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Perspectives on mycotoxin management: occurrence of total aflatoxins in 2018-2019 romanian maize (*Zea Mays* L.) samples

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Abstract

In recent decades, mycotoxin contamination have continued to represent a clear public health concern. Cereals are very susceptible to fungal attacks, both in the field and during storage. Although there are numerous mycotoxins affecting the maize crops, aflatoxins are the most widespread, toxigenic and important mycotoxins in maize. In this context, a maize survey was conducted in Romania, to monitor the occurrence of total aflatoxins in maize samples, collected during the 2018 and 2019 growing seasons from fields located in all counties. A total of 179 maize samples were collected along with information regarding the specific location of fields, the applied agronomic practices and cropping systems. ELISA method was used for the quantification of AFs. Only one sample noted aflatoxin levels higher than the limit of 10.00 µg/kg, settled by the Commission Regulation (EC) No 1881/2006 for maize to be subjected to soring or other physical treatment before human consumption or use as an ingredient in foodstuffs. The highest total afaltoxins level was 77.59 µg/kg, noted by a maize sample from Argeş County (the South-Muntenia development region, macroregion 3). There were gathered information for strategies and solutions to the maize mycotoxin management. When referring to the analysed samples, the total aflatoxin contamination was independent of the type of hybrid, but strongly influenced by the pedo-climatic differences between counties. The southern counties proved to represent critical risk areas for aflatoxin contamination when referring to maize. These results highlight the importance of an effective and sustainable mycotoxin management along the food and feed chain, as well as the need of mapping the mycotoxin risk areas.

Keywords: aflatoxins, maize, Romania

1. Introduction

Cereals are very susceptible to fungal attacks, both in the field and during storage. Depending on environmental conditions, a fungal infection, mainly produced by species of *Aspergillus*, *Fusarium* and *Penicillium*, may result in a mycotoxin contamination of the crop [12].

Mycotoxins are secondary metabolites produced by spore-forming fungi. A wide range of food products could be contaminated with mycotoxins, both pre- and post-harvest [20]. Consequently, a regular contamination can be expected for cereals and cereal-based commodities either in the field, at pre-harvest stage, or post-harvest, during transport or storage [11].

Aflatoxins are a major class of toxic and carcinogenic mycotoxins produced primarily by fungi belonging to *Aspergillus* section Flavi, mainly *Aspergillus flavus* and *A. parasiticus*, which contaminate a wide range of agricultural products at pre- and/or post-harvest stages [3, 13, 18]. The toxin is classified by the International Agency for Research on Cancer as a Group 1 carcinogen [8]. Given their highly toxigenic nature, at European level the presence of aflatoxins is strictly regulated, being imposed maximum levels in various commodities [5].

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Even though there is knowledge of the occurrence of aflatoxins in maize samples collected across Europe [1, 16, 17], there is not so many information on the aflatoxin levels in maize samples cultivated in various Romanian counties, even if maize cultivation is common in Romania [2]. In this context, the current study was undertaken to monitor the occurrence of total aflatoxins in maize samples collected during 2018 and 2019 from fields located in all major maize-producing Romanian counties. Thus, the information on aflatoxin levels in the Romanian counties will be essential to identify hotspot regions in Romania and will aid to design appropriate and cost-effective aflatoxin management strategies to prevent aflatoxin contamination right at the source.

2.Materials and methods

Maize samples. Eighty-four (N_1 = 95; 1 kg/sample) and ninety-five (N_2 = 95; 1 kg/sample) samples of maize were randomly collected in 2018 and 2019, respectively. The sampling was done by inspectors of the County Agriculture Directorates of the Romanian Ministry of Agriculture and Rural Development, according to the European guidelines [4]. All samples were collected from private cereal farmers, immediately after harvest. Upon arrival, all samples were transferred into paper bags and stored in the dark until their assessment. All samples were received along with information regarding the specific location of fields and the applied agronomic practices (hybrid type, previous crops, incorporation of crop residues, sowing date, fertilisation and fungicide information etc.), which were filled in by farmers into a structured questionnaire dedicated to this study.

Geographic Coordinates. In order to reference the origin of samples, the European nomenclature of territorial units for statistics (NUTS) was used, based on the European regulation [6] (Figure 1). The Northern latitude and Eastern longitude of the location of the field of each sample were determined using Google Maps [7], based on the information given by farmers in the questionnaires that accompanied the barley samples.

Figure 1. The NUTS (Nomenclature of Territorial Units for Statistics) regions of Romania: (A) NUTS I – Macroregions; (B) NUTS II – Regions; (C) NUTS III - Counties

Mycotoxin analysis. A competitive enzyme linked immunosorbent assay (ELISA) was selected for the quantitative analysis of total aflatoxins. The assessment was performed with commercially available test kits, according to the manufacturer's instructions (Ridascreen® Aflatoxin Total, R-Biopharm AG, Germany). Thus, all samples were first finely ground using a laboratory mill (MRC Ltd., Israel) and mixed thoroughly to achieve complete homogenization. Furthermore, 2 grams of grinded sample were homogenized in 10 mL methanol / distilled water (70/30; v/v) and mixed vigorously for 10 minutes at room temperature using an orbital shaker (GFL Gesellschaft für Labortechnik mbH, Germany). All extracts were then filtered using a grade 1 filter paper (Whatman™, UK) and the obtained filtrates were further diluted in 600 µL distilled water (100/600; v/v). There were employed 50 µL standard solutions and prepared samples to separate duplicate wells.

A volume of 50 µL of the enzyme conjugate was added to each well, followed by 50 µL of the antibody solution. The plate was gently mixed by hand and incubated for 30 minutes at room temperature in the dark. After the incubation period, the liquid was poured out of the wells and the plate was vigorously taped upside down against absorbent paper to ensure complete removal of liquid from the wells. This was followed by the washing procedure (250 µL washing buffer, repeated three times). There were added 100 µL of substrate/chromogen to each well. The plate was again very well mixed by hand and incubated for 15 minutes at room temperature in the dark. After incubation, 100 µL of the stop solution were added to each well. The absorbance was measured at 450 nm using a Sunrise™ plate reader (Tecan Group Ltd., Switzerland). The RIDA®SOFT Win software was used for the evaluation of the immunoassays. For each sample, two replicates have been used.

The average of these results has been employed in data analysis. A mycotoxin quality control material (Trilogy Reference Material, Naturally Contaminated Aflatoxin Corn, Trilogy Analytical Laboratory, Inc., USA) was used was used for each measurement, to ensure the quality of the analyses.

Data analysis. ELISA tests were run in duplicate for each sample. Results are reported as the mean ± standard deviation and include the recovery of the used quality control material. The uncertainty of the method was 0.34 μg/kg. Statistical analysis was performed using IBM® SPSS® Statistics 20 (IBM Corp., USA). Significance was defined at $P < 0.05$.

3.Results and discussion

Origin of the collected samples. The aim of this study was to monitor the occurrence of total aflatoxins in 179 maize samples collected during the 2018-2019 growing seasons from fields located in different regions of Romania (Figure 2).

In 2018, there were received for analysis 84 maize samples, of which 10 samples (11.90%) from Macroregion 1 (North-West and Central development regions), 17 samples (20.24%) from Macroregion 2 (North-East and South-East development regions), 44 samples (52.38%) from Macroregion 3 (South-Muntenia and Bucharest-Ilfov development regions) and 13 maize samples (15.48%) from Macroregion 4 (South-West Oltenia and West development regions). Thus, the majority of the samples were collected from Macroregion 3, followed by Macroregion 2, respectively.

The South-Muntenia development region registered the highest number of maize samples (43 samples). While on average, there were received 2 maize samples from each County, there were noted 31 maize samples from Călărași County (Macroregion 3, South-Muntenia development region) and 7 samples from Ialomița County (Macroregion 3, South-Muntenia development region), respectively. In 2018, no maize samples were received from counties such as Suceava (Macroregion 2), Mureș, Sibiu (Macroregion 1) and Timiș (Macroregion 4).

A number of 95 maize samples was received for analysis in 2019. There were registered 14 samples (14,74%) from Macroregion 1 (North-West and Central development regions), 24 samples (25,26%) from Macroregion 2 (North-East and South-East development regions), 42 samples (44,21%) from Macroregion 3 (South-Muntenia and Bucharest-Ilfov development regions) and 15 maize samples (15,79%) from Macroregion 4 (South-West Oltenia and West development regions). Again, the highest number of maize samples (42 samples) was received from Macroregion 3, where 20 samples were sent by Ialomița County and 8 samples by Argeș County. In 2018, no maize samples were received from counties such as Satu Mare, Baia Mare, Harghita and Sibiu (Macroregion 1), Suceava and Constanța (Macroregion 2) and Timiș (Macroregion 4).

Prevalence of total aflatoxins in Romanian maize samples. The aim of the present study was to monitor the occurrence of total aflatoxin in 2018 and 2019 maize samples across Romania. When using ELISA method, an accurate quantification is only possible within the range of the calibrators - values of the given standards provided by the kit multiplied by the corresponding dilution factor (e.g. 35 for cereals and feed), which results in a range of 1.75 – 141,75 μg/kg total aflatoxins.

The calculation of the results was done using the cubic spline function for RIDA®SOFT Win software. The analysis of the 179 maize samples revealed that most of the evaluated samples showed no contamination with aflatoxins beyond threshold set by the European regulations, which stipulates 10.00 μg/kg as the maximum level of aflatoxins for maize subjected to sorting or other physical treatment before human consumption [5]. For all samples outside the calibrator range, the mathematical function had to be extrapolated, which increased uncertainty [19]. As samples with negative test results still could contain a total aflatoxin contamination below the limit of detection of the assay, the 'out of range' function of the RIDA®SOFT Win software was applied for these samples, in order to receive a rough estimation of the concentrations of total aflatoxins for the assessed samples. Thus, for the samples noted to have concentrations lower than the minimum value of the given range, there were no actions that could avoid the increased uncertainty obtained after applying the 'out of range' function of the software. When needed, a dilution step was applied for samples with higher concentrations than the given range, for them to lie within the range of the calibrators.

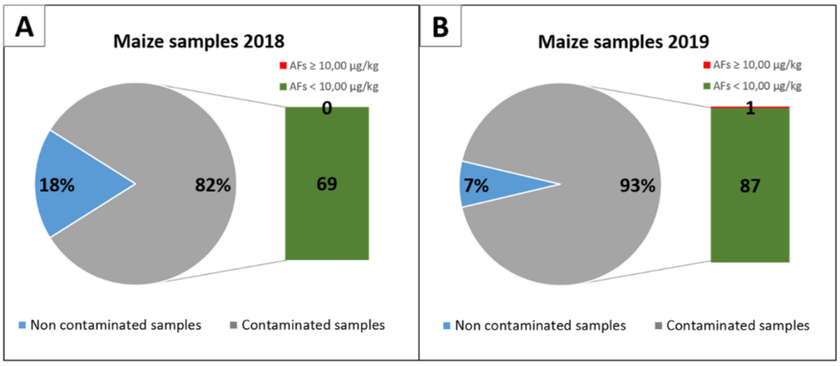

Figure 3. Contaminated maize samples): (A) 2018 harvest; (B) 2019 harvest

In 2018, 66 samples noted concentrations of total aflatoxins lower than the limit of detection of the ELISA kit (1.75 µg/kg). However, for only 4 samples the level of total aflatoxins could not be detected. In 2019, there were 7 maize samples for which the concentrations of total aflatoxins could not be detected, from a total number of 62 samples with aflatoxin levels under 1.75 µg/kg. Thus, only 11 samples (6.15%) were identified as having no detectable concentrations of total aflatoxins, while for 117 samples, the presence of total aflatoxins was confirmed (< 1.75 µg/kg). Taken into account these results, there can be stated that a number of 168 maize samples (93.85%) showed total aflatoxin contamination (Figure 3). Out of the total number of assessed samples (179 maize samples), 51 samples noted concentrations of total aflatoxins over 1.75 µg/kg (28.49%). However, only one sample exceeded the total aflatoxin limit imposed by the European regulations and noted a concentration of 77.59 µg/kg (Table 1).

Table 1. Number (and percent) of 2018 and 2019 maize samples from Romania with various levels of total aflatoxin concentrations

Sample category (based on the relevance of the total aflatoxin concentrations)	Number (and percent) of maize samples		
	2018	2019	Overall
Analysed samples	84 (100.00%)	95 (100.00%)	179 (100.00%)
< 1.75 µg/kg	66 (78.57%)	62 (65.26%)	128 (71.51%)
ND*	4 (4.76%)	7 (7.37%)	11 (6.15%)
0 – 1.75 µg/kg*	62 (73.81%)	55 (57.89%)	117 (65.36%)
1.76 – 5.00 µg/kg	17 (20.24%)	31 (32.63%)	48 (26.82%)
5.01 – 10.00 µg/kg	1 (1.19%)	1 (1.05%)	2 (1.12%)
> 10.00 µg/kg	0 (0.0%)	1 (1.05%)	1 (0.56%)

*Results for which the 'Out of range' function of the RIDA®SOFT Win software was applied; ND – not detected

Several studies on mycotoxin occurrence in cereals suggest that maize is the cereal most frequently contaminated with aflatoxins in Europe [2, 10, 15]. In our study, the concentrations found in most of the examined samples were relatively low. Only one sample noted an extremely high total aflatoxin level (77.59 µg/kg). Similar aflatoxin levels in maize samples have been reported by Kos et al. (2018) [9] in neighbouring Serbia, where the maximum level was 111.2 µg/kg (72.3% contaminated samples) in 2012, a year which noted extreme drought conditions. The same authors noted for 2016, a year characterized by moderate weather conditions in Serbia, a maximum concentration of aflatoxins of 6.9 µg/kg (5.0% contaminated samples). In Romania, Tabuc et al. (2011) [15] noted that aflatoxin contamination was observed for 38% of the assessed maize samples. Within this study, 4.76% of the samples exceeded the EU regulation, while the highest contamination level was 42.60 µg/kg.

Distribution of total aflatoxins across Romania. The occurrence of aflatoxins in maize depends on many parameters, such as susceptibility (mycotoxin content taken as criterion) of a particular type of hybrid to *Aspergillus* infection, pedological characteristics of the soil, climate conditions, particularly temperature, precipitation and relative air humidity, especially during the maize's development stages or harvesting time [10, 14]. Results show a significant difference in terms of aflatoxin occurrence in between growing seasons from fields located in different regions of Romania. The descriptive statistics are shown in Table 2.

Table 2. Incidence and concentration levels of total aflatoxins detected in the 2018-2019 Romanian maize samples

Origin of samples		**Parameter**	**Year**	
NUTS I	**NUTS II**		**2018**	**2019**
Macroregion 1 (*n* = 24)	**North-West** development region	No. of samples	6	6
		Frequency (%)	16.67	33.33
		Mean (± SD) (µg/kg)*	0.82 (± 0.82)	1.82 (± 1.37)
		Range (µg/kg)*	0.13 – 2.30	0.95 – 3.62
		No. (%) of samples > ML	0	0
		Maximum level (µg/kg)	2.30	3.62
	Central development region	No. of samples	4	8
		Frequency (%)	25.00	0.00
		Mean (± SD) (µg/kg)*	1.06 (± 1.78)	1.13 (± 0.48)
		Range (µg/kg)*	0.07 – 3.72	0.65 – 1.63
		No. (%) of samples > ML	0	0
		Maximum level (µg/kg)	3.72	1.63
Macroregion 2 (*n* = 41)	**North-East** development region	No. of samples	8	11
		Frequency (%)	50.00	36.36
		Mean (± SD) (µg/kg)*	1.55 (± 0.95)	1.57 (± 0.86)
		Range (µg/kg)*	0.23 – 3.00	0.68 – 3.06
		No. (%) of samples > ML	0	0
		Maximum level (µg/kg)	3.00	3.06
	South-East development region	No. of samples	9	13
		Frequency (%)	22.22	30.77
		Mean (± SD) (µg/kg)*	1.29 (± 1.03)	1.32 (± 1.21)
		Range (µg/kg)*	0.41 – 3.65	0.54 – 4.28
		No. (%) of samples > ML	0	0
		Maximum level (µg/kg)	3.65	4.28
Macroregion 3 (*n* = 86)	**South-Muntenia** development region	No. of samples	43	39
		Frequency (%)	18.60	43.59
		Mean (± SD) (µg/kg)*	1.10 (± 1.08)	3.63 (± 12.00)
		Range (µg/kg)*	0.03 – 3.98	0.32 – 77.59
		No. (%) of samples > ML	0	1
		Maximum level (µg/kg)	3.98	77.59
	Bucharest-Ilfov development region	No. of samples	1	3
		Frequency (%)	0.00	0.00
		Mean (± SD) (µg/kg)*	0.30 (± 0.00)	0.83 (± 0.27)
		Range (µg/kg)*	n.a.	0.55 – 1.09
		No. (%) of samples > ML	0	0
		Maximum level (µg/kg)	0.30	1.09
Macroregion 4 (*n* = 28)	**South-West Oltenia** development region	No. of samples	7	9
		Frequency (%)	14.29	66.67
		Mean (± SD) (µg/kg)*	1.28 (± 0.88)	2.74 (± 1.92)
		Range (µg/kg)*	0.56 – 3.13	0.42 – 5.67
		No. (%) of samples > ML	0	0
		Maximum level (µg/kg)	3.13	5.67
	West development region	No. of samples	6	6
		Frequency (%)	16.67	0.00
		Mean (± SD) (µg/kg)*	1.75 (± 1.85)	1.10 (± 0.35)
		Range (µg/kg)*	0.60 – 5.48	0.79 – 1.71
		No. (%) of samples > ML	0	0
		Maximum level (µg/kg)	5.48	1.71
Overall (*n* = 179)		No. of samples	84	95
		Frequency (%)	21.43	34.74
		Mean (± SD) (µg/kg)*	1.26 (± 1.06)	2.42 (± 7.88)
		Range (µg/kg)*	0.03 – 5.48	0.32 – 77.59
		No. (%) of samples > ML	0	1
		Maximum level (µg/kg)	5.48	77.59
		ML (µg kg^{-1})	10.00	10.00

n = number of analysed samples; Frequency = the percent of samples ≥ 18.50 µg/kg / total number of samples from that region; Range = minimum and maximum values; Mean = average of the positive results; SD = standard deviation; ML = maximum permitted level set by EC Commission Regulation No. 1881/2006 for maize subjected to sorting or other physical treatment before human consumption e; *Results for which the 'Out of range' function of the RIDA®SOFT Win software was applied; n.a. = not applicable.

When referring to the 2018 contaminated maize samples, results show that Macroregion 2 noted the highest frequency (35.29%) of contaminated samples. At region level, the North-East development region (Macroregion 2) registered the highest percent of total aflatoxin occurrence. However, there were no maize samples exceeding the maximum permitted level of total aflatoxins in 2018. The highest contamination level of aflatoxins was 5.48 µg/kg, registered in Arad County (Macroregion 4, West development region).

In 2019, Argeș County (Macroregion 3, South-Muntenia development region) was identified as the region with the most number of contaminated samples (100%). There were assessed 8 maize samples from this County, which noted aflatoxin levels in the range of 2.75 – 77.59 µg/kg. Only one sample exceeded the total aflatoxin limit imposed by the European regulations and noted a concentration of 77.59 µg/kg. Other studies showed that 37% of maize samples (2002-2004) from the south-eastern region of Romania registered aflatoxin B1 contamination, where the highest contamination level was about 45 µg/kg [15]. Also, for 2008-2010 maize samples from the same region, aflatoxin B1 contamination was observed in 38 % of maize samples. Mean level of contamination was about 3.2 µg/kg and the highest contamination levels observed being about 42.6 µg/kg. Aut of these samples, 4.76% exceeded EU regulation [15].

Aflatoxin occurrence in correlation with the applied agronomic practices and cropping systems. When referring to the analysed samples, the total aflatoxin contamination was independent of the type of hybrid. Also, no hybrid type showed statistically significant ($P > 0.05$) differences in total aflatoxin content between the different levels of used nitrogen fertilisation during the 2018 and 2019 growing seasons in the assessed regions in Romania.

However, variations in total aflatoxin levels in maize samples, even when they originate from the same geographical region, could be attributed to the type of farming systems. Along with differences in temperature, humidity, soil and hybrid type, these factors are thought to play an important role in the observed aflatoxin concentrations. Our results showed that the southern counties proved that they may act as hotspot regions for aflatoxins depending on climate conditions. Taking into consideration the information provided by the monitoring questionnaires, the same counties noted poor agricultural practices, also. In order to prevent the occurrence of aflatoxins, management strategies to reduce mycotoxin contamination in the field are required.

Thus, farmers from mycotoxin hotspot regions could alternate maize with other crops like common beans and potatoes to support little to no growth of aflatoxin-producing fungi [13].

4. Conclusions

The present study showed that maize is a potential source of aflatoxin exposure in certain regions of Romania. Our results indicate that high concentrations of aflatoxins are independent of the hybrid type as well as the different levels of nitrogen fertilisers. Hotsport regions for aflatoxins were identified in areas where environmental conditions are favourable for the occurrence of toxigenic fungi. Also, not correctly applied agronomic practices represent a favourable factor for aflatoxin contamination.

The results showed that only a small fraction of the analysed samples contained unsafe aflatoxin levels. However, the detected level of total aflatoxins which exceeded the threshold set by the European regulations (10.00 µg/kg) was 77.59 µg/kg (Argeș County, South-Muntenia development region, Macroregion 3). Thus, more research is needed in the development of risk maps and mycotoxin management strategies for the maize crops located in hotspot regions for aflatoxin contamination, in order to result safe maize crops that will enhance trade and increase income and welfare of farmers and consumers.

Compliance with Ethics Requirements. Authors declare that they respect the journal’s ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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