

# Contamination of Plant Substrates Evaluated by Mycological and Mycotoxicological Investigations

Ioana Poroşnicu<sup>1,3\*</sup>, Nicolae I. Bădilaş<sup>2</sup>, Mădălina A. Davidescu<sup>2,3</sup>, Bianca M. Mădescu<sup>2,3</sup>, Ioan Coman<sup>1</sup>

<sup>1</sup> The "Stefan S. Nicolau" Institute of Virology, Romanian Academy, 030304, Street "Mihai Bravu", no. 285, Bucharest, Romania

<sup>2</sup> Faculty of Animal Sciences, University of Agricultural Sciences and Veterinary Medicine of Iasi, 700490, Mihail Sadoveanu Alley, no.3, Romania

<sup>3</sup> Research and Development Station for Cattle Breeding, 707252, Iasi-Ungheni no.9, Dancu, Iasi, Romania

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## Abstract

Mycologically and mycotoxicologically, 26 samples were analyzed (corn grains, corn flour, wheat, soybeans, sunflower seeds, pumpkin seeds), representing plant substrates. The highest fungal load was recorded for corn grains ( $35 \times 10^{-3}$  and  $360 \times 10^{-3}$  cfu/g) and wheat grains ( $190 \times 10^{-3}$  and  $240 \times 10^{-3}$  cfu/g) and the lowest was highlighted in soybeans ( $3 \times 10^{-3}$  and  $23 \times 10^{-3}$  cfu/g). For corn flour, the values did not exceed  $45 \times 10^{-3}$  cfu/g and for sunflower and pumpkin seeds the degree of fungal contamination was average, the number of colony-forming units remaining confined between  $11 \times 10^{-3}$  and  $65 \times 10^{-3}$  and  $19 \times 10^{-3}$  and  $80 \times 10^{-3}$  cfu/g, with insignificant differences between these two assortments. The purpose of this research was to discover the dominant fungal flora in the geographical area of the Moldavian Plateau, thus concluding that the genera *Aspergillus* (68.0%), *Fusarium* (59.7%) and *Cladosporium* (51.3%) predominated in the higher proportion, and from a mycotoxicological viewpoint, the mycotoxins identified in plant substrates were zearalenone and ochratoxin.

**Keywords:** contamination, fungi, mycotoxins, plant substrates

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## 1. Introduction

Fungi are a microbial hazard and by the fact that they produce secondary metabolites, such as mycotoxins, cause problems both economically [1] and in terms of human and animal health [2]. In indoor and outdoor environments, where temperature and humidity are favorable, fungi grow everywhere [3]. Regarding this growth of fungi, they were classified according to their stage of appearance and moisture content in three categories, so in the first category are the fungi grown in the field that have a high moisture content ( $a_w=1$ ) and are called hydrophilic fungi or

pre-harvest fungi, belonging to the genera *Alternaria*, and *Fusarium* [4]. The second category includes mesophilic or intermediate fungi, optimal for harvesting because they have reached the appropriate maturity and have a water activity of 0.95-1.0 (genera *Cladosporium* and *Verticillium*). The third category includes xerophilous or post-harvest fungi ( $a_w = 0.6-0.95$ ), belonging to the genera *Aspergillus* and *Penicillium* [5]. While species belonging to the genera *Aspergillus* and *Penicillium* grow frequently on food and feed for storage, species of the genus *Fusarium* often contaminate field crops (wheat, barley and corn) [6]. Non-compliant harvesting and storage practices contribute to fungal contamination, resulting in metabolism products-mycotoxins [3]. Mycotoxins are the most common contaminants of food and feed, the most important being aflatoxins (AF), ochratoxins

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\* Corresponding author: Ioana Poroşnicu, 0756803047, [ioana.porosnicu@yahoo.com](mailto:ioana.porosnicu@yahoo.com)

(OTA), fumonisins, patulin, zearalenone (ZEA) and trichothecene, including deoxynivalenol (DON) and toxin T-2 [7]. Also, aflatoxins are included in the category of the most toxic, with negative economic influence for agriculture [4]. Consumption of food or feed contaminated with mycotoxins can induce acute or chronic toxicity in humans and animals [8]. In order to determine mycotoxins, several screening methods have been validated and used such as thin layer chromatography (TLC) [9], high performance liquid chromatography (HPLC), gas chromatography (GC), enzyme-linked immunosorbent assay (ELISA) and many others [2]. Contamination of food with mycotoxins has been and is a continuing global concern, so it is necessary to control these planetary pollutants, because they affect the quality, safety and productivity of agricultural products.

## 2. Materials and methods

The research consisted of the mycological analysis of 26 samples (corn grains, corn flour, wheat, soybeans, sunflower seeds, pumpkin seeds), the degree of fungal contamination being evaluated by standardized working technique according to SR EN ISO 6887-1 2017. The culture medium of the micromycetes was PDA (Potato Dextrose Agar), the antibiotic used was Penstrept, and the plates were thermostated at 25°C. Only plates containing less than 150 colonies were considered. Regarding the qualitative mycological examination, to identify the colonies of micromycetes that were to be taxonomically framed, preparations were made between the blade and the lamella, and then they were examined under a microscope [10]. The peculiarities of the mycelial apparatus were analyzed, thus identifying the genera and even the species of micromycetes that contaminated the food substrates.

Mycotoxins were highlighted by the technique of thin layer chromatography (TLC), having as principle the extraction of mycotoxins with specific organic solvents and their separation, their identification in ultraviolet light with a wavelength of 254–366 nm [11]. The first step (actual extraction) consisted of degreasing twice every 60 min with petroleum ether (150 ml per 50-g sample), followed by cold extraction with

acetonitrile (8 h in the dark), filtration and evaporation to dryness [9].



Figure 1. Dry evaporation at the rotary evaporator

In the second stage, in the first phase, the plates were maintained for 60 min at 105°C. After the migration columns of the mycotoxins were sketched with a sharp tip, 30–50 µl of each dissolved extract was spotted at the starting line of the plate. The same was done with standard mycotoxins. Also, 100 ml of developing solvent consisting of 50 ml of ethyl acetate, 40-ml toluene and 10 ml of formic acid were introduced into the chromatographic tank. The chromatographic plate was introduced in an inclined position in this solvent tank. The solvent migrated and when it reached the limit of 2 cm from the upper edge of the plate, it was removed from the tank and dried in a stream of warm air for 5–6 min [12].



Figure 2. Chromatographic plate in the developing solvent

The plate thus prepared was examined in UV light with a wavelength of 366 nm. The migration distances of all spots were noted, but also of mycotoxins, expressed in cm. Mycotoxins were

identified on the basis of spots fluorescence color and Rf value.

$$Rf = d / D$$

d = distance in cm of spot migration

D = distance in cm of migration of the developing liquid

If the Rf value of this spot was identical to that of the standard mycotoxin, then it is considered that in the crude extract obtained from a sample there was a standard mycotoxin [13]. For confirmation, the in situ derivatization technique is used.

### 3. Results and discussion

The degree of fungal contamination of the 26 samples representing vegetal substrates that enter human and animal food was high, the number of colony forming units (cfu/g) varying in wide limits, with maximum values of  $360 \times 10^{-3}$ . The highest fungal load was recorded in cereals - corn and wheat, respectively. In the 12 grain corn samples, the number of colony forming units ranged between  $35 \times 10^{-3}$  and  $360 \times 10^{-3}$  cfu/g and in the wheat samples the variation limits were between  $190 \times 10^{-3}$  and  $240 \times 10^{-3}$  cfu/g (table 1).

**Table 1.** Results of quantitative mycological exam

Analyzed samples (26)	Cfu/g $\times 10^{-3}$	Asp.		Pen.		Fus.		Clad.		Alt.		Stc.		Muc.		Lev.	
		Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%	Nr.	%
C.grains <sup>1</sup>	89	29	32.5	-	-	53	59.5	5	5.6	-	-	2	2.2	-	-	-	-
C.grains <sup>1</sup>	340	80	23.5	50	14.7	190	55.8	20	5.8	-	-	-	-	-	-	-	-
C.grains <sup>1</sup>	140	30	21.4	-	-	20	14.2	80	57.1	-	-	-	-	-	-	-	-
C.grains <sup>1</sup>	170	90	52.9	30	17.6	-	-	-	-	-	-	20	11.7	-	-	-	-
C.grains <sup>1</sup>	35	-	-	10	28.5	-	-	20	57.1	5	14.2	-	-	-	-	-	-
C.grains <sup>1</sup>	220	-	-	-	-	190	86.3	20	9.09	-	-	-	-	-	-	-	-
C.grains <sup>1</sup>	80	10	12.5	30	37.5	10	12.5	-	-	-	-	-	-	10	12.5	-	-
C.grains <sup>1</sup>	47	32	68.0	10	21.2	5	10.6	-	-	-	-	-	-	-	-	-	-
C.grains <sup>1</sup>	360	300	83.3	40	11.1	-	-	-	-	-	-	-	-	-	-	-	-
C.grains <sup>1</sup>	62	24	40.0	-	-	18	29.0	-	-	3	4.8	-	-	4	6.4	-	-
C.grains <sup>1</sup>	38	9	23.6	-	-	11	28.9	4	10.5	-	-	1	2.6	-	-	5	13.1
C.grains <sup>1</sup>	101	80	79.2	1	0.9	-	-	-	-	-	-	5	4.9	3	2.9	-	-
C. flour <sup>2</sup>	12	-	-	-	-	5	41.6	-	-	2	16.6	-	-	5	41.6	-	-
C. flour <sup>2</sup>	45	21	46.6	-	-	7	15.5	8	17.7	-	-	-	-	-	-	3	6.6
Wheat	190	70	36.8	30	15.7	10	5.2	80	42.1	-	-	-	-	-	-	-	-
Wheat	240	60	25.0	30	12.5	10	4.1	-	-	20	8.3	20	8.3	-	-	50	20.8
Soybeans	23	8	34.7	15	65.2	-	-	-	-	-	-	-	-	-	-	-	-
Soybeans	15	4	26.6	-	-	2	13.3	6	40.0	-	-	-	-	-	-	-	-
Soybeans	3	3	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soybeans	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. seeds <sup>3</sup>	64	4	6.2	21	32.8	-	-	15	23.4	-	-	-	-	2	3.1	14	21.8
S. seeds <sup>3</sup>	65	-	-	37	56.9	4	6.15	-	-	2	3.0	1	1.5	-	-	15	23.0
S. seeds <sup>3</sup>	11	-	-	-	-	2	18.1	5	45.4	-	-	-	-	3	27.2	-	-
P. seeds <sup>4</sup>	32	7	21.8	-	-	-	-	14	43.7	-	-	-	-	3	9.3	8	25.0
P. seeds <sup>4</sup>	19	-	-	2	10.5	-	-	-	-	1	5.2	2	10.5	5	26.3	-	-
P. seeds <sup>4</sup>	80	15	18.7	-	-	-	-	3	3.75	-	-	-	-	6	7.5	34	42.7

Asp = *Aspergillus spp.*, Pen = *Penicillium spp.*, Fus = *Fusarium spp.*, Clad = *Cladosporium spp.*, Alt = *Alternaria spp.*, Stc = *Stachybotrys spp.*, Muc = *Mucoraceae*, Lev = *Yeasts*.

<sup>1</sup>C. grains = Corn grains.

<sup>2</sup>C. flour = Corn flour.

<sup>3</sup>S. seeds = Sunflower seeds.

<sup>4</sup>P. seeds = Pumpkin seeds.

It was observed that the structural element most exposed to fungal attack was the tegument because for corn flour (in which the grain shell is removed by grinding and sifting) the fungal load had lower values that did not exceed  $45 \times 10^{-3}$  cfu/g. Sunflower and pumpkin seeds showed a medium degree of fungal contamination, the

number of colony-forming units remaining between  $11 \times 10^{-3}$  and  $65 \times 10^{-3}$  and  $19 \times 10^{-3}$  and  $80 \times 10^{-3}$  cfu/g, with insignificant differences between these two assortments. The lowest fungal load was highlighted in soy samples ( $3 \times 10^{-3}$  and  $23 \times 10^{-3}$  cfu/g), because its processing includes as a mandatory technological phase a heat treatment

at high temperatures. The number of unidentified colonies, which is not mentioned in the table, is represented by other unknown genera.

Table 2 shows the incidence and percentage expression of the main genera of micromycetes inventoried and identified in the analyzed substrates.

**Table 2.** Results of the qualitative mycological exam

Analyzed samples	NSA <sup>5</sup>	Asp.		Pen.		Fus.		Clad.		Alt.		Stc.		Muc.		Lev.	
		PS <sup>6</sup>	%	PS	%	PS	%	PS	%	PS	%	PS	%	PS	%	PS	%
C. grains <sup>1</sup>	12	10	83.3	7	58.3	8	66.6	6	50.0	2	16.6	4	33.3	3	25.0	1	8.3
C. flour <sup>2</sup>	2	1	50.0	-	-	2	100	1	50.0	1	50.0	-	-	1	50.0	1	50.0
Wheat	2	2	100	2	100	2	100	1	50.0	1	50.0	1	50.0	-	-	1	50.0
Soybeans	4	3	75.0	1	25.0	1	25.0	1	25.0	-	-	-	-	-	-	-	-
S. seeds <sup>3</sup>	3	1	33.3	2	66.6	2	66.6	2	66.6	1	33.3	1	33.3	2	66.6	2	66.6
P. seeds <sup>4</sup>	3	2	66.6	1	33.3	-	-	2	66.6	1	33.3	1	33.3	3	100	2	66.6

Asp = *Aspergillus* spp, Pen = *Penicillium* spp, Fus = *Fusarium* spp, Clad = *Cladosporium* spp, Alt = *Alternaria* spp, Stc = *Stachybotrys* spp, Muc = *Mucoraceae*, Lev = *Yeasts*.

<sup>1</sup>C. grains = Corn grains.

<sup>2</sup>C. flour = Corn flour.

<sup>3</sup>S. seeds = Sunflower seeds.

<sup>4</sup>P. seeds = Pumpkin seeds.

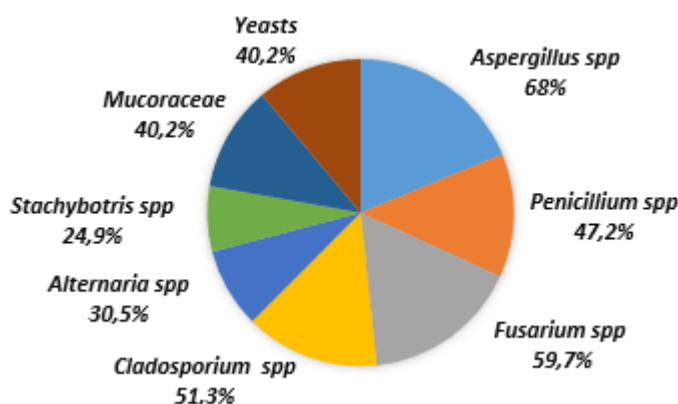
<sup>5</sup>NSA = Number of samples analyzed.

<sup>6</sup>PS = Positive samples.

In the corn samples, the genera *Aspergillus* (83.8%) and *Fusarium* (66.6%) predominated and in the corn flour the highest percentage was represented by the genus *Fusarium* (100%). In the wheat samples, dominant were the genera *Aspergillus*, *Penicillium*, *Fusarium* (100%), in soybeans (heat treated), the highest value was the genus *Aspergillus* (75%), in sunflower seeds dominated the genera *Penicillium*, *Fusarium*, *Mucor* and yeasts, while in pumpkin seeds the highest fungal load was represented by the *Mucoraceae* family (100%). Mycologically analyzed plant substrates can be grouped into two categories, as follows: cereal grain products (corn,

wheat, soybeans) in which the dominant fungal flora is represented by higher filamentous fungi taxonomically classified in the genera *Penicillium*, *Aspergillus*, *Fusarium*, *Cladosporium* and products with a higher degree of humidity (sunflower seeds, pumpkin seeds), in which the yeasts and filamentous fungi of the *Mucoraceae* family are dominant.

The synthetic graphic highlighting of the fungal genera frequently involved in human and animal pathology (figure 3) offered us the possibility of interpreting with more objectivity the obtained data.



**Figure 3.** Graphical representation of the genera of micromycetes isolated from plant substrates

It can be seen that the genus *Aspergillus* dominated the entire mycotic mosaic that

characterizes the examined products, being present in proportion of 68%. The dominant

fungal flora was followed by the genus *Fusarium*, with a participation rate of 59.7% and the genus *Cladosporium* by 51.3%. The fourth place in this hierarchy was represented by the genus *Penicillium* (47.2%), but it is not to be missed the quite high incidence of yeasts and fungi of the *Mucoraceae* family, which without reaching figures that had propelled them into the category of dominant fungi, were present in a percentage of 40.2%. The other taxonomic groups (*Alternaria*-30.5%, *Stachybotris*-24.9%) do not have too high percentages of representation, but their destructive

potential is just as high. The vegetal substrates studied differ from each other by the content in trophins, but also by other physico-chemical properties that can influence the multiplication of micromycetes and implicitly the elaboration of mycotoxins. The presence of mycotoxins was highlighted by a fast and accurate thin-layer chromatography (TLC) test, and for their identification the color of fluorescence in UV light (with  $\lambda = 366$  nm) was compared with the Rf value (table 3).

**Table 3.** Mycotoxicological exam results

Analyzed samples (26)	NSF <sup>1</sup>	Color	Rf	Identified mycotoxins	NSF <sup>1</sup>	Color	Rf	Identified mycotoxins
Corn grains	3	Yellow	0.42	N.I. <sup>2</sup>	3	Blue	0.32	N.I. <sup>2</sup>
		Yellow	0.46	N.I. <sup>2</sup>		Green-blue	0.55	Zearalenone
		Brick green	0.58	N.I. <sup>2</sup>		Blue	0.82	N.I. <sup>2</sup>
Corn grains	4	Violet	0.40	N.I. <sup>2</sup>	4	Red	0.42	N.I. <sup>2</sup>
		Yellow	0.48	N.I. <sup>2</sup>		Black	0.56	N.I. <sup>2</sup>
		Bright yellow	0.61	N.I. <sup>2</sup>		Blue	0.75	N.I. <sup>2</sup>
Corn grains	2	Brick red	0.79	N.I. <sup>2</sup>	0	Brick	0.82	N.I. <sup>2</sup>
		Green	0.44	N.I. <sup>2</sup>		-	-	-
		Blue	0.62	N.I. <sup>2</sup>		Green	0.32	N.I. <sup>2</sup>
Corn grains	3	Yellow	0.38	N.I. <sup>2</sup>	3	Chartreuse	0.67	N.I. <sup>2</sup>
		Teal	0.49	N.I. <sup>2</sup>		Violet	0.72	N.I. <sup>2</sup>
		Ocher	0.72	N.I. <sup>2</sup>		Green	0.38	N.I. <sup>2</sup>
Corn grains	5	Green	0.38	N.I. <sup>2</sup>	4	Red	0.44	N.I. <sup>2</sup>
		Green-blue	0.51	N.I. <sup>2</sup>		Brick-red	0.78	N.I. <sup>2</sup>
		Green-yellow	0.66	N.I. <sup>2</sup>		Yellow	0.81	N.I. <sup>2</sup>
		Black	0.72	N.I. <sup>2</sup>		Bright yellow	0.86	N.I. <sup>2</sup>
		Black-olive	0.89	N.I. <sup>2</sup>		Green	0.32	N.I. <sup>2</sup>
Corn grains	3	Chartreuse	0.67	N.I. <sup>2</sup>	0	-	-	-
		Violet	0.72	N.I. <sup>2</sup>		-	-	-
Corn flour	2	Blue	0.54	N.I. <sup>2</sup>	0	-	-	-
		Blue	0.61	N.I. <sup>2</sup>		-	-	-
Wheat	3	Pale blue	0.52	N.I. <sup>2</sup>	4	Green	0.38	N.I. <sup>2</sup>
		Purple blue	0.64	N.I. <sup>2</sup>		Mauve	0.41	N.I. <sup>2</sup>
		Yellow straw	0.86	N.I. <sup>2</sup>		Blue-green	0.59	N.I. <sup>2</sup>
Soybeans	3	Yellow	0.76	N.I. <sup>2</sup>	2	Yellow	0.64	N.I. <sup>2</sup>
		Green	0.28	N.I. <sup>2</sup>		Mauve	0.32	N.I. <sup>2</sup>
		Green	0.64	N.I. <sup>2</sup>		Indigo	0.64	N.I. <sup>2</sup>
Soybeans	0	-	-	-	0	-	-	-
Sunflower seeds	3	Blue	0.45	N.I. <sup>2</sup>	2	Green	0.72	N.I. <sup>2</sup>
		Blue-green	0.71	N.I. <sup>2</sup>		Yellow-green	0.84	N.I. <sup>2</sup>
		Indigo blue	0.80	N.I. <sup>2</sup>		-	-	-
Sunflower seeds	0	-	-	-	-	-	-	-
Pumpkin seeds	3	Bright green	0.45	N.I. <sup>2</sup>	3	Bright blue	0.42	Ochratoxin
		Blue	0.60	N.I. <sup>2</sup>		Green	0.56	N.I. <sup>2</sup>
		Pale blue	0.74	N.I. <sup>2</sup>		Blue	0.75	N.I. <sup>2</sup>
Pumpkin seeds	1	Bright yellow	0.75	N.I. <sup>2</sup>	-	-	-	-

<sup>1</sup>NSF = Number of fluorescent spots

<sup>2</sup>N.I. = Unidentifiable

As can be seen from the data listed in tables 3 and 4, not all samples examined were mycotoxinically

contaminated, the fluorescent spots varying in number, color and Rf depending on the nature of

the assortment analyzed. Also, for each assortment the fungal load. were presented in detail the limits of variation of

**Table 4.** Results of mycological and mycotoxicological exam

Sample	Number of samples analyzed	Cfu variation limits / g x 10 <sup>-3</sup>	Qualitative mycological exam	Mycotoxicological exam	
			Dominant genera of micromycetes	Fluorescent spotlights (limits)	Identified mycotoxins
Corn grains	12	35-360	<i>Asp, Fus, Pen, Cld, Stc, Muc, Alt, Lev</i>	0-5	Zearalenone
Corn flour	2	12-45	<i>Fus, Asp, Cld, Alt, Muc, Lev</i>	0-2	0
Wheat	2	190-240	<i>Asp, Pen, Fus, Cld, Alt, Stc, Lev</i>	3-4	0
Soybeans	4	3-23	<i>Asp, Pen, Fus, Cld</i>	0-3	0
Sunflower seeds	3	11-65	<i>Asp, Fus, Cld, Alt, Pen, Muc, Lev, Stc</i>	0-3	0
Pumpkin seeds	3	19-80	<i>Asp, Cld, Alt, Pen, Muc, Lev, Stc</i>	1-3	Ochratoxin

*Asp* = *Aspergillus* spp, *Pen* = *Penicillium* spp, *Fus* = *Fusarium* spp, *Cld* = *Cladosporium* spp, *Alt* = *Alternaria* spp, *Stc* = *Stachybotrys* spp, *Muc* = *Mucoraceae*, *Lev* = *Yeasts*.

Thus, it was observed that mycotoxins were present in corn samples (zearalenone) and pumpkin seeds (ochratoxin). Zearalenone is produced by species of the genus *Fusarium* (*F. graminearum* and *F. semitectum*) and is an estrogenic mycotoxin [7]. Among the most frequently contaminated products are corn and wheat, this contamination being favored by high humidity and low temperature [6]. Regarding the consumption of contaminated feed in cattle, ZEA can trigger infertility, hyperestrogenism and reduced milk production. IARC has also classified ZEA as a group 3 carcinogen [3]. Ochratoxin (OTA) is produced by species belonging to the genera *Aspergillus* and *Penicillium*, being one of the most widespread fungal toxins [14]. It is active in low concentrations, of the order of ppm, it accumulates in the body, affects its vital organs (liver, kidneys), causing irreversible structural changes, proving to be carcinogenic, mutagenic and carcinogenic [8].

#### 4. Conclusions

The mycological exam showed that the level of micromycete contamination of the vegetal substrates subjected to the analyzes did not exceed the maximum limit allowed by the European legislation, even if an intense fungal contamination was highlighted, with variation limits between 0 and 360 x 10<sup>-3</sup> cfu/g. It was noted that in the case of cereal grains (corn, wheat, soybeans), the dominant fungal flora was represented by filamentous fungi belonging to the

genera *Penicillium*, *Aspergillus*, *Fusarium* and *Cladosporium*. In contrast, the yeasts and filamentous fungi of the *Mucoraceae* family were dominant in the products with a higher degree of humidity (sunflower seeds, pumpkin seeds). The mycotoxicological exam allowed the isolation, separation and identification of the fluorescent fractions present in the crude extracts, their number varying between 0 and 5. The color of the fluorescence of the spots in UV light with  $\lambda = 366$  nm, the Rf value and in situ derivatization were the techniques that allowed the identification of mycotoxins with a high epidemiological risk, namely zearalenone and ochratoxin.

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