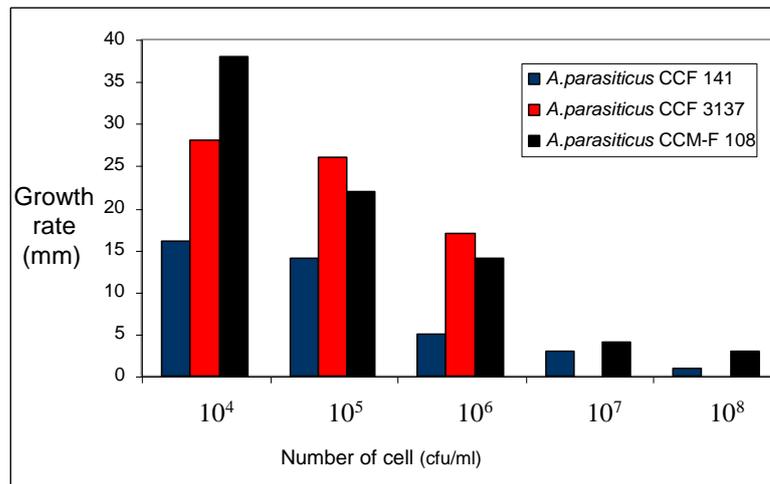


Figure 2 The dependence of the fungal growth rate on the number of *Flavobacterium* cells (BHI, 30 °C, 72 h).

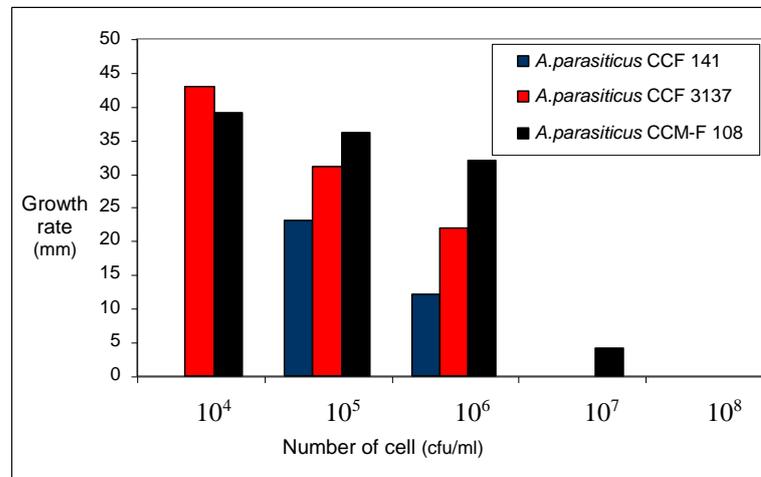


Figure 3 The dependence of the fungal growth rate on the number of *Rhodotorula* cells (MALT, 25 °C, 72 h).

Table 6 The growth rate of fungi *A. parasiticus* in the presence of different microorganisms after 72 hours.

Microorganisms [cfu/ml]	<i>A. parasiticus</i>	<i>A. parasiticus</i>	<i>A. parasiticus</i>
	CCF 141	CCF 3137	CCM F- 108
	[mm]	[mm]	[mm]
<i>Lactococcus 10⁸</i> (MRS, 30 °C)	2 ±0.7	2 ±1.4	2 ±0.7
<i>Rhodotorula 10⁸</i> .(MALT, 25 °C)	0	0	0
<i>Rhodotorula 10⁷</i> .(MALT, 25 °C)	0	0	4 ±0.7
<i>Rhodotorula 10⁶</i> .(MALT, 25 °C)	12 ±1.4	22 ±0.7	32 ±1.4
<i>Rhodotorula 10⁵</i> .(MALT, 25 °C)	23 ±1.4	31 ±2.1	36 ±0.7
<i>Rhodotorula 10⁴</i> .(MALT, 25 °C)	0	43 ±1.4	39 ±0.7

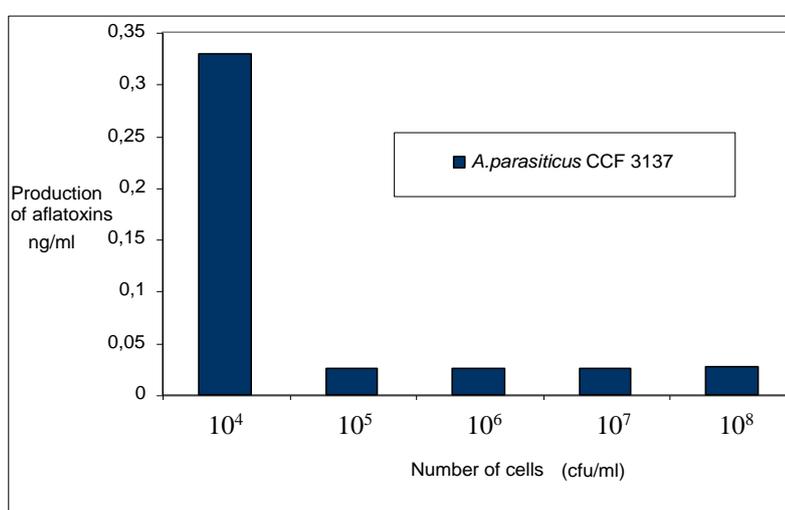


Figure 4 The dependence of aflatoxin production by *A. parasiticus* CCF 3137 on the number of *Rhodotorula* cell (MALT, 25 °C, 72 h).

Aflatoxins production inhibition by different microorganisms

In the presence of *Lactococcus*, no tested fungi produced any aflatoxins. The authors **Smiley and Draughon (2000)** tested the inhibition of aflatoxin production by protein extracts of *Flavobacterium*. *Aspergillus flavus* and *Aspergillus parasiticus* isolated from spices with protein extract of *Flavobacterium* in BHI broth at 30° C for 5 days were cultivated. Inhibition of aflatoxin production by the bacteria, according to these authors 80% (**Smiley and Draughon, 2000**).

In the presence of *Lactococcus* and *Rhodotorula*, others the strains do not produced of aflatoxins. Only *Rhodotorula* strain in density of 10⁴ cfu/mL inhibited of production of aflatoxins at least. 0,329 ng/mL of AFB1 and 0,293 ng/mL of AFG1 were produced by *A. parasiticus* CCF 3137 (Figure 4).

The aflatoxin production by *Aspergillus* sp. in the presence of peptone and sucrose was observed by the HPLC method. In all applied peptone concentrations, the strains were considerably inhibited.

Results revealed that *Pythium oligandrum* caused a total inhibition of growth and aflatoxin biosynthesis in the toxigenic *Aspergillus* sp.

A strong effect on limiting the growth of aflatoxigenic fungi and aflatoxin biosynthesis was also observed when using the *Rhodotorula*.

Also *Lactococcus* had an inhibiting effect, although only in its highest concentration (10⁸ cfu/mL).

CONCLUSION

This work also aimed to detect aflatoxins by the HPLC method and to determine factors influencing their production.

The collection cultures' aflatoxin production was quantitatively determined by the optimized HPLC method.

Chemical factors influencing growth and production of aflatoxins were tested. Tested preservatives (propionic acid, citric acid and potassium sorbate) showed different effect on different strains.

The most sensitive to preservatives was *A. flavus* CCM F-108. The most efficient growth inhibition of fungi and

aflatoxin biosynthesis was determined already for the concentration of 100 mg/kg.

REFERENCES

- Abarca, M. L., Bragulat, M. R., Bruguera, M. T., Cabañes, F. J. 1988. Comparison of some screening methods for aflatoxigenic moulds. *Mycopathologia*, vol. 104, no. 2, p. 75-79. [PMid:3146701](#)
- Bennett, J. W., Papa, K. E., 1988. The aflatoxigenic *Aspergillus* spp. *Adv. Plant Pathol.*, vol. 6, p. 263-280. <http://dx.doi.org/10.1016/B978-0-12-033706-4.50022-0>
- Chourasia, H. K. 1993. Growth, sclerotia and aflatoxin production by *Aspergillus parasiticus* influence of food preservatives. *Lett. Appl. Microbiol.*, vol. 17, no. 5, p. 204-207. <http://dx.doi.org/10.1111/j.1472-765X.1993.tb01447.x>
- Čonková, E., Para, L., Košičová, A. 1993. Inhibition of the moulds growth by selected organic acids. *Vet. Med.*, vol. 38, no. 12, p. 723-727. [PMid:8122343](#)
- Ehrlich, K. C., Montalbano, B. G., Cotty, P. J. 2005. Divergent regulation of aflatoxin production at acidic pH by two *Aspergillus* strains. *Mycopathologia*, vol. 159, no. 4, p. 579-581. <http://dx.doi.org/10.1007/s11046-005-1150-7> [PMid:15983745](#)
- Gqaleni, N., Smith, J. E., Lacey, J. and Gettinby, G. 1997. Effects of temperature, water activity, and incubation time on production of aflatoxins and cyclopiazonic acid by an isolate of *Aspergillus flavus* in surface agar culture. *Appl. Environ. Microbiol.*, vol. 63, no. 3, p. 1048-1053. [PMid:16535539](#)
- Gunterus, A., Roze, L. V., Beaudry, R., Linz, J. E. 2007. Ethylene inhibits aflatoxin biosynthesis in *Aspergillus parasiticus* grown on peanuts. *Food Microbiol.*, vol. 24, no. 6, p. 658-663. <http://dx.doi.org/10.1016/j.fm.2006.12.006> [PMid:17418318](#)
- Huang, J. Q., Jiang, H. F., Zhou, Y. Q., Lei, Y., Wang, S. Y., Liao, B. S. 2009. Ethylene inhibited aflatoxin biosynthesis is due to oxidative stress alleviation and related to glutathione redox state changes in *Aspergillus flavus*. *Int. J. Food Microbiol.*, vol. 130, no. 1, p. 17-21. <http://dx.doi.org/10.1016/j.ijfoodmicro.2008.12.027> [PMid:19162358](#)
- Kady, I., Maraghy, S. 1994. Mycotoxin producing potential of some isolates of *Aspergillus flavus* and *Eurotium* groups from meat products. *Microbiol. Res.*, vol. 149, no. 3, p. 279-307. [PMid:7987615](#)
- Le Floch, G., Vallenge, J., Benhamou, N., Rey, P. 2009. Combining the oomycete *Pythium oligandrum* with two other antagonistic fungi: Root relationships and tomato grey mold biocontrol. *Biol. Control*, vol. 50, no. 3, p. 288-298. <http://dx.doi.org/10.1016/j.biocontrol.2009.04.013>
- Liewen, M. B., Marth, E. H. 1984. Inhibition of *Penicillia* and *Aspergilli* by Potassium Sorbate. *J. Food Prot.*, vol. 7, p. 554-556.
- Malir, F., Ostry, V., Grosse, Y., Roubal, T., Skarkova, J., Ruprich, J. 2006. Monitoring the mycotoxins in food and their biomarkers in the Czech Republic. *Mol. Nutr. Food Res.*, vol. 50, no. 6, p. 513-518. <http://dx.doi.org/10.1002/mnfr.200500175> [PMid:16676375](#)
- Nesci, A., Rodriguez, M., Etcheverry, M. 2003. Control of *Aspergillus* growth and aflatoxin production using antioxidant at different conditions of water activity and pH. *J. Appl. Microbiol.*, vol. 95, no. 2, p. 279-287. <http://dx.doi.org/10.1046/j.1365-2672.2003.01973.x> [PMid:12859759](#)
- Nigam, P., Singh, D. 2000. *Production of Secondary metabolites-Fungi. Encyclopedia of Food Microbiology*, (Robinson, R. K., Batt, C. A., Patel, P. D., Eds.), Academic Press, San Diego, 2000, ISBN 0-12-227070-3.
- Paster, N., Lecong, Z., Menashrov, M., Shapira R. 1999. Possible synergistic effect of nisin and propionic acid on the growth of the mycotoxigenic fungi *Aspergillus parasiticus*, *Aspergillus ochraceus* and *Fusarium moniliforme*. *J. Food Prot.*, vol. 62, no. 10, p. 1223-1227. [PMid:10528732](#)
- Razzaghi-Abyaneh, M., Shams-Ghahfarokhi, M., Allameh, A., Kazeroon-Shiri, A., Ranjbar-Bahadori, S., Mirzahoseini, H., Rezaee, M. B. 2006. A survey on distribution of *Aspergillus* section Flavi in corn field soils in Iran: Population patterns based on aflatoxins, cyclopiazonic acid and sclerotia production. *Mycopathologia*, vol. 161, no. 3, p. 183-192. <http://dx.doi.org/10.1007/s11046-005-0242-8> [PMid:16482391](#)
- Rey, P., Le Floch, G., Benhamou, N., Salerno, M.-I., Thuillier, E., Tirilly, Y. 2005. Interactions between the mycoparasite *Pythium oligandrum* and two types of sclerotia of plant-pathogenic fungi. *Mycol. Res.*, vol. 109, no. 7, p. 779-788. <http://dx.doi.org/10.1017/S0953756205003059> [PMid:16121563](#)
- Scherm, B., Palomba, M. 2004. Detection of transcripts of the aflatoxin genes by RT-PCR allows differentiation of *Aspergillus parasiticus*. *Int. J. Food Microbiol.*, vol. 98, no. 2, p. 201-210. <http://dx.doi.org/10.1016/j.ijfoodmicro.2004.06.004> [PMid:15681047](#)
- Scherm, B., Palomba, M., Serra, D., Marcello, A., Migheli, Q. 2005. Detection of transcripts of the aflatoxin genes aflD, aflO, and aflP by reverse transcription-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *Int. J. Food Microbiol.*, vol. 98, no. 2, p. 201-210. <http://dx.doi.org/10.1016/j.ijfoodmicro.2004.06.004> [PMid:15681047](#)
- Smiley, R. D., Draughon, F. A. 2000. Preliminary evidence that degradation of aflatoxin B1 by *Flavobacterium aurantiacum* is enzymatic. *J. Food Prot.*, vol. 63, no. 3, p. 415-418. [PMid:10716576](#)
- Sweeney, M. J., Pamies, P., Dobson, A. D. 2000. The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in *Aspergillus parasiticus* 439. *Int. J. Food Microbiol.*, vol. 56, no. 1, p. 97-103. [http://dx.doi.org/10.1016/S0168-1605\(00\)00277-4](http://dx.doi.org/10.1016/S0168-1605(00)00277-4) [PMid:10857929](#)
- Tsai, W.-Y. J., Shao, K. P. P., Bullerman, L. 1984. Effects of Sorbate and Propionate on Growth and Aflatoxin Production of Sublethally injured *Aspergillus parasiticus*. *J. Food Sci.*, vol. 49, no. 1, p. 86-93. <http://dx.doi.org/10.1111/j.1365-2621.1984.tb13676.x>
- Weidenbömer, M. 2001. *Encyclopedia of Food Mycotoxins*. Springer-Verlag Berlin, Heidelberg, ISBN 3-540-67556-6. <http://dx.doi.org/10.1007/978-3-662-04464-3>
- Zachová, I., Vyřasová, J., Pejchalová, M., Červenka, L., Tavčar-Kalcher, G. 2003. Detection of aflatoxinogenic fungi in feeds using the PCR method. *Folia Microbiol.*, vol. 48, no. 6, p. 817-821. <http://dx.doi.org/10.1007/BF02931519>

Acknowledgments:

This work was supported by Research Project MSMT No. 0021627502 and Project No. 203/09/0148 of the Grant Agency of the Czech Republic.

Contact address:

Iveta Brožková, University of Pardubice, Faculty of Chemical Technology, Department of Biological and Biochemical Sciences, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: iveta.brozkova@upce.cz.

Petra Šmahová, University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: iveta.brozkova@upce.cz.

Jarmila Vytřasová, University of Pardubice, Faculty of Chemical Technology, Department of Biological and Biochemical Sciences, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: jarmila.vytrasova@upce.cz.

Petra Motřková, University of Pardubice, Faculty of Chemical Technology, Department of Biological and Biochemical Sciences, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: petra.motkova@upce.cz.

Marcela Pejchalová, University of Pardubice, Faculty of Chemical Technology, Department of Biological and Biochemical Sciences, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: marcela.pejchalova@upce.cz.

David Šilha, University of Pardubice, Faculty of Chemical Technology, Department of Biological and Biochemical Sciences, Studentská 573, 532 10 Pardubice, Czech Republic, E-mail: david.silha@upce.cz.