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ISOLATION AND IDENTIFICATION OF MYCOTOXIGENIC FUNGI FROM WHEAT GRAINS STORED IN BIHOR COUNTY

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Abstract
The purpose of this study was to identify mycobiota and mycotoxins content of wheat grains. For this purpose, 15 samples were collected from wheat grains depositing in several storages from Bihor county, during period October - March. Analyses were done using the conventional methods, on fungal species isolated of Aspergillus and Fusarium, in order to determine their mycotoxigenic potential, while mycotoxins analysis for aflatoxin B1, ochratoxin A and zearalenone were assessed using thin layer chromatography and high performance liquid chromatography. Two different genera including Fusarium and Aspergillus were isolated from wheat samples. Of the fungal spp. isolated, Aspergillus was the most predominant and was succeeded by species of Fusarium. Two dominant species from Aspergillus genus: Aspergillus ochraceus and Aspergillus flavus were isolated from the wheat samples. Mycotoxins were identified and quantified with HPLC and TLC techiques.

Key words: TLC, HPLC, fungi, Aspergillus, Fusarium.

INTRODUCTION

A variety species of microorganisms often contaminate the wheat grains before harvesting or after harvesting and other species may develop when grains are in silo conditions. In all cases they decrease quality of wheat (Magan, N., et al., 2003).

There are a lot of factors that affects wheat grains health, but from all of them, the most important are fungi. Some of their effects on wheat grains consists in fact that they reduces seed germination and vigor and cause decrease quality during storage (Doohan, F.M., et al., 2003).

Fungi produce mycotoxins under stressful conditions such as temperature, moisture or aeration. There are some conditions which influence the fungal growth such as : species, a high temperature and moisture. Fungi grow at temperatures between 20–30°C. Normally, fungi grow in storage conditions at a 13–18% moisture (Novošinskas H. et al., 2005).

There are two important groups of fungi: field fungi and storage fungi. First one are those that invade the seeds while the seccond is still in the field and require high moisture conditions (20-21%) (CAST, 2003).

Amongst the fungal genera, Fusarium and Alternaria are considered most important because of their toxigenic ability to produce mycotoxins and they are classified by some authors as field fungi, while, Aspergillus and

Penicillium species are often considered storage fungi (Roige M. et al, 2009).

Fumonisin, aflatoxin, ochratoxin, zearalenone and trichothecenes such as deoxynivalenol, T-2 toxin and nivalenol are appreciate as most important mycotoxins (Shepard G. S., 2008).

The important mycotoxins produced by Aspergillus species include aflatoxin B1, B2, G1 and G2, ochratoxin A, sterigmatocystin and cyclopiazonic acid. Aflatoxins are produced mainly by A. flavus, A. parasiticus and A. nominus.

Zearalenone is a phenolic resorcyclic acid lactone mycotoxin produced by several Fusarium species, particularly Fusarium graminearum. The presence of Zerealenone in cereals is often accompanied with other Fusarium toxins including trichothecenes and fumonisins.

MATERIAL AND METHODS

Most currently used techniques for mycotoxins determination are chromatography including high performance liquid chromatography (HPLC), thin layer chromatography (TLC), gas chromatography, mass spectroscopy (GCMS) and ELISA techniques. Analytical determination is important for the assessment of mycotoxins. It is an innovative alternative to conventional technique (Mahendra K. Rai et al., 2012).

In our study, for fungal isolation and identification we have used a HPLC method. We have done the separation and purification using normal and reversed-phase columns.

For the study purpose, we took samples consisting of 40 grains. We have introduced malt extract agar medium in Petri dishes and after that we have plated the samples in those Petri plates and we have incubated them at 25°C for 10 days. After this period, the fungal colonies that have developped on the surface of medium, were counted (Collins, C. H., 1989) for determining colony forming unit per gram of sample (cfu/g). Sub-culturing of isolated colonies was done on potato dextrose agar, Czapek yeast agar and malt extract agar for 14 days at 28°C followed by the micro- and macroscopic identification of fungal species using the keys described for fungi genera (Bradshaw, J. L., 1992).

In order to extract OTA from the isolates belonging to Aspergillus genus we have used High Performance Liquid Chromatography method. The extracts were analyzed by HPLC equipped with a fluorescence detector. Development of analysis methods was according to International Official Methods of Analysis.

The isolated fungal spp. of Aspergillus were analysed for their ability to produce the aflatoxin AFB1. For this aim, the fungal subspecies

were re-inoculated on yeast extract sucrose agar and incubated for 2 weeks at 28ºC. The colonies isolated were removed from the Petri dish with culture medium and extracted in dichloromethane. The solution was vortexed, filtered and spotted on a two-dimensional TLC plate. The analysis was done along side with mycotoxin standard. The intensities of fluorescence of mycotoxin spots were estimated by using a fluoro densitometer. The fluorescing colour and retardation factor value of the spot of the extracts were compared with that of the mycotoxin standard. According to studies of other authors from literature (Bennett G.A. et al., 1979), in order to determine the zearalenone we took samples and we have followed next stages: extraction, partial purification of the extract by column chromatography, liquid-liquid partitioning, subsequent measurement.

RESULTS AND DISCUSSION

Mycotoxins are not distributed uniformly in all amount of stored grains. In most cases, the toxin level varies from one seed to another. Moreover, it is possible that highly contaminated seeds to be next to seeds in which the toxin cannot be detected. Because of this variable distribution, it is very difficult to obtain representative samples to analyze.

The results obtained after we have analyzed the 15 samples of stored wheat grains indicate an important infestation with all three categories of mycotoxins that we have determined in samples: aflatoxin B1, Ochratoxin A and Zearalelone. The levels of infestations are relatively high, but the increased values compared with the tolerance level are more significant for aflatoxin B1 and Ochratoxin A.

The levels of ZEA in this study were higher than the EU acceptable level (350 µg/kg) for unprocessed wheat grains. The levels of Aflatoxin B1 and Ochratoxin A determined for samples of wheat grains were also higher than the EU acceptable level (5 µg/kg for Aflatoxin B1 and 5 µg/kg for Ochratoxin A).

Although the number of analyzed sample were not high, the results of this study indicate some significant contamination of wheat grains based on production of mycotoxins and emphasizes the need for further and regular studies of wheat grains stored in order to identify toxins, to evaluate and to prevent the risks in human and animal.

Table 1

Toxigenicity of fungal strains isolated from wheat samples

Fungal species	Type of toxin produced	Intensity of toxin produced
Aspergillus flavus	AFB1	++
Aspergillus ochraceus	OTA	++
Fusarium graminearum	ZEA	++

Table 2

Values of wheat samples for extracting mycotoxins

Type of mycotoxin isolated from samples	AFB1	OTA	ZEA
Incidence rate (%)	80	73	46
Range (μg/kg)	0-72	0-60	0-854
Mean (μg/kg)	31	27	201

Table 3

Results for mycotoxins determined with HPLC

Number of sample	AFB1 (μg/kg)	OTA (μg/kg)	ZEA (μg/kg)
1	55.1	60	0
2	15.2	13.7	0
3	72	56.4	0
4	0	0	360.2
5	36.2	33.8	0
6	0	0	358.4
7	41.8	42.6	854
8	18.6	28.4	352.3
9	0	0	347.6
10	12.4	18.2	0
11	13.4	37.6	0
12	58.7	0	368.4
13	66.5	25.8	0
14	48.5	33.6	390.5
15	22.3	57.3	0

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