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## MOLDS AND MYCOTOXINS IN FRESHLY HARVESTED MAIZE

**ABSTRACT:** Incidence of toxigenic fungi (molds) and concentration of mycotoxin aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), deoxynivalenol (DON), zearalenone (ZON) and fumonisin (FB<sub>1</sub>) were studied in the maize grains collected immediately after harvesting in 2012. A total of 29 maize samples were analyzed and the highest incidence was determined for fungal species of *Rhizopus* (56.41%), *Aspergillus* (43.66%) and *Fusarium* (14.97%) genera. Significantly lower incidence was obtained for species of genus *Penicillium* (3.31%), and especially for species of genera *Acremonium* (1.38%), *Alternaria* (0.75%) and *Cladosporium* (0.14%). Among toxigenic fungi *Aspergillus flavus* (36.69%) was the most common species of *Aspergillus* genus, whereas the *Fusarium verticillioides* with 14.69% of incidence was the predominant species of *Fusarium* genus. In all studied maize samples, the presence of AFB<sub>1</sub>, ZON and FB<sub>1</sub> mycotoxins was established, except for DON which was established in 75.86% samples. AFB<sub>1</sub> was detected in average concentration of 13.95 µg kg<sup>-1</sup> for 44.83% of samples, and average concentration higher than 40 µg kg<sup>-1</sup> for 55.17% of samples. The average concentrations of DON which was detected was 235 µg kg<sup>-1</sup>, while it was 98.38 µg kg<sup>-1</sup> and 3590 µg kg<sup>-1</sup> for the presence of ZON and FB<sub>1</sub>, respectively. Moderate positive correlation was obtained between concentrations of AFB<sub>1</sub> and FB<sub>1</sub> (r=0.35), while weak positive correlation was established between concentrations ZON and DON (r=0.02).

**KEYWORDS:** maize grains, molds, mycotoxins

## INTRODUCTION

Maize is one of the major crops in Serbia, and it plays an important role in rotation with wheat, in animal feed and direct human consumption. Maize grain as energy source is widely used in both human and animal nutrition (cows, sheep, goat, poultry and fish etc.). The nutritive value of maize grain depends on the nutrient contents and digestibility (A l p t e k i n et al., 2009). Maize grains are subject to infection by a variety of toxigenic fungi, most

commonly *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp. and *Penicillium* spp. (K r n j a j a et al., 2006, 2007, and 2011). Toxigenic *Alternaria* and *Fusarium* species are often classified as field fungi, while *Aspergillus* and *Penicillium* species are considered as storage fungi (L o g r i e c o et al., 2003).

Although contamination with fungi diminishes the quality of grain, toxigenic fungi species can produce highly toxic compounds known as mycotoxins. Fungal growth and toxin production in maize have been found to depend on several interacting factors which stress maize plants. Stress factors include low moisture content of soil, high daytime maximum temperatures, high nighttime minimum temperatures, and nutrient-deficient soils (A b b a s et al., 2006).

The fusariotoxins (fumonisins, zearalenone – ZON and deoxynivalenol – DON) and aflatoxins have an important economic impact on the grain industry. Fumonisins are mycotoxin group produced by *F. verticillioides*, *F. proliferatum* and *A. niger* (M o g e n s e n et al., 2009) while ZON and DON were produced by *Fusarium* species, mainly of *F. graminearum* and *F. culmorum* (Y l i – M a t t i l a, 2010). Aflatoxins are potent carcinogenic and toxic metabolites produced by fungal species *A. flavus* and *A. parasiticus* (M e d i n a – M a r t í n e z and M a r t í n e z, 2000).

The negative effect of mycotoxins on the growth and health of livestock make them a major problem for many production systems. Food and feed safety and hygiene represent a significant problem, and great attention is directed towards diseases that are closely related to different mycotoxicoses. Mycotoxins cause a whole range of disorders in the body of animals, ranging from biochemical changes, through the functional and morphological damages of different tissues and organs, to the appearance of clinical signs of mycotoxicoses with even possible fatal consequences (J a k i ć – D i m i ć et al., 2009).

The objectives of this study were to identify the fungi with special focus on *Aspergillus* and *Fusarium* species in freshly harvested maize grains and to quantify the associated mycotoxins.

## MATERIAL AND METHODS

A total of 29 maize grain samples were collected in the experimental maize fields of the Institute for Animal Husbandry (Belgrade) immediately after the harvest in 2012. Samples were collected according to the Commission Regulation (EC) No 401/2006 (E u r o p e a n C o m m i s s i o n, 2006). The total moisture content was determined according to the drying method.

For mycological analysis, kernels were surface-sterilized for 5 min in 5% NaOCl and rinsed three times in sterile water. Fifty kernels (5 kernels per plate 90 mm) from each sample (1450 kernels in total) were transferred to 2% water agar (WA) and the agar plates were incubated at 25°C in the dark for 5-7 days. The morphological characteristic of isolated genera and species were

identified based on macroscopic (colony appearance) and microscopic (spores size and shape) traits (W a t a n a b e, 1994).

The presence of ZON, DON, FB1 and AFB1 was tested by *enzyme-linked immunosorbent assay* (ELISA) method. Five grams of sample were mixed with 1 g of NaCl and homogenized in 25 ml of 70% methanol in a 250 ml Erlenmeyer flask on the orbital shaker (GFL 3015, Germany) for 30 minutes. Homogenate was filtered through a Whatman filter paper 1. The filtrate was further analyzed according to the manufacturer's instructions Celery Techna ® ELISA kits. Absorbance was measured at a wavelength of 450 nm on an ELISA reader spectrophotometer (Biotek EL x 800TM, USA).

The incidence (I) of certain fungi was estimated according to L e v i ć et al. (2012):  $I (\%) = (\text{number of seeds in which a species occurred} / \text{total number of seeds}) \times 100$ .

Correlation between the concentrations of the investigated mycotoxins was determined using Pearson's correlation coefficient.

## RESULTS AND DISCUSSION

In mycological study of 1450 maize kernels (50 kernels / sample) with average moisture content of 11.74%, seven fungal genera, *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Rhizopus* were identified. The most common isolated species were from the genera *Rhizopus* (66.41%), *Aspergillus* (43.66%) and *Fusarium* spp. (14.97%), followed by species of genera *Penicillium* (3.31%), *Acremonium* (1.38%), *Alternaria* (0.75%) and *Cladosporium* (0.14%) (Table 1). From the genus *Aspergillus*, species *A. flavus* was more dominant (36.69%) than *A. niger* (6.97%), while from the genus *Fusarium*, two species were identified, *F. verticillioides* with presence of 14.69% which was more dominant than *F. graminearum* (0.28%) (Table 2).

Tab. 1. – Incidence of toxigenic fungi in maize grain samples

Fungal genera	Number of infected kernels/Total number of investigated kernels	% infected kernels
<i>Acremonium</i> spp.	20/1450	1.38
<i>Alternaria</i> spp.	11/1450	0.75
<i>Aspergillus</i> spp.	633/1450	43.66
<i>Cladosporium</i> spp.	2/1450	0.14
<i>Fusarium</i> spp.	217/1450	14.97
<i>Penicillium</i> spp.	48/1450	3.31
<i>Rhizopus</i> spp.	818/1450	56.41

Tab. 2. – Incidence of toxigenic species from *Aspergillus* and *Fusarium* genera in samples of maize grain

Fungal species	Number of infected kernels/Total number of investigated kernels	% infected kernels
<i>Aspergillus flavus</i>	532/1450	36.69
<i>Aspergillus niger</i>	101/1450	6.97
<i>Fusarium verticillioides</i>	213/1450	14.69
<i>Fusarium graminearum</i>	4/1450	0.28

According to the data from the Republic Hydrometeorological Service of Serbia for 2012, extremely high temperatures (daily temperatures above 25°C) and mostly dry weather with little precipitation (49 mm) were recorded during the reproductive period of maize. These climatic conditions were favorable for the development of some fungal species on maize grain, especially species from the *Aspergillus* and *Fusarium* genera.

Mycotoxycological study of 29 samples of maize grains revealed natural occurrence of four mycotoxins, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), zearalenone (ZON), deoxynivalenol (DON) and fumonisin B<sub>1</sub> (FB<sub>1</sub>). Average concentrations of fusariotoxins were 98.38 µg kg<sup>-1</sup> for ZON in 100% positive samples, 235 µg kg<sup>-1</sup> for DON in 75.68% positive samples and 3590 µg kg<sup>-1</sup> for 100% FB<sub>1</sub> positive samples (Table 3). All investigated samples of maize grain were 100% AFB<sub>1</sub> positive and 26 of total 29 samples of maize grain showed concentration >5 µg kg<sup>-1</sup>. AFB<sub>1</sub> was detected in 44.83% of the samples with an average concentration of 13.95 µg kg<sup>-1</sup>, while in other samples (55.17%) AFB<sub>1</sub> was present in average concentrations of >40 µg kg<sup>-1</sup>.

Tab. 3. – Concentration of mycotoxins in samples of maize grain

Item	AFB <sub>1</sub>	ZON	DON	FB <sub>1</sub>
Sample size <sup>a</sup>	13/29+16/29 = 29/29	29/29	22/29	29/29
Incidence (%)	44.83 + 55.17 = 100	100	75.86	100
Average (µg kg <sup>-1</sup> ) <sup>b</sup>	13.95 + >40	98.38	235	3590.00

<sup>a</sup> Number of positive samples/Number of total samples

<sup>b</sup> Average concentration in positive samples

Positive but not significant ( $r=0.35$ ) co-occurrence of AFB<sub>1</sub> and FB<sub>1</sub> was established and it was weaker between ZON and DON ( $r=0.02$ ).

Based on the investigations carried out over the last decade in Serbia, there are more data about natural occurrence of fusariotoxins (J a j i ć et al., 2008a,b; K o k i ć et al., 2009; S t a n k o v i ć et al., 2011), than of AFB<sub>1</sub> in maize grains. This can be explained with the fact that *Aspergillus* spp. was not so common in freshly harvested maize during the investigated period. Thirty years ago, there were no significant changes in the distribution of genera *Aspergillus* (5.0%), *Fusarium* (41.0%) and *Penicillium* (46.0%) in maize grain in Serbia (L e v i ć et al., 2004), except in the species composition and its frequency. In recent years, the genus *Fusarium* has been the most common

fungus isolated from freshly harvested maize grains, followed by species from genera *Alternaria*, *Aspergillus*, *Rhizopus* and *Penicillium*. It was reported that among the *Fusarium* species the most dominant were *F. verticillioides* and *F. graminearum* (K r n j a j a et al., 2006, 2011).

In Serbia, there are numerous data about the high presence of *Aspergillus* spp. isolated from different kinds of feed which contained maize. From the samples for feeding dairy cattle K r n j a j a et al. (2008), *Aspergillus* spp. and *Fusarium* spp. were isolated in ground maize samples in the highest percentage (85.70%, 71.40%), while in samples of maize grain this percentage was lower for these fungal species, *Aspergillus* spp. (52.90%) and *Fusarium* spp. (64.70%). The most common species was *A. flavus* with 71.40% in samples of ground maize and 47.10% in samples of maize grain (K r n j a j a et al., 2008). Furthermore, from the samples of feed for dairy cows, the widest spectrum of fungi species was isolated in autumn and the most present were species of *Aspergillus* genus (68.0%), whereas *Penicillium* species were mostly present in summer (94.0%) and winter period (68.0%) (Š k r i n j a r et al., 2008). According to these authors, the presence (56.0%) of species from genera *Fusarium*, *Mucor* and *Penicillium* was equal in spring.

According to the National Regulation in the Republic of Serbia, all tested samples did not show an increase in average concentrations of ZON and DON above the maximum allowed limits, while concentration of FB1 was rather high comparing with maximum allowed limit (4000  $\mu\text{g kg}^{-1}$ ) in unprocessed maize. In addition, according to the Regulation on Amendments to the Regulation on the maximum allowed levels of residue of pesticides in food and animal feed, for which maximum allowed quantities of residue of products for plant protection are regulated (Sluzbeni Glasnik RS, 2011), concentrations of AFB1 in 26 of total 29 maize samples exceeded the maximum allowed limit ( $>5 \mu\text{g kg}^{-1}$ ) in unprocessed maize.

In the first report on DON content in crops in Serbia, J a j i ć et al. (2008a) detected its presence in 44.7% of 76 investigated maize samples from the harvest in 2004, with a range of 40-2460 (on average 536  $\mu\text{g kg}^{-1}$ ). In only three samples concentrations were above the maximum level adopted by the European Commission. During the harvest from 2005 to 2007, there were 32.4% DON positive samples in a range of 27-2210 (on average 223  $\mu\text{g kg}^{-1}$ ) (J a j i ć et al., 2008b). Fumonisin and DON were found in 75 and 25% of the maize samples collected in 2009, respectively, but none of the samples was contaminated with aflatoxins, ochratoxins and ZON (K o k i ć et al., 2009). FB1 in a range of 750-4300 (on average 1225.7  $\mu\text{g kg}^{-1}$ ) were detected in 72% of 203 maize samples collected during 2006-2009 (S t a n k o v i ć et al. 2011). In Turkey, it was reported that incidence of *Penicillium* spp. was significantly higher than *Fusarium* and *Aspergillus* spp. in 28 tested maize samples. Twelve samples demonstrated the level of total aflatoxins of  $>5 \mu\text{g kg}^{-1}$ . Aflatoxin levels ranged from 7.70-108.86  $\mu\text{g kg}^{-1}$ . To summarize, 43% of samples were contaminated with AFB1 (A l p t e k i n et al., 2009). Based on the data from R e d d y et al. (2007) and according to the USA Regulation, aflatoxin was above the regulatory limit ( $^320 \mu\text{g kg}^{-1}$ ) in maize grain which was harvested in

2004, while fumonisin showed even higher level ( $4000 \mu\text{g kg}^{-1}$ ). According to the data from Rogé et al. (2009), *Penicillium* (70%), *Fusarium* (47%) and *Aspergillus* (34%) were the most frequent and abundant genera in maize grains produced in the Southeastern region of the Buenos Aires province. In all samples, DON levels ranged from 240 to  $1000 \mu\text{g kg}^{-1}$ , while ZON ranged from  $>100$  to  $1560 \mu\text{g kg}^{-1}$  in six positive samples and AFB1 was detected in two of 58 maize samples (Rogé et al., 2009). In Malaysia, *A. flavus* (87%), *A. niger* (83%), *F. verticillioides* (47%), *F. graminearum* (43%), *F. proliferatum* (42%), *F. equiseti* (30%) and *Penicillium* spp. (5%) were the prevalent fungi in 80 maize grain samples used for animal feeds. Eighteen (22.5%) samples exceeded AFB1 above the international regulatory limits (legal limits of Argentina, Australia, Brazil, India, Netherlands and USA) of animal feeds ( $>20 \mu\text{g kg}^{-1}$ ) ranging from 20.6-135  $\mu\text{g kg}^{-1}$ . Fumonisin were detected in all maize samples (100%) ranging from 261-2420  $\mu\text{g kg}^{-1}$  (Reddy and Salleh, 2011). In Argentina average values of AFB1 for freshly harvested samples of maize were between 0.38 and 2.54  $\mu\text{g kg}^{-1}$  during the period from 1999 to 2010. The average values and frequency of contamination with ZON and DON were low for all the investigated years. The average ZON and DON contaminations showed values from 0 up to 83  $\mu\text{g kg}^{-1}$  (ZON) and from 0 up to 140  $\mu\text{g kg}^{-1}$  (DON) (Garrido et al., 2012).

The co-occurrence of mycotoxins is very important because the combined effects of the toxins can have synergistic or antagonistic effects on animals. In this study, relatively low co-occurrence of AFB1 and FB1 can be explained because *A. flavus* and *F. verticillioides* infect maize ears by different routes. *A. flavus* is a nonpathogenic fungus colonizing through the silk ears and cracks in the pericarp of maize grain and do not need to be seedborne. *F. verticillioides* is endophytic to maize, entering the grain through the pedicle to occupy the internal space distal to the tip cap and is primary seedborne (Medina-Martínez and Martínez, 2000).

Weather conditions influence aflatoxin and fumonisin contamination of maize, although *Fusarium* spp. are found in a wider range of climate conditions. Heat stress during the period of kernel development, particularly nighttime temperatures of above  $20^{\circ}\text{C}$ , is a major factor in mycotoxin contamination (Abbas et al., 2006). Aflatoxin production by the *Aspergillus* spp. is triggered by drought and high temperature during grain fill. Nitrogen deficiency, excessive plant population, poor root development and insect damage of grains may also induce aflatoxin production in the field. When the weather conditions are favorable for the development of fungi, the fungus may produce aflatoxins at any stages of production and transformations (Alptekin et al., 2009). The optimal conditions for *Fusarium* species, which causes maize ear rot, tend to be hot and dry weather (Dohan et al., 2003). According to Miller (2001), the incidence of *Fusarium* kernel rot (*F. verticillioides* and *F. proliferatum*) is higher in warmer climates under dry conditions. In such environments, insect damage is well recognized as a collateral factor (Miller, 2001). There is no doubt that the climate is the principal factor triggering the fungal attacks, but several other factors may intervene and consequently affect

the incidence of the fungal infection in cereal crops over the regions such as the influence of location, cultivar properties and agricultural practices (crop rotation and management) (G u e r i f e t a l., 2001). Differences in geographical and environmental conditions might be responsible for differences in fungal distributions and concentration observed among different locations (R o i g é e t a l., 2009).

## CONCLUSION

The results obtained from this study showed that the most presented toxigenic fungi were from the genera *Aspergillus* and *Fusarium*. It could be assumed that their high incidence in the field resulted in high average concentration of AFB1 and FB1 in maize grain samples. This was also the consequence of exceptional favorable weather conditions during the period of maize maturation. Data obtained in this study indicated that all the subjects included in the technology of maize production should be responsible for the good quality of maize and its products.

Therefore, it is very important for farmers, feed manufacturers, agronomists, plant pathologists, and other human participants to be informed about the risks of consuming moldy maize and maize products. Emphasis should be put on designing the strategies that will develop awareness about the undesirable effects of contaminated grain in human and animal foods and feeds, and on organizing interventions with appropriate crop and commodity management methods to reduce the risk of contamination at the farm gate.

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## ПЛЕСНИ И МИКОТОКСИНИ У ЗРНУ КУКУРУЗА ПОСЛЕ БЕРБЕ

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### Резиме

Учесталост токсигених гљива (плесни) и концентрација микотоксина афлатоксина В<sub>1</sub> (AFB<sub>1</sub>), деоксиниваленола (DON), зеараленона (ZON) и фумонизина В<sub>1</sub> (FB<sub>1</sub>) је проучавана у узорцима зрна кукуруза прикупљеним одмах после бербе у 2012. години. У испитиваних 29 узорака највећу заступљеност имају врсте из родова *Rhizopus* (56,41%), *Aspergillus* (43,66%) и *Fusarium* (14,97%). Значајно нижа учесталост установљена је за врсте рода *Penicillium* (3,31%), а посебно за врсте из родова *Alternaria* (0,75%) и *Cladosporium* (0,14%). Међу токсигеним врстама, *A. flavus* (36,69%) је била најучесталија врста из рода *Aspergillus*, док је *F. verticillioides* са присуством од 14,69% била доминантна врста рода *Fusarium*. У свим испитиваним узорцима кукуруза установљено је присуство микотоксина AFB<sub>1</sub>, ZON-а и FB<sub>1</sub> (100%), осим DON-а који је био присутан у 75,86% узорака. AFB<sub>1</sub> је детектован у просечној концентрацији од 13,95 µg kg<sup>-1</sup> у 44,83% узорака и у просечној концентрацији од више од 40 µg kg<sup>-1</sup> у 55,17% узорака. Просечна концентрација DON-а је била 235 µg kg<sup>-1</sup>, ZON-а 98,38 µg kg<sup>-1</sup> и FB<sub>1</sub> 3590 µg kg<sup>-1</sup>. Средња позитивна корелација установљена је између концентрација AFB<sub>1</sub> и FB<sub>1</sub> (r=0,35), док је слаба позитивна корелација утврђена између концентрација ZON-а и DON-а (r=0,02).

КЉУЧНЕ РЕЧИ: зрна кукуруза, плесни, микотоксини

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