The Effect of Feed Contamination with Mycotoxins on Animals and Ways for Prevention and Degradation of Mycotoxins

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Abstract

Mycotoxins are secondary metabolites produced by fungi that are capable of causing illness and sometimes death to animals and not only animals even humans. In 1960 it was established that some fungal metabolites, now called mycotoxins, that have a destructive effect on animal health, since then people were interested on the effect and the way to stop it. Among them, aflatoxins, B1, B2, G1 & G2 synthesized mainly by *Aspergillus flavus/ Aspergillus parasiticus* are known to induce severe effects on animal: can cause liver damage, decreased milk production, reduced reproductively and suppressed immunity in animals consuming low dietary concentrations, decreased feed intake and efficiency, weight loss, jaundice, drop in milk production, nervous signs, bleeding and death. The aim of this work was the isolation of aflatoxin producing fungi in order to investigate new ways that can determinate, inhibit or degradation of aflatoxin, ochratoxin, using lactic bacteria and yeast. A number of 14 *Aspergillus spp.* isolates were selected from wheat, barley, triticale, oats, and sunflower seeds and identified, based on macroscopic and microscopic features as *A.flavus/A.parasiticus.* The ability of aflatoxin biosynthesis was detected on PDA medium with β cyclodextrine and sodium deoxycholate were evaluated by TLC and RIDA Screen R-biopharm. At this stage of experiments 3 fungal isolates, designated as GE2, G32, T11 were selected as aflatoxin B1, B2, G1 and used for further analysis (molecular identification, interactions with LAB and yeasts).

Keywords: animal illness, *Aspergillus flavus,* mycotoxins

1. Introduction

The contamination of animal feed with mycotoxins represents a worldwide problem for farmers. These toxins are from molds that growth on living and stored plants is almost unavoidable particularly under moist conditions. Among them, aflatoxins, B1, B2, G1 & G2 synthesized mainly by *Aspergillus flavus/ Aspergillus parasiticus* are known to induce severe effects on animal: can cause liver damage, decreased milk production, reduced reproductively and suppressed immunity in animals consuming low dietary concentrations, decreased feed intake and efficiency, weight loss, jaundice, drop in milk production, nervous signs, bleeding and death [1].

Aspergillus is a group of fungi, of which about 200 species have been identified. *Aspergillus* are found throughout the world and are the most common type of fungi in the environment. About 16 species of *Aspergillus* molds are known to be dangerous to humans, causing disease and infection. The aflatoxin LD50 rate (the dosage level that causes 50% of a group to die) for animals is between 0.5 and 10 mg/kg of the animal's weight.

Aflatoxin is principally hepatotoxic and hepatocarcinogen [2], but it causes a myriad of other effects either directly or indirectly associated with this toxicity: immunosuppressant, reduced growth rate, lowered milk and egg production,

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reduced reproduction, reduced feed utilization and efficiency and anemia. AFB1 has been shown to induce hepatocellular carcinoma in many species of animals including fish (rainbow trout, sockeye salmon, and guppy), poultry (turkeys, ducks, and geese),

Animal species respond differently in their susceptibility to the chronic and acute toxicity of aflatoxins. The toxicity can be influenced by environmental factors, exposure level, and duration of exposure, age, health, and nutritional status of diet. Aflatoxin B1 is a very potent carcinogen in many species, including nonhuman primates, birds, fish, and rodents. In each species, the liver is the primary target organ of acute injury. Metabolism plays a major role in determining the toxicity of aflatoxin B1; studies show that this aflatoxin requires metabolic activation to exert its carcinogenic effect.

2. Materials and methods

Biological material

Three collection strains of *Aspergillus flavus, A.niger (An5)* and *A.ochraceus* (ochra 5) as well as new fungal isolates were used in experiments.

Isolation of fungal strains: 10-20 seeds of wheat, barley, triticale, oats, or sunflower (from USAMVBT experimental fields) were added to 10ml of sterile water and incubated, with occasionally stirring, at room temperature for 1h. Dilutions prepared from the supernatant were placed on Petri plates containing Dichloran Rose Bengal Agar medium supplemented with chloramphenicol (DRBC) or Dichloran Glycerol Agar (DG-18)(0,1 ml/plate). The inoculated plates were incubated at room temperature (22-24°C) for 3-5 days. The isolated fungal colonies were recovered, cultivated on PDA (Potato dextrose agar) and used in further experiments.

Preliminary identification of fungal strains

Morphological features of *Aspergillus* cultures were studied, the major and remarkable macroscopic features in species identification were the colony diameter, color (conidia and reverse), exudates and colony texture. Microscopic characteristics for the identification were conidial heads, stipes, color and length vesicles shape, covering, conidia size, shape and roughness also colony features including diameter after 7 days, color of conidia, mycelia, exudates and reverse, colony texture and shape. As a final we compared the morphological characteristics of tested *Aspergillus* isolates with those of the standard species. Microscope used Zeiss Stemi 2000-C and Olympus URFLT 50.

Screening for mycotoxin (aflatoxin) producing strains

Three different media were used for detection of mycotoxigenic abilities of fungal strains: Potato Dextrose Agar (PDA) supplemented with 0.3% methyl-β-Cyclodextrin and 0.6% Sodium Deoxycholate(PDAC) [3], YES [4] with 0.3% β Cyclodextrin and 0.6% Sodium Deoxycholate (CCD) [5, 6], and Coconut agar medium [7].

The presence of a beige opaque ring surrounding the fungal colonies cultivated on PDACD and CCD is associated to aflatoxin production [8, 9]. On coconut medium the production of mycotoxins was revealed by examination on UV light (365nm): blue florescence is associated with aflatoxin production, and blue-green fluorescence with ochratoxin production [9, 10].

TLC for mycotoxin separation

Blocks of aprox.1 cm^2 were cut from the edges of fungal colonies grown of PDA medium and extracted with 5 ml of chloroform (2 times) for 30 min. Samples were centrifuges and the supernatant was subjected to evaporation. The sediment was dissolved in 0,1ml of chloroform and used for TLC on silica gel 60 Plates (10x10cm). The extracts were applied on silica gel 60 plates, dried and the separation was carried out using toluene: ethylic acetate: formic acid (15/14/1 v/v) as mobile phase. The detection of aflatoxin/ ochratoxin specific spots was observed under UV light (365nm).

Quantitative analysis of aflatoxin was performed with RIDA® QUICK Aflatoxin RQS kit from R-Biofarm AG, according to kit protocol using 4 days old liquid fungal culture in APA broth [10]

3. Results and discussion

3.1. Isolation of new fungal strains

A number of 44 fungal distinct colonies were isolated from seeds used in experiments. The largest contamination was observed in barley seeds, both with aspergilla and other fungal contaminants (fig.1).

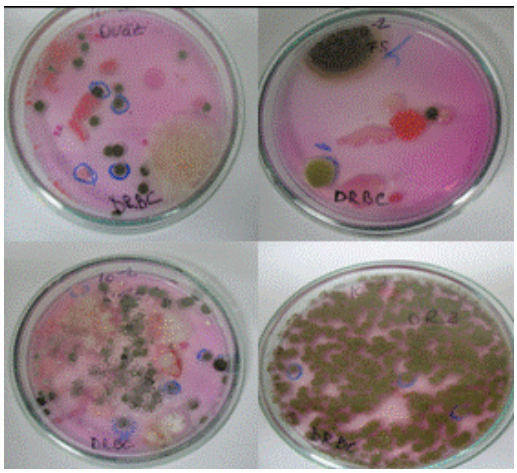

Figure 1. Macroscopic aspect of the fungal colonies

However, among the fungal isolates 14 colonies presented the phenotypic characteristics specific for *Aspergillus* section *flavi* (fig.2).

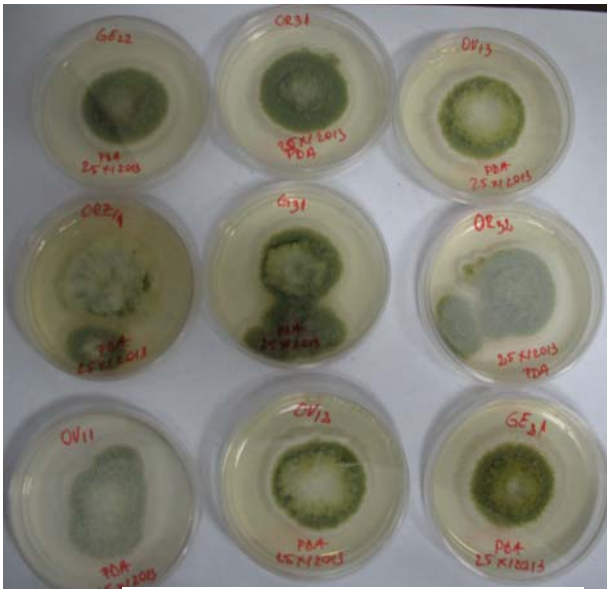

Figure 2. Phenotypic differences among Aspergillus spp. colonies

3.2. Screening of aflatoxin producing fungi

The ability of aflatoxin biosynthesis was detected on various culture media, supplemented with β-cyclodextrine and sodium deoxycholate. The best results (clear visualization of opaque ring around the fungal colonies) were obtained with PDA medium with methyl β-cyclodextrine and sodium deoxycholate after 7 days of incubation in the dark, at 26°C (fig.3).

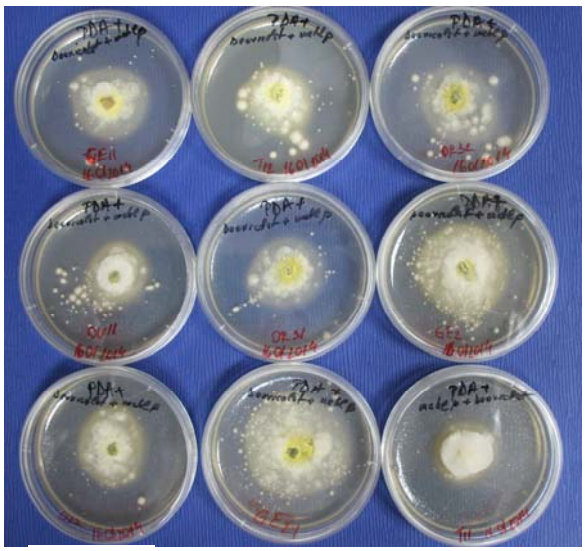

Figure 3. Ring surrounding the fungal colonies is associated to aflatoxin production

Clear differences among the ring diameter and the moment of apparition were observed among the fungi: the largest rings were obtained with the isolates designated as T11, GE2, OR31, G32 after 10 days of incubation, comparing with the strains ORZ11, ORZ12, T12 were the ring was hard to detect. Moreover, for the strains T11, G32 the ring started to be observed after 24h of incubation, instead the rest of strains were the ring was visible after 3 days (fig 4).

		2	3	4	5	6	7	8	9	10
GE2	0	0.2	0.3	0.5	0.7	1	1	1	1	1
GE21	0	0	0.2	0.3	0.3	0.7	0.7	1	1	1
GE22	0	0	0.1	0.5	0.5	0.5	0.5	0.5	0.5	0.5
GE11	0	0	0	0.5	0.5	0.8	1	1	1	1
OR31	0	0.1	0.2	0.5	0.7	0.7	1	1.2	1.2	1.2
OR32	0	0	0.2	0.3	0.5	0.5	1	1	1	1
OV11	0	0	0	0.2	0.5	0.5	0.7	0.7	0.7	0.7
OV12	0	0	0.2	0.2	0.3	0.5	0.5	0.7	0.7	0.7
T11	0.1	0.3	0.5	0.7	0.7	1	1	1.2	1.4	1.4
T12	0	0	0.2	0.2	0.5	0.5	0.7	0.9	0.9	0.9
G32	0.1	0.5	0.5	0.5	0.5	0.9	0.9	0.9	1.2	1.2
G31	0	0	0.2	0.5	0.5	0.6	0.6	0.6	0.7	0.7
ORZ12	0	0	0.2	0.2	0.2	0.5	0.5	0.5	0.7	0.7
ORZ11	0	0	0	0	0.2	0.2	0.5	0.5	0.5	0.5

Figure 4. Ring diameter surrounding the fungal colonies associated to aflatoxin production

On coconut medium, blue fluorescence (associated with aflatoxin production) on reverse side of some fungal colonies, as well as green-blue fluorescence (associated with ochratoxin production) was observed in UV light (Fig. 5).

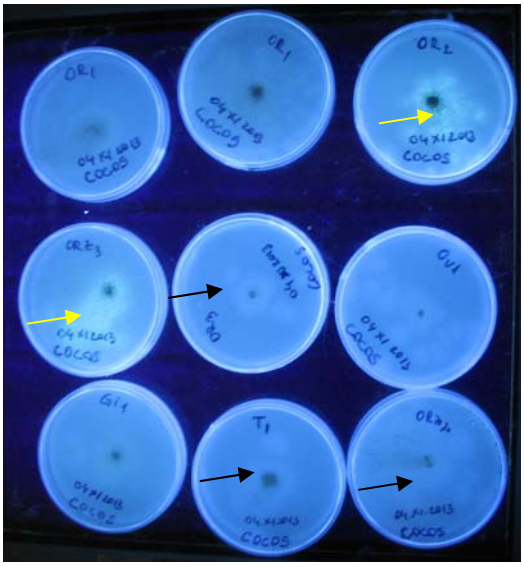

Figure 5. Specific fluorescence associated with ochratoxin (yellow arrow) or aflatoxin production (black arrow)

The level of aflatoxin produced by these strains, after 4 days of cultivation in APA broth was evaluated using RIDA® QUICK Aflatoxin RQS kit (R-Biofarm AG). The results confirm the observations on cyclodextrine and deoxycholate containing media: the level of aflatoxins was significant: between 6.1-6.5 ppb, comparing with 5.2 ppb detected for the collection *A. flavus* used as reference.

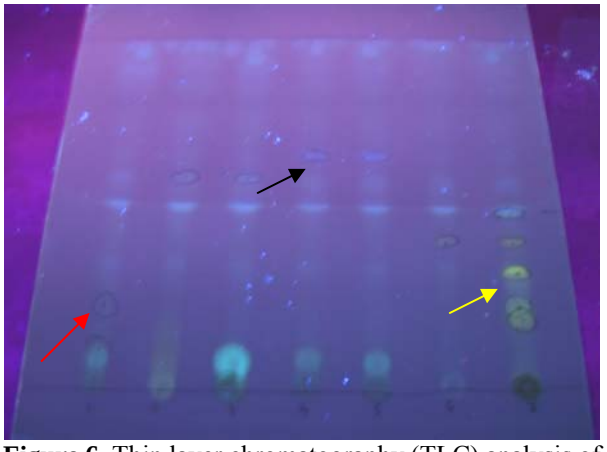

Figure 6. Thin layer chromatography (TLC) analysis of chloroform extracts from potential aflatoxin producing fungal isolates. The arrows indicate the position of aflatoxin corresponding spots (red arrow-AFB1, the black arrow-AFB2 and yellow arrow-AFG2).

When TLC analysis was performed, the results obtained (based on Rf calculation and comparison with the data from literature) [11], confirm the presence of aflatoxins in several strains: among seven fungal isolates with clear fluorescent or opaque halo, the isolates designated GE2,OR31 presented AFB1, the isolates A. Flavus presented AFB2 and the isolate AN5 presented AFG2 (fig.6).

At this stage of experiments three fungal isolates, designated as GE2, G32, T11 were selected as aflatoxin B1, B2, G1 and used for further analysis (molecular identification, interactions with LAB and yeasts).

4. Conclusions

The isolation procedure allows the selection of several contaminant fungi from seeds, the highest contamination with aspergilla being detected in barley seeds.

A number of 14 *Aspergillus spp.* isolates were selected from wheat, barley, triticale, oats, and sunflower seeds and identified, based on macroscopic and microscopic features as *A.flavus/A.parasiticus.* Their ability to produce aflatoxins was evaluated using three methods: selective media, UV examination of fungal colonies, and TLC.

Three out the aspergilla, designated as GE2, G32, T11 were selected as aflatoxin B1, B2, G1 and will be used for further analysis (molecular identification, interactions with LAB and yeasts).

Furthermore, the assessment of the quality of foods using this method provides an acceptable, accurate, and alternative method to establish guidelines and to evaluate the status of aflatoxins contaminated grains.

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