RESEARCH ON FUNGAL CONTAMINATION AND TOTAL AFLATOXIN CONTENT IN SOME LOCAL MAIZE (*ZEA MAYS*) POPULATIONS

CERCETĂRI PRIVIND CONTAMINAREA FUNGICĂ ȘI CONȚINUTUL TOTAL DE AFLATOXINĂ LA UNELE POPULAȚII LOCALE DE PORUMB (*ZEA MAYS*)

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Abstract. *Mycotoxins are toxic chemicals produced by fungal species like Aspergillus, Fusarium, Penicillium and Alternaria which could reach into macro-organisms by food and fodder administration. Mycotoxins can reach the human body not only through the consumption of cereals or food prepared from contaminated seeds, but also through the consumption of milk, meat or eggs from animals fed contaminated feed. The paper presents researches carried out in order to establish contamination with potentially toxic fungi of maize, taking into account climatic and storage conditions. It was analyzed, on the one hand an evaluation of epiphytes and endophytes mycological flora that occurs on the seeds, after medium-term storage, and on the other hand a toxicological characterization in order to avoid or reduce the damage caused by the fungal pathogens.This study led to a better understanding of the cause-effect relationship, where the cause is represented by the action of specific micromycetes during the growing period or storage period, and the effect is quantified by the level of viability and seed health according to the duration of conservation, respectively the degree of infection with mycotoxins. The experiments performed represent a special material basis for growers and processors (milling and bakery units), by highlighting the risk factors that determine contamination with toxic fungi and the production of mycotoxins. It is a unanimous requirement to evaluate the quality of cereals intended for human and animal consumption. The results of the study show that prevention of mycotoxins is possible through creation of disease-resistant hybrids and agro technical measures: crop rotation, tillage system, fertilization, choice of varieties and phytosanitary treatments (diseases, pests, weeds, etc.), infestation control with mycotoxins prior to harvesting, ensuring appropriate storage conditions.*

Key words: mycotoxins, local populations, total aflatoxin

Rezumat. *Micotoxinele sunt substanțe chimice toxice produse de specii de ciuperci precum Aspergillus, Fusarium, Penicillium și Alternaria care ar putea ajunge în macroorganisme prin alimentație și furaje. Micotoxinele pot ajunge în organismul uman nu doar prin consumul de cereale sau alimente*

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preparate din semințe contaminate, ci și prin consumul de lapte, carne sau ouă de la animale hrănite cu furaje contaminate. Lucrarea prezintă cercetări efectuate în vederea stabilirii contaminării cu fungi potențial toxici ai porumbului, ținând cont de condițiile climatice și de depozitare. S-a analizat, pe de o parte, o evaluare a florei micologice epifite și endofite care apare pe semințe, după depozitare pe termen mediu, iar pe de altă parte o caracterizare toxicologică pentru a evita sau reduce daunele cauzate de agenții patogeni fungici. Acest studiu a condus la o mai bună înțelegere a relației cauză-efect, unde cauza este reprezentată de acțiunea unor micromicete specifice în perioada de creștere sau perioada de depozitare, iar efectul este cuantificat prin nivelul de viabilitate și sănătatea semințelor în funcție de durata. de conservare, respectiv gradul de infectare cu micotoxine. Experimentele efectuate reprezintă o bază materială deosebită pentru cultivatori și procesatori (unități de morărit și panificație), prin evidențierea factorilor de risc care determină contaminarea cu fungi toxici și producerea de micotoxine. Este o cerință unanimă evaluarea calității cerealelor destinate consumului uman și animal. Rezultatele studiului arată că prevenirea micotoxinelor este posibilă prin crearea de hibrizi rezistenți la boli și prin măsuri agrotehnice: rotația culturilor, sistemul de lucrare a solului, fertilizare, alegerea varietăților și tratamente fitosanitare (boli, dăunători, buruieni etc.), controlul infestării cu micotoxine înainte de recoltare, asigurându-se condiții adecvate de păstrare.

Cuvinte cheie: micotoxine, populații locale, aflatoxină totală

INTRODUCTION

Mycotoxins are an alarm signal by their simple presence in food, even in low concentrations endanger animal health and, implicitly, human health, by affecting the immune response, and their determination in caryopsis of cereals intended for genetic preservation is a primary activity in gene banks, thus preventing their contamination by regenerating or multiplying germplasm in experimental fields or by use by various external users (Miller, 1998).

Maize is the main food for a large part of the population and contains a significant part of the protein, energy, and mineral requirements, but unfortunately, we are not aware of the risk of the introduction into the human body of the secondary metabolites produced by the diseases attacking or molds that can be installed on stored grains (Muntean, 1995). The undesirable effect of these mycotoxins does not occur immediately, except in cases of serious poisoning, they are causing damage to human and animal health after a gradual accumulation process. The impact is complex, the interactions between mycotoxins and the organism reach the genome level by denaturing cellular DNA, the impact being difficult to quantify, and due to the reduced molecular size, some mycotoxins can circulate throughout the food chain: feed, animal products, final consumer (Pop, 2013).

Mycotoxins are toxic secondary metabolites developed by phytopathogenic agents on various substrates through a series of enzyme-

catalyzed reactions under specific conditions: genetic capacity, substrate, humidity, substrate pH, temperature, aeration, brightness, competing microorganisms, time period to contamination. They are found in spores of fungus or as their secretion products in substrates where they develop. There are about 300-400 mycotoxins belonging to 24 chemical groups of toxins that may occur under different conditions in agricultural crops and in the various foodstuffs obtained from them.

Generally, high humidity and temperatures promote the growth of pathogenic agents and the production of mycotoxins. Issues that arise in the field of production technologies, poor harvesting and storage, transport and marketing conditions also contribute to the installation of a wide range of molds, from the least harmful to the ones, which develops highly pathogenic mycotoxins (Șara and Borobil, 2007).

Recent studies have indicated the existence of high concentrations of mycotoxins in cereals and cereal products. From the cereals, maize is the most strongly affected by fungal infestation and mycotoxin contamination. These results require a vigilant attitude and the taking of measures to protect consumers and to increase their confidence in products that maximize the nutritional potential of cereals (Ittu, 2008).

The study aimed to perform a mycotoxicological screening that was completed by analyzing 30 maize samples from the Suceava Gene bank collection. The total aflatoxin content (B1 + B2 + G1 + G2) was determined by ELISA enzyme immunoassay.

The purpose of this research was to:

- phytopathological evaluation of epifite and endophyte mycoflora from preserved seeds at different time intervals, in controlled atmosphere storage (T = + 4°C, relative air humidity = 30-40 %), by indicating the number of infected cariopses and genus of isolated pathogens;
- identification of fungal microorganisms according to the storage period of analyzed cariops;
- determination of the influence of CGA medium substrate type (potato - dextrose - agar), Sabouraud medium (yeast extract - dextrose - chloramphenicol - agar), blotting paper on the evolution of fungal pathogens;
- identification of the correlations between the evolution of the identified micromycetes, the seed storage periods and the type of substrate used.
- quantitative determination of the total aflatoxin content secreted by the fungal microorganisms to the samples taken in this study.

The information obtained from the analyzes and determinations carried out are presented in tables and graphs, and to some of them were used statistical methods to relieve some correlations and significant regressions between different elements.

The calculation of the results for the quantitative determination of the fungi was performed according to the STAS ISO 7954/2001 method.

Quantitative determination of mycotoxins was performed using Ridawin mycotoxin kit software for total aflatoxin.

MATERIAL AND METHOD

As biological material, was studied 30 local maize populations, with different origins, 300 seed/ sample from the Suceava Genebank collection, namely conserved seeds for 17 and 8 years at T = +4 °C.

Experimental conditions can be grouped as follows:

- specific storage conditions for medium-term conservation: T = +4 °C (± 1 °C), humidity relative air

= 30-40 %, and seed moisture percentage between 5-8%;

- phytopatological analysis (by determining the presence of specific micromycetes on the analyzed maize seeds, after different storage periods, by classical methods of phytopathological analysis (Ulster method on CGA medium - potato - dextrose - agar), blotting paper method and Sabouraud method (yeast extract - dextrose - chloramphenicol - agar).
- toxicological analyzes, the mycotoxin content being determined by the thin layer chromatography method (C.S.S.) and the ELISA immunoenzymatic method, a method of rapid quantitative screening which is based on the antigen - antibody reaction, using the aflatoxin - specific kit for analysis.

The experiments regarding the fungal contamination of the maize samples taken in the study were performed in the phytopathology laboratory of the Suceava Genebank.

Toxicological experiments for the quantitative determination of aflatoxins were carried out with the support of Residue Control and Toxicology Laboratory from Suceava.

Several species of fungi of the genera *Fusarium, Penicillium* and *Aspergillus* have been identified in naturally contaminated maize. The samples were analyzed from a mycological and mycotoxicological point of view.

The statistical calculation of the data from this study was performed by the method of analysis of correlations and regressions between the analyzed elements (Ceapoiu, 1968).

RESULTS AND DISCUSSIONS

The purpose of this study was:

- phytopathological evaluation of the epiphytic and endophytic mycoflora on the seeds preserved at different time intervals, in the controlled conditions (T = + 4 °C; relative air humidity = 30 - 40%), by indicating the number of infected caryopsis and genera of isolated pathogens.
- identification of fungal microorganisms depending on the storage period of the analyzed caryopsis.

- establishing the influence of the type of substrate medium CGA (potato - dextrose - agar), Sabouraud medium (yeast extract - dextrose - chloramphenicol - agar), blotting paper on the evolution of fungal pathogens.
- highlighting the correlations between the evolution of the identified micromycetes, the seed storage periods and the type of substrate use.
- quantitative determination of the total aflatoxin content secreted by fungal microorganisms.

Regarding the phytopathological evaluation, the micromycetes were evaluated by counting infected seeds and the frequency of attack was expressed as a percentage, by visual estimation of the seed area.

Maize seeds, placed on CGA medium and blotting paper, after the incubation period, showed different characteristics regarding the presence of fungal microorganisms.

On 30 maize samples, kept at +4°C, but for different conservation periods (17 and 8 years), the largest number of micromycetes was found on agar medium compared with other mediums (Sabouraud, blotting paper).

Samples in different conservation periods were infected in different proportions by fungal pathogens, but the total number of infected seeds is much higher on CGA medium. For those seeds kept at + 4°C for 8 years, the micromycetes infected 112 seeds from a total of 150, resulting a 75 % infection rate, while those stored for 17 years were infected a total number of 443 from 750 taken in this study, the infection rate being 59 % (tab. 1).

Table 1

Proportion of micromycetes isolated on *Zea mays* seeds placed on CGA medium

Experimental conditions	**Seeds conserved at T=+ 4 ^{0}C, for 8 years**	**Seeds conserved at T= +4 ^{0}C, for 17 years**
Isolated micromycetes	Frequency of attack (%)	
Penicillium sp.	32	20.5
Aspergillus sp.	4	3.1
Rhizopus sp	11.3	23.2
Mucor sp.	4	2
Cladosporium herbarum	2	1.5
Alternaria alternata	2	1.5
Trichothecium roseum	2	1.1
Chaetomium sp.	0	1.5
Fusarium moniliforme	14.6	4.8
Oedocephalum sp.	2.6	0
TOTAL	74.5	59.2

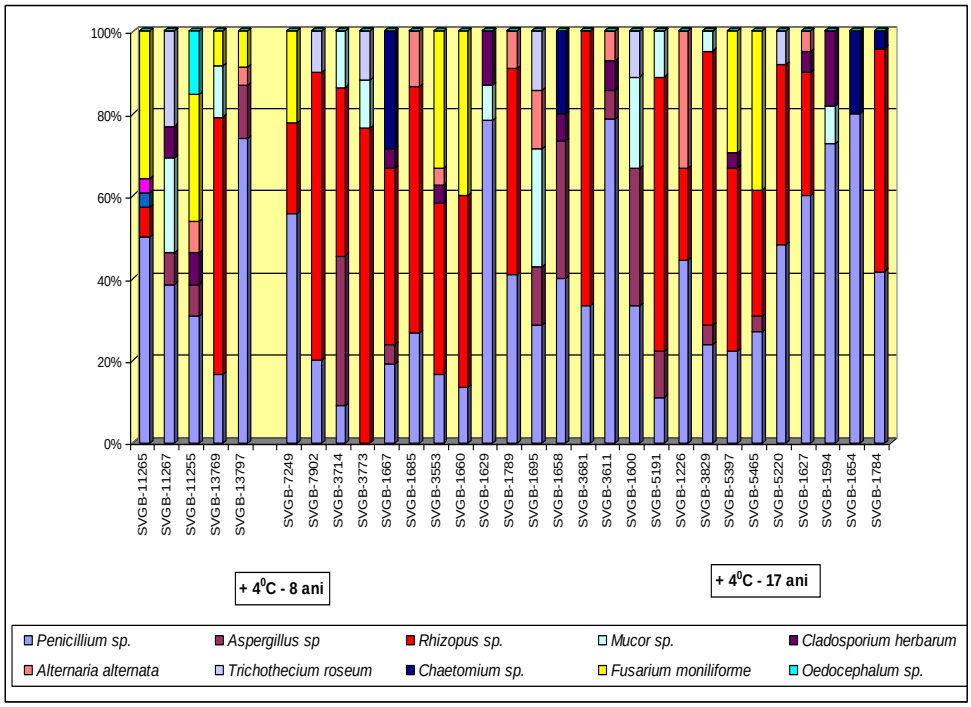

Fig. 1 Infection percentages of fungal pathogens isolated on CGA medium in *Zea mays* samples stored under controlled environmental conditions (+ 4 ^{0}C, for a period of 8 and 17 years)

The genera *Penicillium* and *Rhizopus* were dominant, infecting the largest number of seeds, manifesting themselves much more strongly on the CGA medium, compared to the Sabouraud medium. The other genera of fungal microorganisms were isolated on a small number of samples, the number of infected seeds in each sample being very small (fig. 1).

In order to establish the complementary action of some micromycetes identified on the maize seeds in the two storage periods, the correlation coefficients between the action of the fungal pathogens identified on the samples studied were determined.

The results obtained indicate a small number of correlations statistically assured at both storage periods. After 17 years of storage of *Zea mays* caryopsis at + 4°C, there are only two significant positive correlations between the action of fungal pathogens *Muco*r sp. x *Aspergillus* sp. and *Cladosporium herbarum x Penicillium sp*. (tab.2).

Table 2

Correlation coefficients between the actions of micromycetes identified on *Zea mays* samples stored at +4 ° C for 17 years

Characteristics corelated	*Penicillium sp.*	*Aspergillus sp.*	*Rhizopus sp.*	*Mucor sp.*	*Cladosporium herbarum*	*Alternaria alternata*	*Trichothecium roseum*	*Chaetomium sp.*	*Fusarium moniliforme*
Penicillium sp.	1								
Aspergillus sp.	-0.2822	1							
Rhizopus sp.	-0.2251	-0.1388	1						
Mucor sp.	-0.2430	0.5338*	-0.1111	1					
Cladosporium herbarum	0.5856*	-0.0894	-0.4089	0.0793	1				
Alternaria alternata	-0.0092	-0.2180	-0.1408	-0.2439	-0.1216	1			
Trichothecium roseum	-0.1768	-0.1072	0.2336	0.2017	-0.2765	-0.1853	1		
Chaetomium sp.	-0.0882	0.1808	-0.0572	-0.2167	0.1702	-0.1854	-0.1602	1	
Fusarium moniliforme	-0.0637	-0.1652	0.1633	-0.3048	0.0028	-0.1291	-0.2254	-0.1615	1

Regarding the quantitative toxicological determination of mycotoxins, the total aflatoxin concentrations (B1 + B2 + G1 + G2) obtained for the 30 analyzed samples are represented in table 3.

Table 3

Values of total aflatoxin concentration by ELISA method

Nr. crt.	Number of accession	Total aflatoxin concentration (µg/kg (ppt)	Nr. crt.	Number of accession	Total aflatoxin concentration (µg/kg (ppt)
1	SVGB-7249	2855.82	16	SVGB-11265	3763.05
2	SVGB-7902	2614.71	17	SVGB-13769	4943.09
3	SVGB-3714	3114.45	18	SVGB-11267	3728.55
4	SVGB-13797	5634.46	19	SVGB-11255	3119.77
5	SVGB-3773	2807.16	20	SVGB-1600	3448.57
6	SVGB-1667	2650.51	21	SVGB-5191	2984.46
7	SVGB-1685	2855.82	22	SVGB-1226	2823.84
8	SVGB-5353	2686.44	23	SVGB-3829	3160.51
9	SVGB-1660	2356.90	24	SVGB-5397	2725.96
10	SVGB-1629	2519.97	25	SVGB-5465	3025.38
11	SVGB-1789	3383.35	26	SVGB-5220	3255.94
12	SVGB-1695	3867.69	27	SVGB-1627	3320.42
13	SVGB-1658	2997.90	28	SVGB-1594	2764.88
14	SVGB-3681	2840.07	29	SVGB-1654	3087.35
15	SVGB-3611	3318.89	30	SVGB-1784	3562.65

By evaluating the action of the deposit mycoflora encountered in the samples of *Zea mays*, it is observed that the samples that had a total aflatoxin

concentration above the allowed limit were kept for 8 years at a temperature of +4°C. The SVGB-13769 sample had the highest degree of infection caused by *Penicillium* sp. out of 30 analyzed seeds, 20 being infected by *Penicillium* sp. The SVGB-13797 sample had a high degree of infection with *Penicillium* sp. and *Aspergillus* sp., so that of the 30-caryopsis analyzed, 17 were infected with *Penicillium* sp., and 3 were attacked by *Aspergillus* sp. (tab. 2).

The maximum limit allowed and recommended by the EU for the total aflatoxins identified in cereals and products derived from them (provided in order 145/2004) is 4 µg/kg (ppb), respectively 4000 µg/kg (ppt).

Analyzing the results obtained by determining the total aflatoxin concentration in maize samples, it is observed that only 2 samples had a total aflatoxin concentration above the maximum limit allowed by the EU, being considered positive (SVGB-13797 - the sample no. 4 with concentrations 5634.46 µg/kg ppt (5.64 µg/kg ppb) and SVGB-13769 - the sample no. 17 with concentration 4943.09 µg/kg ppt (4.94 µg/kg ppb) (table 3, 4, 5). In this case, aflatoxins were secreted by fungal microorganisms of the genera *Penicillium* and *Aspergillus.*, the most common of these genera being *A. flavus, A. ochraceus, A. niger, A. fumigatus.*

Table 4

Determination of total aflatoxin concentration by ELISA method (P1-P10)

Standards

Ser. No.	Concentration ppt	Absorbance (Mean)	(CV)	B/B0 (%)	calculated ppt	Deviation (%)
1	0.0	1.872	3.7	100.0		
2	50.0	1.693	0.8	90.4	50.10	0.2
3	150.0	1.411	1.6	75.4	149.70	0.2
4	450.0	0.873	7.2	46.6	450.30	0.1
5	1350.0	0.402	3.7	21.5	1351.70	0.1
6	4050.0	0.139	9.2	7.4	4044.50	0.1

Samples

Ser. No.	ID	Absorbance (Mean)	(CV)	(%)	calculated ppt	*	=	ppt
1	P1 porumb	1.595	1.2	85.2	81.59	35.00		2855.82
2	P2 porumb	1.615	0.6	86.3	74.71	35.00		2614.71
3	P3 porumb	1.574	1.2	84.1	88.98	35.00		3114.45
4	P4 porumb	1.382	0.6	73.8	160.98	35.00		5634.46
5	P5 porumb	1.599	0.4	85.4	80.20	35.00		2807.16
6	P6 porumb	1.612	0.1	86.1	75.73	35.00		2650.51
7	P7 porumb	1.595	0.1	85.2	81.59	35.00		2855.82
8	P8 porumb	1.609	0.7	86.0	76.76	35.00		2686.44
9	P9 porumb	1.637	0.3	87.4	67.34	35.00		2356.90
10	P10 porumb	1.623	0.0	86.7	72.00	35.00		2519.97

Table 5

Determination of total aflatoxin concentration by ELISA method (P11-P20)

Standards						
Ser. No.	Concentration ppt	Absorbance (Mean)	(CV)	B/B0 (%)	calculated ppt	Deviation (%)
1	0.0	1.983	1.4	100.0		
2	50.0	1.752	1.1	88.4	50.00	0.0
3	150.0	1.419	0.7	71.6	149.90	0.1
4	450.0	0.937	0.6	47.3	449.70	0.1
5	1350.0	0.432	1.3	21.8	1352.70	0.2
6	4050.0	0.152	2.8	7.7	4043.30	0.2

Samples								
Ser. No.	ID	Absorbance (Mean)	(CV)	(%)	calculated ppt	*	=	ppt
1	P11 porumb	1.569	0.3	79.1	96.67	35.00		3383.35
2	P12 porumb	1.526	0.6	77.0	110.51	35.00		3867.69
3	P13 porumb	1.606	0.2	81.0	85.65	35.00		2997.90
4	P14 porumb	1.622	0.5	81.8	81.14	35.00		2840.07
5	P15 porumb	1.575	0.8	79.4	94.83	35.00		3318.89
6	P16 porumb	1.535	1.2	77.4	107.52	35.00		3763.05
7	P17 porumb	1.441	0.8	72.7	141.23	35.00		4943.09
8	P18 porumb	1.538	0.4	77.6	106.53	35.00		3728.55
9	P19 porumb	1.594	0.6	80.4	89.14	35.00		3119.77
10	P20 porumb	1.563	0.8	78.8	98.53	35.00		3448.57

CONCLUSIONS

1. From the period of growth and development and later in storage, both the vegetative part and the final product are constantly attacked by a variety of phytopathogenic fungi and molds produced by different species of fungi. Fungi, through the action of secreted enzymes, break down nutrients from the food and feed they meet.

2. The main pathogenic fungi that cause significant damage to cereal crops, turning crops into sources of mycotoxin contamination, are part of the following genera*: Fusarium*, *Tilettia, Ustilago, Puccinia*, *Claviceps purpurea*, *Penicillium* and *Aspergillus*. Microbiological analyzes reveal the high load of *Penicillium* fungi present on the grains of maize populations. Of the total toxicogenic fungi identified, 43% belong to the genus *Penicillium,* 20 % belong to the genus *Fusarium*, 5 % to the genus *Aspergillus*.

3. The studied populations represent a diversified biological material with a high variability. The deposit mycoflora developed on the seeds studied was analyzed according to the genotype, the conservation period of the seeds, the type of substrate

used and thus some correlations were established between the evolution of micromycetes isolated on seeds and the influence of action on the germination of seeds.

4. The conservation period is negatively correlated with the longevity of fungal pathogens, in the sense that the higher it is, the lower the degree of infection.

5. The viability of the samples under study was not greatly influenced by the degree of infection of the seeds with fungal pathogens, which shows that by preserving the samples under controlled atmosphere conditions for different periods of time, the decrease in germination is not only determined by the appearance of micromycetes. The increase in the frequency of micromycetes on seeds is determined by a complex of factors (genotype, chemical composition, genetic resistance) that together can lead to decreased seed viability.

6. By evaluating the action of the deposit mycoflora encountered in the samples studied, it was observed that all samples that had a high concentration of total aflatoxins were stored for 8 years at a temperature of + 4°C.

7. Samples with many fungal pathogens showed a high concentration of total aflatoxin (5634. 46/4943.09 (µg/kg (ppt)) which demonstrates the correlation between mycotoxins secreted by toxin-containing fungal microorganisms and the number of infected seeds.

8. The determination of mycotoxins secreted by toxic microorganisms has played an important role in the more accurate determination of some species of *Aspergillus* and *Penicillium.*

9. The results show that maize is frequently contaminated with fungi and mycotoxins. Prevention of mycotoxins is possible in three very important ways: creation of disease-resistant hybrids, agro technical measures: crop rotation, tillage system, fertilization, choice of varieties and phytosanitary treatments (diseases, pests, weeds, etc.), infestation control with mycotoxins prior to harvesting, ensuring appropriate storage conditions.

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