Mycological and Mycotoxinic Investigations of Plant Substrates in the Moldavian Plateau Area

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Abstract
Mycological and mycotoxinic contamination of plant substrates endangers the most important sectors globally - agriculture, animal husbandry and the food industry. The starting point of contamination is the spread of pathogens in the field, transport and even handling before storage. Increasing the temperature and humidity of these seeds can be an important factor in the development of fungi and mycotoxins. In this paper, we aimed to evaluate the fungal and mycotoxinic potential in the samples that characterize the area of the Moldavian Plateau. From agricultural farms, samples such as corn grains, wheat, barley, soybeans, rapeseed, peas and sunflowers were collected and analyzed and a series of 10 determinations / sample were performed in order to establish the fungal and mycotoxinic load. The results clearly indicate the presence on the seed coat of fungal spores of the genera *Penicillium* (60%), *Aspergillus* (52.8%), *Fusarium* (48.5%) together with species from the *Mucoraceae* family (38.5%). The most contaminated samples were corn, wheat and barley grains. The climatic conditions in this geographical area are favorable for these species of micromycetes and regarding the mycotoxinic examination performed by the TLC technique, the mycotoxins identified in the 70 plant substrates were aflatoxins B_1, G_2, ochratoxin and zearalenone.

Keywords: contamination, mycotoxins, micromycetes, plant substrates

1. Introduction

Various cereals, especially maize and wheat, which are considered to be key agricultural commodities worldwide in relation to the nutrition of farm animals but also other fodder plants are particularly important and belong to the category of substrates susceptible to fungal contamination [1]. In the agricultural or zootechnical units it is essential to pay more attention to the vegetal substrates, needing to be of good quality when introduced in the food of animals but also of people [2]. In different climatic conditions, the vegetal substrates do not correspond entirely from the qualitative point of view, being attacked by different micromycetes even in the vegetative phase and thus they spread large amounts of spores, which are carried by the wind, almost always ensuring their presence in the atmospheric air [3]. Certain eco-physiological factors, especially the temperature and water activity of the crops and products attacked greatly influences the growth and production of mycotoxins [4]. Mycotoxins are secondary toxic metabolites of micromycetes and are commonly found in raw materials [5]. Ingestion of food contaminated with mycotoxins by farm animals would favor the appearance of unwanted residues in animal products (milk, meat), being a public health

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problem, because they are in the food chain and can cause adverse effects on human health [6]. Mycotoxin contamination has been and is a continuing global concern, so it is necessary to control these planetary pollutants as they affect the quality, safety and productivity of agricultural products [7].

2. Materials and methods

The vegetal substrates represented by corn grains, wheat, barley, soybean, rapeseed, pea and sunflower grains, which characterize the Moldavian Plateau area, were analyzed for the fungal and mycotoxinic load.The number of filamentous micromycetes in the plant substrates was determined based on the serial dilutions method, according to the SR EN ISO 16140-2 standard. The Petri dishes were thermostated at 25^{o}C, and will be examined at 3 and 5 days to assess and describe the cultural features of the colonies, morphological markers particularly important for the taxonomic classification of micromycetes. Evaluation of mycotoxin samples was performed by TLC technique [8]. The working principle consisted in isolating the mycotoxins present in the substrate by extraction with organic solvents and then separating them on chromatographic plates and identifying them based on fluorescence color and Rf value, in UV light with a wavelength of 254 nm-366 nm. Also, before being subjected to the working technique, the samples were analyzed at first sight with a UV lamp with a wavelength of 254-366 nm, to see if the sample as such shows fluorescence. Regarding the degreasing, extraction and purification of the sample, 50 g of sample was weighed into glasses, over which 150 ml of degreasing solvent (petroleum ether) was added and the homogenizer was kept for 30 minutes. After decantation, the solvent was removed and the operation was repeated. An amount of 150 ml of extraction solvent (acetonitrile) were added to the degreased sample, the beakers were sealed, the contents of the beakers were shaken and left in the dark for 8 hours (cold extraxtion) [9]. The supernatant was filtered through Wattman 2 filter paper. The obtained extract was evaporated to dryness on a rotary evaporator with the water temperature kept constant at 85°C, the temperature at which the acetonitrile evaporated. After the plate was spotted, it was introduced into the chromatographic tank, and the chromatographic plate was then examined in UV light with a wavelength of 254-366 nm. The migration distances of the spots were noted and expressed in cm. Mycotoxins were also identified based on 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). For confirmation, the in situ derivatization technique was used, which consisted in spraying the developed plate with derivatization solution [10].

3. Results and discussion

Results of quantitative mycological examination

During the experimental period, the 7 categories of samples of 10 determinations each (Table 1) were subjected to quantitative and qualitative mycological control.

Table 1. Results of quantitative and qualitative mycological examination of plant substrates

Nr. crt.	Analyzed sample	Number of samples analyzed	Limits of variation (cfu/g x 10^{-3})	Maximum allowed limits (cfu/g x 10^{-3})	Dominant genera of micromycetes
1.	Peas	10	10-40	50	*Asp., Pen., Fus., Mucor.*
2.	Corn grains	10	22-100	50	*Asp., Pen., Fus.,Clad., Alt., Mucor., Trich.*
3.	Wheat	10	15-80	50	*Asp., Pen., Fus.,Clad., Alt., Mucor., Trich.*
4.	Barley	10	20-78	50	*Asp., Pen., Fus.,Clad., Alt., Mucor.*
5.	Soybeans	10	14-75	50	*Asp., Pen., Fus.,Clad., Trich.*
6.	Sunflower	10	12-45	50	*Asp., Pen., Fus., Mucor.*
7.	Rapeseed	10	13-60	50	*Asp., Pen., Fus.,Clad., Mucor.*
Total samples		70			

Asp.-Aspergillus spp, *Pen.-Penicillium* spp, *Fus.-Fusarium* spp, *Cld.-Cladosporium* spp, *Alt.-Alternaria* spp, *Trich.-Trichosporon* spp, *Muc.-Mucoraceae*

Table 1 shows for each category of plant substrate the limits of variation of the fungal load, the highest value being recorded for maize grains (100 x 10^{-3} cfu/g). Corn grains, which are mainly attacked by micromycetes, are a cereal component that often enters animal feed. The degree of fungal contamination largely depends on the conditions under which the harvesting, transport and storage were carried out. Thus, the fungal contamination of corn grains recorded the highest values. In the background, on the 2nd and 3rd places are the wheat with variation limits between 15 x 10^{-3} and 80 x 10^{-3} cfu/g, respectively barley with variation limits between 20 x 10^{-3} and 78 x 10^{-3} cfu/g. The average fungal load is highlighted in the soybeans samples with variation limits between 14 x 10^{-3} and 75 x 10^{-3} cfu/g and in the rapeseed samples with variation limits between 13 x 10^{-3} and 60 x 10^{-3} cfu/g. The following samples belong to the category of vegetal substrates with the lowest fungal load: peas with variation limits between 10 x 10^{-3} and 40 x 10^{-3} cfu/g and sunflower with variation limits between 12 x 10^{-3} and 45 x 10^{-3} cfu/g, the latter falling within the maximum allowed limits.

Results of qualitative mycological examination

Table 2 shows the incidence and percentage expression of the main genera of micromycetes inventoried and identified at the level of the dominant fungal genus in the 70 analyzed plant substrates.

Tabelul 2. Results of qualitative mycological examination of plant substrates

Sample	*Aspergillus*		*Penicillium*		*Fusarium*		*Cladosporium*		*Alternaria*		*Mucoraceae*		*Trichosporon*	
	P.s	%	P.s	%	P.s	%	P.s	%	P.s	%	P.s	%	P.s	%
Peas (10 samples)	3	30	2	20	2	20	-	-	-	-	2	20	-	-
Corn grains (10 samples)	8	80	9	90	7	70	2	20	1	10	6	60	4	40
Wheat (10 samples)	7	70	7	70	6	60	2	20	4	40	7	70	2	20
Barley (10 samples)	4	40	8	80	6	60	2	20	2	20	7	70	-	-
Soybeans (10 samples)	4	40	6	60	7	70	1	10	-	-	-	-	3	30
Sunflower (10 samples)	6	60	4	40	2	20	-	-	-	-	4	40	-	-
Rapeseed (10 samples)	5	50	6	60	4	40	2	20	-	-	1	10	-	-
TOTAL (70 samples)	37	52.85	42	60	34	48.57	9	12.85	7	10	27	38.57	9	12.85
	Aspergillus		*Penicillium*		*Fusarium*		*Cladosporium*		*Alternaria*		*Mucoraceae*		*Trichosporon*	

P.s. - Positive samples

The highest degree of contamination was represented by corn grains - 90% with species belonging to the genus *Penicillium* and 80% with species belonging to the genus *Aspergillus*. The genus *Penicillium* had a higher percentage of contamination (90%) in the 10 samples analyzed,

unlike the other samples, but it is noteworthy that it includes many adapted strains, which seem to prefer geographical areas of the Moldavian Plateau. Species of this genus were detected as contaminants in 42 samples, respectively 2 in peas, 9 in corn grains, 7 in wheat, 8 in barley, 4 in sunflower, 6 in soybeans and rapeseed. The samples were ranked second and third in terms of contamination: wheat with a percentage of 70% with species of the genus *Aspergillus* and *Penicillium* and barley with 80% with species of the genus *Penicillium* and 70% with species of the family *Mucoraceae*. The average level of contamination is the soybeans and rapeseed samples, which offer optimal conditions for the multiplication of micromycetes belonging to the genera *Penicillium* (60%) and *Fusarium* (70%), respectively *Aspergillus* (50%) and *Penicillium* (60%). *Cladosporium* and *Trichosporon* species were not identified in the peas and sunflower samples, the latter showing the lowest percentages of micromycete contamination. The more eloquent interpretation of the obtained data is highlighted by a graphical representation of the fungal genera, so we distinguish that the genus *Penicillium* dominates the entire mycotic mosaic that characterizes the examined plant substrates, being present in 60% of the analyzed samples. The genus *Aspergillus* follows with a fungal flora with a participation rate of 52.85% and the genus *Fusarium* (48.57%). It is interesting to note that the very high incidence of *Mucoraceae* occupying 38.57 percent, the fourth place in this hierarchy (in plant substrates) indicates a higher degree of humidity which could be a signal of a potential alteration process (Figure 1).

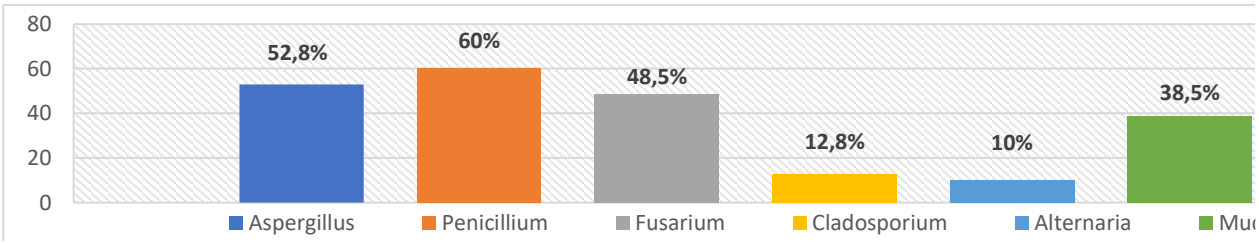

Figure 1. Graphic representation of the genera of micromycetes isolated from plant substrates

The qualitative mycological examination aims at identifying the strains of isolated micromycetes and their taxonomic classification. The taxonomic classification of the fungi that frequently contaminate the vegetal substrates was made corroborating the aspects of the colonies developed with the morpho-structural particularities of the fruiting bodies of the micromycetes: whorls at *Penicillium*, aspergillation heads at *Aspergillus*, macro and microconidia in *Fusarium*, sporangiophores in *Mucoraceae*, respectively bicellular pores in *Cladosporium*. It is essential that each micromycete colony is carefully examined, in order to highlight the special cultural aspects, specific to each genus. Thus, the fungal load was represented by species belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Rhizopus*, *Alternaria* and *Trichosporon* (Figures 2,3).

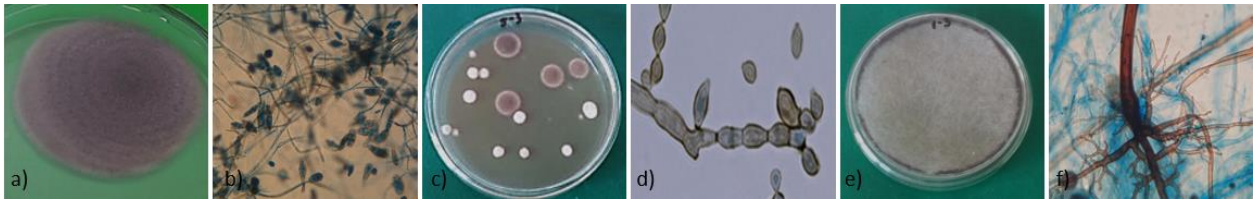

Figure 2. Identified genera-macroscopic and microscopic appearance (a, b-Alternaria; c, d-Cladosporium; e, f-Rhizopus)

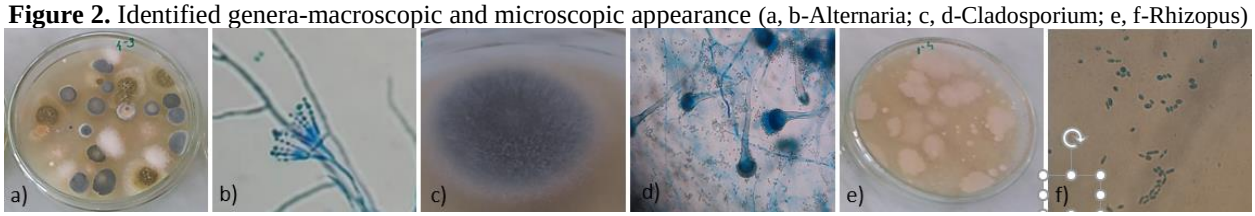

Figure 3. Identified genera-macroscopic and microscopic appearance (a,b-*Penicillium*; c,d-*Aspergillus*; e,f-Trichosporon)

The samples transferred from the reception room, through the communication window, are tested for the detection of infested grains and contaminated with fluorescent mycotoxins. For this purpose, a certain amount of each sample is placed in a Petri dish with a diameter of 12 cm and viewed in a niche, in the dark field, with a UV radiation lamp, with a wavelength of 254-366. nm. The contaminated samples will show a variable fluorescence, in intensity and especially color (blue, green, yellow, red), depending on the nature and structure of the contaminant. The present samples did not show fluorescence in UV light (Figure 4). Next, they are also examined individually to determine the degree of mycotoxins infestation.

Results of mycotoxinic examination

Mycotoxins contamination of the 70 plant substrates, as can be seen from the data in Table 3, was not found in all the samples examined.

Table 3. Results of qualitative mycotoxinic examination performed by the TLC technique from vegetal substrates

Nr. Crt.	Analyzed sample	Number of samples analyzed	Positive samples		Mycotoxins identified		
			N.s.	%	Aflatoxin	Ochratoxin	Zearalenone
1.	Peas	10	0	0	0	0	0
2.	Corn grains	10	7	70	4	2	1
3.	Wheat	10	6	60	3	3	0
4.	Barley	10	3	30	2	0	1
5.	Soybeans	10	1	10	1	0	0
6.	Sunflower	10	1	10	1	0	0
7.	Rapeseed	10	0	0	0	0	0
TOTAL		70	18 (25.71%)		11 (15.71%)	5 (7.14%)	2 (2.85%)

N.s.-Number samples

The strongest mycotoxin load and therefore a major risk to animal health was represented by corn grains and wheat. Of the 10 representative samples of corn grains, in 7 samples (70%) found aflatoxins B_1, G_2 (4), ochratoxin A (2), and respectively zearalenone (1). Simultaneous contamination with aflatoxin, ochratoxin and zearalenone was detected in a corn grains sample. Of the 10 wheat samples, 6 (60%) were contaminated with aflatoxins (3) and ochratoxin A (3). The other vegetal substrates showed a medium degree of contamination. In 2 samples of barley and in other samples of soybean and sunflower, aflatoxins B_1 and G_2 were identified and in the barley sample zearalenone was felt. Fluorescent spots were not detected in the peas and rapeseed samples (Figure 5).

Figure 4. Highlighting samples in UV light (soybeans, corn grains, wheat, peas, sunflower, barley)

Figure 5. Working technique (a-degreasing of samples; b, c-cold extraction; d, e-dry evaporation in rotavapor; f, g-plate staining; h, i-mycotoxins identified)

We can estimate that out of the 70 samples of plant substrates that enter the animal feed and not only, 18 were positive, respectively a percentage of 25.71%, of which 11 (15.71%) of positive samples with aflatoxins. These results are for guidance only, because the positive samples do not exceed the maximum limits allowed by the regulations in force, being necessary to confirm these results with at least approximately quantitative methods. For confirmation, the in situ derivatization technique was used, which consisted in spraying the developed plate with derivatization solution. After spraying, the uncertain spot acquired the same color characteristic of mycotoxin (Table 4).

Table 4. In situ derivatization test

Nr. crt.	Standard mycotoxin	Value Rf	UV spot color with λ = 366 nm	Derivatizing agent	366 nm UV color after sprayers
1.	Aflatoxin B_1	0.21	Bright blue	H_2SO_4 20%	Greenish yellow
2.	Aflatoxin G_2	0.12	Bright green	H_2SO_4 20%	Yellow
3.	Zearalenone	0.55	Blue-green	Diazoting mixture	Visible dark brown
4.	Ochratoxin A	0.42	Bright blue	0,1 N NaOH 50% H_2SO_4 in methanol	Intense bright blue Bright green blue

Mycotoxins are one of the main food safety issues, as they are widely reported contaminants in both raw materials and feed for various animal species [11]. Mycotoxin legislation does not take into account the frequently reported and worrying scenario of simultaneous mycotoxins contamination of individual goods and feed [12]. Studies aiming at the contamination of feed materials and fodder with mycotoxins recognize the concomitant occurrence of mycotoxins as an increasingly relevant issue [13]. In any case, worldwide legislation (including European legislation) only considers mycotoxin mono-exposure data and does not address relevant combinations of mycotoxins [14]. Therefore, there is a regulatory gap that should be addressed as soon as possible. On the other hand, there are developing mycotoxins that are widespread in feed and have not yet been regulated.

4. Conclusions

At the quantitative mycological examination the highest values at the level of contamination were represented by corn grains (22 x 10^{-3} and 100 x 10^{-3} cfu/g) and wheat (15 x 10^{-3} and 80 x 10^{-3} cfu/g) and the least contaminated were peas (10 x 10^{-3} and 40 x 10^{-3} cfu/g) and sunflower (12 x 10^{-3} and 45 x 10^{-3} cfu/g). Regarding the qualitative mycological examination, the first place in terms of contamination was held by the genus *Penicillium* with a percentage of 90% in corn grains, and in second and third place were placed the samples: wheat with a percentage of 70% with species of the genus *Aspergillus* and *Penicillium* and 80% barley with species of the genus *Penicillium*. The color of the spot fluorescence in UV light with λ = 366 nm, the Rf value and the in situ derivatization were the techniques that allowed the identification of some mycotoxins with high epidemiological risk, namely aflatoxins B_1, G_2 (corn grains, wheat, barley, soybeans, sunflower), ochratoxin (corn grains, wheat) and zearalenone (corn grains, barley).

It seems that these species of micromycetes prefer the Moldovan Plateau area, but they are widespread everywhere, so it is essential to take precautions during harvesting, transporting and storing feed to reduce/prevent fungal contamination.

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