IMPACTS OF CLIMATIC CONDITIONS ON AFLATOXIN B_1 AND FUMONISINS CONTAMINATION OF MAIZE KERNELS AND THEIR CO-OCCURRENCE

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Abstract: Agroecological and climatic conditions in Serbia greatly affected the development of toxigenic fungi and occurrence of mycotoxins in the maize. The presence of fungal toxigenic *Aspergillus* and *Fusarium* species and levels of aflatoxin B_1 (AFB_1) and sum of fumonisins B_1, B_2 and B_3 (FBs) were established in 127 maize kernel samples harvested during 2012 (37 samples) and 2013 (90 samples). The periods of silking and grain filling of the maize in 2012 in comparison to 2013 were characterised with extremely dry spells, with high temperatures and low precipitation sums. The mean incidences of *A. flavus* and *F. verticillioides* were 50.4 and 11.7% in 2012 and 18.9 and 33.4% in 2013, respectively. According to the regulations of the World Health Organisation, unacceptable levels of AFB_1 (>20 µg kg^{-1}) and FBs (>2000 µg kg^{-1}) were established in the 30.6 and 24.1% samples of 2012 and 16.7 and 40% maize kernel samples of 2013, respectively.

It can be concluded that high temperatures and low precipitation sums in 2012 favoured the development of *A. flavus* affecting the high level of AFB_1 , in comparison with *F. verticillioides* and the production of FBs. There was no positive correlation between the incidences of *A. flavus* and *F. verticillioides*, while a statistically significant positive correlation has been found between AFB_1 and FBs levels, in both investigated years (2012-2013). This indicates that the mycotoxin production depended more on weather conditions than on the distribution of corresponding toxigenic fungal species.

Key words: toxigenic fungi, *A. flavus*, *F. verticillioides*, aflatoxin B_1, fumonisins

Introduction

Recently, the variability of climatic conditions together with global climate changes contributes to higher biosynthesis of mycotoxins in maize, which causes economic losses in the production and risk for human and animal health. It is well known that aflatoxins and fumonisins are threat to human health, and in some cases, directly cause a disease and even death. Aflatoxins are hepatotoxins, teratogens, mutagens and carcinogens. Fumonisins cause more frequently diseases in animals, such as leukoencephalomalacia in horses and pulmonary oedema in swine and they are also tumour promoters in rats (*Abbas et al., 2002; Fandohan et al., 2004*). Aflatoxins are secondary metabolites of fungi *Aspergillus flavus* and *Aspergillus parasiticus*, while fumonisins are produced by species *Fusarium verticillioides* and *Fusarium proliferatum* (*Abbas et al.,* 2002).

Increased temperatures and drought stress are the principal factors causing high levels of aflatoxins in maize. High temperatures, especially night-time temperatures above 20°C, and drought favour the development, sporulation and distribution of *A. flavus* in the period from plant silking to grain filling. It is considered that night-time temperatures are more important for the growth of mycotoxin producing fungi, because the energy balance of the plant is negative during the night and its ability to defend itself is lower (*Abbas et al., 2002, 2006*). Likewise, drought, extremely high temperatures, insect damages and water activity (a_w) are the main factors causing the risk of the *Fusarium* infection and later fumonisin contamination in maize kernels. Furthermore, maize contamination with fumonisin-producing *Fusarium* species depends on the geographical area and agroecological conditions (*Medina et al., 2015*).

Maize contamination with aflatoxins is of a great concern, since this crop is a major source of food and feed worldwide. Until recently, aflatoxins have not been signalled as a matter of concern for the primary production in Europe. Under agroecological conditions of many European countries, such as Germany (*Curtui et al., 2004*), Hungary (*Varga et al., 2004*), Belgium (*Chandelier et al., 2004*), Poland (*Perkowski et al., 2004*), Austria (*Öhlinger et al., 2004*), *Aspergillus* cereal infection and aflatoxin contamination in fields are rare and usually with low mycotoxin levels. However, years 2003 and 2012 have to be mentioned, for Italy and south Europe, because of the alarming aflatoxin contamination in maize (*Battilani et al., 2016*). During 2013, in Italy, the intensive occurrence of *A. flavus* and aflatoxins in maize was observed as a result of extremely high temperatures during the growing season, as well as of conditions of insufficient moisture/drought stress in the period from May to September (*Moretti et al., 2004*). In south-eastern Romania, *Tabuc et al. (2009)* recorded approximately 30% maize samples, collected between 2002 and 2004, contaminated with aflatoxin B_1 (AFB_1). *Aspergillus* species are considered poor maize pathogens under average climatic

conditions of Serbia. However, due to extreme high temperatures and low precipitation sums in Serbia in the 2012 summer, the incidence of *A. flavus* species was high in many plant species (soya bean, maize, sunflower, barely, wheat), which resulted in a high aflatoxins level. The intensity of *Aspergillus* spp. infestation in 2012 was up to 95.3% in maize kernels (*Lević et al., 2013*) with a high AFB_1 level (>40 µg/kg) in 55.17% of analysed maize samples (*Krnjaja et al., 2013*).

Agroecological conditions in Serbia are suitable for the intensive development of pathogenic and toxigenic species of the genus *Fusarium* (*Lević et al., 2004*). It is considered that in the past few years there has been an increasing occurrence of species of the section *Liseola* (*F. verticillioides, F. proliferatum* and *F. subglutinans),* and in particular *F. proliferatum* in cereal grain (*Stanković et al., 2008*). These species produce fumonisin B, of which FB_1 is mostly distributed under natural conditions (*Nelson et al., 1993*). It has been established that these fungi synthesised FB_1 more in maize kernels than in wheat and barley kernels (*Visconti and Doko, 1994*). In Serbia, there is less data on the presence of fumonisins in cereals in comparison with other toxins (*Stepanić et al., 2011*).

There are different results on the correlation between occurrences of aflatoxins and fumonisins, as well as their producers in maize kernels. Results obtained by *Abbas et al. (2006)* indicated that the natural infection with *Fusarium* spp. did not affect the occurrence of *Aspergillus* spp. nor aflatoxin production (*Widstrom et al., 1994*). Some authors reported a negative correlation between *Aspergillus* spp. and *Fusarium* spp. infections in maize kernels (*Marin et al., 1998*), so it could be expected that the levels of aflatoxins and fumonisins would be negatively correlated. However, results obtained by *Abbas et al. (2006)* showed a positive correlation between these two mycotoxins. Moreover, *Scudamore (1997)* determined that maize samples were predominantly contaminated with several mycotoxins. Aflatoxins and fumonisins were the most common combination (28%) in total of 60% of maize samples positive for more than one mycotoxin.

Taking into account that agroecological conditions in Serbia are favourable for the development of toxigenic species of the genus *Fusarium*, and in some years even species of the genus *Aspergillus*, the aim of this study was to determine the impact of climatic factors on the incidence of fungal toxigenic *Aspergillus* and *Fusarium* species and the level of their mycotoxins, AFB_1 and FBs. The correlation between maize grain contamination with AFB_1 and FBs was observed in particular and the frequency of their co-occurrence was established.

Materials and Methods

A total of 127 kernel samples were collected during the maize harvests in 2012 (37 samples) and 2013 (90 samples) in the production plots in Zemun Polje

(in the vicinity of Belgrade, Serbia). Each sample contained 1 kg of the maize kernels, out of which subsamples of 200 g were drawn and used in the further mycological and mycotoxicological analyses.

In mycological analyses, the maize kernels was disinfected with the solution of sodium hypochlorite (NaOCl) and water (1:3) for 3 minutes, rinsed with distilled water and dried on sterile filter paper. Then, 100 kernels of each sample were plated on potato dextrose agar (PDA) in Petri dishes (five kernels per Petri dish). The kernels were incubated in the dark during 7 days at 25°C. According to the fungal keys of *Singh et al. (1991)* and *Burgess et al. (1994)* the identification of toxigenic species, *A. flavus* and *F. verticillioides*, was performed by morphological criteria. The incidence was calculated by percentage of fungal species in each maize kernel sample.

A total of 100 g of maize kernels from each sample were dried at 50°C for 72h. After drying, the samples were ground to the fine powder using the basic analytical mill (A11, IKA, Germany). The samples were analysed for the presence of AFB_1 and FBs (sum of FB_1, FB_2 and FB_3) using ELISA (Enzyme linked immunosorbent assay). The mycotoxin analysis was performed according to the manufacturer's instructions (Tecna S.r.l., Italy, Celer AFLA B_1 Test Kit and Celer Fumo Test Kit). The quantitative determination of mycotoxins was performed at 450 nm wavelength using an ELISA reader (BioTek EL x 800^{TM}). The detection limit values for AFB_1 and FBs were 1 μg kg^{-1} and 750 μg kg^{-1}, respectively.

The obtained results were processed in the software package Statistica 12.0. The Pearson's coefficient of correlation (Pearson Product-Moment Correlation) was used to establish the relationship between the species of *A. flavus* and *F. verticillioides*, as well as, between the concentrations of AFB_1 and FBs. The t-test was used to determine the significance level of the coefficient of correlation.

Results

Meteorological data for the Zemun Polje area have been provided by the meteorological station of the Maize Research Institute, Zemun Polje. In 2012, high mean daily temperatures in July and August (>26°C), very low precipitations (4 mm) and a very low precipitation factor (0.15) in August, caused an extreme drought in comparison to the corresponding period in 2013. At the end of the growing season, the higher recorded precipitations and the precipitation factor were, recordedin 2013 than in 2012 (Table 1).

In the mycological analyses, the incidence of toxigenic species belonging to the genera *Aspergillus* and *Fusarium* varied depending on the year of investigation. In 2012, *A. flavus* and *F. verticillioides* were isolated in the range of 6.6-95.5% (mean 50.4%), and 0-44.4% (mean 11.7%), respectively. The statistically very significant negative correlation (r=-0.55**) was established

between distributions of these two species. In 2013, the incidence of the species *A. flavus* was lower (18.9%) and of *F. verticilioides* was higher (33.4%) in comparison with incidences recorded in 2012, while the correlation between distributions of these two species was statistically insignificantly negative (r=-0.08) (Table 2).

Table 1. Total monthly precipitation, mean daily temperature and precipitation factors per month from May to September in the Zemun Polje area (in the vicinity of Belgrade) in 2012 and 2013

Month	Year					
	2012			2013		
	Rainfall (mm)	Temperature (°C)	Pf*	Rainfall (mm)	Temperature (°C)	Pf*
May	127.5	17.9	7.12[SH]	93.9	19.68	4.77[SA]
June	13.9	24.56	0.57[A]	37.8	21.89	1.73[A]
July	39.0	27.08	1.45[A]	16.0	23.78	0.67[A]
August	4.0	26.21	0.15[A]	12.7	23.72	0.53[A]
September	31.4	22.14	1.42[A]	70.1	16.87	4.15[SA]

*Pf = precipitation / average monthly temperature: A- arid climate (Pf <3.3), SA- semi-arid climate (Pf 3.4-5.0), SH- semi-humid climate (Pf >5) (Gračanin, 1950, cit. Lević, 1987)

Table 2. Incidence (%) of toxigenic species *A. flavus* and *F. verticillioides* in maize kernel samples collected in 2012 and 2013 and their correlation coefficient (r)

Year	Number of total samples	*A. flavus*	*F. verticillioides*	*A. flavus*	*F. verticillioidies*	Correlation coefficient
		Range		Mean		
2012	37	6.6 –95.5	0 – 44.4	50.4	11.7	-0.55**
2013	90	0 – 79	7 – 88	18.9	33.4	-0.08[ns]

**significant at the 0.01 level of probability; *significant at the 0.05 level of probability; ns, not statistically significant.

Table 3. Sample size (number of positive samples/number of total samples), incidence rate and levels of AFB_1 (µg kg^{-1}) and FBs (µg kg^{-1}) in tested maize samples in 2012 and 2013

Year	2012		2013	
Items	AFB_1	FBs	AFB_1	FBs
Sample size	36/37	29/37	12/90	30/90
Incidence (%)	97.3	78.4	18.9	33.3
Range (µg kg^{-1})	0 – 491.7	0 – 10790	0 – 27.9	0 – 10860
Mean (µg kg^{-1})	60.3	1300	1.3	2800
Correlation coefficient (r)	0,40*		0,61**	

**significant at the 0.01 level of probability; *significant at the 0.05 level of probability; ns, not statistically significant.

Table 4. Distribution of AFB$_1$ and FBs levels in investigated maize kernel samples harvested in 2012 and 2013

	Year 2012	Year 2013		Year 2012	Year 2013
AFB$_1$ (µg kg^{-1})	% of total samples		FBs (µg/kg)	% of total samples	
< 20	69.4	83.3	< 2000	75.9	60.0
> 20	30.6	16.7	> 2000	24.1	40.0

In the mycotoxicological analyses, AFB$_1$ was detected in 97.3% (2012) and 18.9% (2013) of maize kernel samples, and FBs was recorded in 78.4% (2012) and 33.3% (2013) of maize kernel samples. In 2012, levels of AFB$_1$ ranged from 0 to 491.7 µg kg^{-1} (mean 60.3 µg kg^{-1}), while FBs ranged from 0 to 10790 µg kg^{-1} (mean 1300 µg kg^{-1}). The corresponding values in 2013 amounted to 0-27.9 µg kg^{-1} (mean 1.3 µg kg^{-1}) and 0-10806 µg kg^{-1} (mean 2800 µg kg^{-1}), respectively. Statistically significantly positive (r = 0.40*) and statistically very significant (r = 0.61**) correlations were established between AFB$_1$ and FBs in 2012 and 2013, respectively (Table 3).

According to the regulation of World Health Organisation (WHO) unacceptable levels of AFB$_1$ (>20 µg/kg) and FBs (>2000 µg/kg) were detected in 30.6 and 24.1% maize kernel samples in 2012 and 16.7 and 40% maize kernel samples in 2013, respectively (Table 4).

Discussion

The incidence of toxigenic species, *A. flavus* and *F. verticillioides*, and production of AFB$_1$ and FBs in investigated maize harvested during the growing seasons of 2012 and 2013 had significantly depended of climate factors (temperatures and precipitation). It was determined that high temperatures, low precipitations and a low precipitation factor during the summer in 2012 favoured the intensive development of toxigenic species *A. flavus* and a high AFB$_1$ production in maize kernels. However, high mean daily temperatures in July and August (>20°C) favoured the development of the species *F. verticillioides* in both years of investigation, while increased precipitations prior to harvest resulted in a greater FBs production in 2013. Similarly, *Magan et al. (2011)* were established that temperatures at different levels of water availability (a$_w$) affected the scope of the development of toxigenic fungi and the mycotoxin concentration. According to the results of *Miraglia et al. (2009)* the ability of fungi to produce mycotoxins was most dependent on temperatures, relative air humidity, insect damages and stress in host plants. *Abbas et al. (2006)* also observed that climatic conditions in 1998 favoured high levels of both, aflatoxins and fumonisins, while in 2001, a high level of fumonisins and a low level of aflatoxins in maize kernels were favoured. These authors concluded that climatic conditions, high temperature stress during the kernel development period, especially night-time temperatures above 20°C, were

significant for the explanation of the stated differences. High risk for maize contamination with fumonisins is caused by high temperatures, drought and abundant precipitation during the growing season (*Miller, 2008*).

The species *A. flavus*, i.e. *F. verticillioides* was dominant in 2012, i.e. 2013, respectively. Although both species were present in both years of investigation the coefficient of their co-occurrence was negative (Table 2). However, the correlation between AFB_1 and FBs levels was positive and statistically significant in both years of investigation (Table 3). Similar to our study, *Sun et al. (2011)* were established 100% AFB_1 positive and 85.3-100% FB_1 positive maize samples with the co-occurrence of AFB_1 and FB_1 in 90.3-100% maize samples. Likewise, *Abbas et al. (2006)* established a positive correlation between the levels of aflatoxins and fumonisins, emphasising that the natural infection of maize with *Fusarium* spp. did not provide protection against the aflatoxin production. In addition, the greater incidence of *F. verticillioides* compared to *A. flavus* in maize kernel samples drawn in the 1999 and 2000 harvests was explained by *Bush et al. (2003)* by the competitive relationship between these two species. Contrary, *Sreenivasa et al. (2011)* isolated a high percentage of species, *F. verticillioides* (86%) and *A. flavus* (84.9%), from 86 maize kernel samples. According to *Zorzete et al. (2008)* in analysed maize samples the species *F. verticillioides* was isolated in a greater percentage (34.6%) than the species *A. flavus* (0.5%) and the correlation coefficient between these two species was negative. *Rocha et al. (2009)* established a statisticaly significant negative correlation (r=-0.61) between isolated *Fusarium* and *Aspergillus* genera. *Marin et al. (1998)* observed that activities of the species *F. verticillioides* and *F. proliferatum* reduced the presence of the species *A. flavus*, *A. niger* and *A. ohraceus*, particularly under conditions of high a_w and temperatures of 15°C. Species *F. verticillioides* and *F. proliferatum* tend to increase the production of fumonisins in the presence of *A. niger* and *A. flavus* under certain environmental conditions.

In this study, unacceptable AFB_1 and FBs levels were established in 30.6 and 16.7% and 24.1 and 40% maize samples in 2012 and 2013, respectively. In China, according to the results of *Sun et al. (2011)* the unacceptable levels of AFB_1 and FB_1 were recorded in 35% and in 58% of the maize samples, respectively. These authors concluded that the climatic conditions in the location of Huaian favoured the development of toxigenic fungi and a greater accumulation of mycotoxins in observed maize samples. Similarly, in Malaysia, *Reddy and Salleh (2011)* observed 80 maize samples and detected AFB_1 and FB_1 in 81.2% and 100% of analysed samples, respectively. Eighteen (22.5%) samples had an AFB_1 level above the internationally prescribed limit (20 µg/kg) ranging from 20.6 to 135 µg/kg.

In conclusion, the present study confirmed an exceptionally significant effect of climatic factors on the presence and incidence of toxigenic fungi of

Aspergillus and *Fusarium* genera as well as on AFB$_1$ and FBs levels. In order to recommend preventive measures in the management risks of the mycotoxin presence in maize and other cereals, it is necessary to provide constant and relevant information on the impact of abiotic and biotic factors on cereal contamination with toxigenic fungal species and on the production of their mycotoxins. This information should contain data on health safety of cereals in the field and later during their storage. Considering the differences in the incidence of toxigenic species established in this study during the two growing seasons, it can be concluded that the meteorological, weather conditions have a crucial role in the development of toxigenic fungi in the maize. It is therefore important to improve the monitoring of weather conditions under conditions of global climate changes that can radically alter the distribution and the spectrum of toxigenic fungal species in maize worldwide. Maize is used as a staple food in many countries around the world. Animal husbandry as an economic branch is also largely dependent on maize as a main source of feed. The mycotoxin accumulation in cereal crops in fields and later during storages is a risk to human and animal health. Setting appropriate tolerance values for mycotoxins in cereals will be challenges for current and future researchers. These tolerances should have a significant impact on the improvement of public health, especially children's health. Further research should be focussed on studying the effects of major factors affecting the intensive occurrence of mycotoxins and, based on their results, on the development and application of integrated measures that will contribute to better management practices for these harmful agents and on the provision of food safety.

Uticaj klimatskih uslova na kontaminaciju zrna kukuruza sa aflatoksinom B$_1$ i fumonizinima i njihova združena pojava

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Rezime

Agroekološki i klimatski uslovi u Srbiji veoma su pogodni za razvoj toksigenih gljiva i njihovih mikotoksina u kukuruzu. Prisustvo toksigenih vrsta gljiva iz rodova *Aspergillus* i *Fusarium,* kao i nivoi aflatoksina B$_1$ (AFB$_1$) i ukupnih fumonizina B$_1$, B$_2$ i B$_3$ (FBs) utvrđeni su u 127 uzoraka zrna kukuruza iz berbe tokom 2012 (37 uzoraka) i 2013. godine (90 uzoraka). U fenofazama svilanja i nalivanja zrna kukuruza u 2012. u odnosu na 2013. godinu zapaženi su ekstremno sušni periodi sa visokim temperaturama i niskim količinama padavina. Prosečne vrednosti za učestalost vrsta *A. flavus* i *F. verticillioides* bile su 50,4 i 11,7% u

2012., odnosno 18,9 i 33,4% u 2013. godini. Prema pravilniku Svetske zdravstvene organizacije (WHO) nedozvoljeni nivoi AFB_1 (>20 µg/kg) i FBs (>2000 µg/kg) utvrđeni su u 30,6% i 24,1% uzoraka u 2012., odnosno u 16,7% i 40% uzoraka u 2013. godini.

Na osnovu dobijenih rezultata zaključeno je da su visoke temperature i niske količine padavina u 2012. godini uslovile značajno veću učestalost *A. flavus* i visoku produkciju AFB_1 u poredjenju sa vrstom *F. verticillioides* i produkcijom FBs. U obe ispitivane godine (2012-2013), između učestalosti *A. flavus* i *F. verticillioides* nije ustanovljena pozitivna korelacija, dok je statistički značajna pozitivna korelacija ustanovljena između nivoa AFB_1 i FBs. Ovo ukazuje da produkcija mikotoksina je više zavisna od vremenskih uslova u odnosu na distribuciju toksigenih vrsta gljiva.

Ključne reči: toksigene gljive, *A. flavus*, *F. verticillioides*, aflatoksin B_1, fumonizini

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