

THE PRESENCE OF TOXIGENIC *Fusarium* SPECIES AND FUSARIOTOXINS DEOXYNIVALENOL AND ZEARELENONE IN WINTER WHEAT

V. Krnjaja¹, S. Stanković², J. Lević²

¹Institute for Animal Husbandry, Autoput 16, P. Box 23, 11080, Belgrade-Zemun, Republic of Serbia

²Maize Research Institute „Zemun Polje”, Slobodana Bajića 1, 11185, Belgrade-Zemun, Republic of Serbia

Corresponding author: VesnaKrnjaja.IZS@gmail.com

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Abstract: The frequency of fungi and mycotoxin concentrations of deoxynivalenol (DON) and zearalenone (ZON) were studied in winter wheat grains harvested in 2009. The most frequently isolated species belonged to genera *Alternaria* (81.55%) and *Fusarium* (12%), followed by *Rhizopus* spp. (3.75%), *Acremoniella* spp. (1.15%) and other fungi (*Acremonium* spp., *Arthrimum* spp., *Aspergillus* spp., *Bipolaris* spp., *Chaetomium* spp., *Nigrospora* spp., *Penicillium* spp. and *Ramichloridium* spp.) isolated in less than 1%. The following species of the genus *Fusarium* were identified: *F. graminearum* (82.50%), *F. sporotrichioides* (5.42%), *F. proliferatum* (4.17%), *F. subglutinans* (4.17%), *F. poae* (1.66%), *F. semitectum* (1.25%), and *F. verticillioides* (0.83%). In 100% of wheat grain samples DON was detected (110–1200 µg kg⁻¹, average 490 µg kg⁻¹), while ZON was detected in 10% of samples and in the lower average of 70 µg kg⁻¹ with the limit values ranging from 60 to 80 µg kg⁻¹. Statistically significant positive correlations were established between the concentration of ZON with the frequency of *F. graminearum* ($r = 0.63^{**}$) or with the frequency of *Fusarium* spp. ($r = 0.58^{**}$). A negative insignificant correlation was determined between the DON level and the percentage of present *Fusarium* species.

Key words: wheat, moulds, *Fusarium* spp., DON, ZON

Introduction

The contamination of agricultural commodities by fungi able to produce toxic metabolites is often unavoidable and of worldwide concern. The relatively high intake of raw materials with the diet of livestock can lead to nutrient losses and have adverse effects on animal health and on productivity (Biagi, 2009). Pathogenic microorganisms and their secondary metabolites (mycotoxins) in a general chain of nutrition represent the most important potential risk to animal and

human health. Food safety is important problem requiring all-inclusive approach "from field to fork", including all sectors, from the production of livestock feed, primary production, food processing, storage, transport and placing on market (Radin et al., 2005). Wheat is one of the most important crops in Serbia; it is cultivated on approximately 600,000 ha with the average grain yield of 3600 kg ha⁻¹ (Statistical Yearbook, 2010). For livestock nutrition wheat grain can be used as concentrated livestock feed, while whole plants can be used as fodder. During the last twenty years, the increased level of colonisation and infection by *Fusarium*, particularly of ripening ears of cereals, has been attracting much attention. Firstly, because of the significant effects on the yield and quality of harvested grains, and secondly, because of the ability of *Fusarium* species to produce a wide range of mycotoxins which can enter the human and animal food chains (Magan et al., 2002).

The genus *Fusarium* is important in agriculture as it includes a number of important plant pathogens that can produce a wide spectrum of mycotoxins (Edwards et al., 2002). Many of toxigenic *Fusarium* species are also major pathogens of cereal plants, causing head blight in wheat and barley, for example, and ear rot in maize. A relationship between the level of crop infection with these pathogens and mycotoxin contamination of harvested grain is to be expected (Placinta et al., 1999). The *Fusarium* mycotoxins are primarily found in cereal grains, where trichothecenes, moniliformin, fumonisins and ZON are the predominant mycotoxins (D'Mello et al., 1999). Many toxigenic *Fusarium* species have been associated with infected wheat grains, but the predominant pathogens found worldwide are *F. graminearum* Schw. (sexual state *Gibberella zeae* (Schw.) Petch), and *F. culmorum* (W. G. Sm) Sacc. (Edwards et al., 2002). These species are primary causing agents of grain fusariosis known as Fusarium head blight (FHB). FHB is most easily recognised on immature ears where one or more spikelets in each ear become prematurely bleached. Sometimes large areas of ears may be affected and, where infection is severe, pink or orange spore masses occur on diseased spikelets. FHB can cause yield losses of 30-70% when conditions favour the disease, but, more importantly, grain from affected crops may be less palatable to stock than healthy grain and may contain mycotoxins (Bai and Shaner, 1994). The most important toxins produced by *F. graminearum* and *F. culmorum* are the trichothecene deoxynivalenol (DON) and the oestrogenic mycotoxin zearalenone (Bottalico, 1998). These mycotoxins have been linked to livestock toxicoses and feed refusal (Lemmens et al., 2004). In nonruminants, DON contaminated feed can reduce growth rates, while zearalenone can cause reproductive problems (Turkington et al., 2002).

Danger from pathogen fungi and their mycotoxins starts with the food chain. Therefore, in order to avoid harmful consequences of the impact of mycotoxins, it is necessary to conduct control of all procedures in the production and processing

of grain. Spike fusariosis in wheat is an economically significant problem in the wheat production in Serbia. The evaluation of grain quality after harvesting is one of the main steps in the control of the grain health status. This is the reason why the incidence of pathogenic fungi was investigated in this study, with a special focus on species of the *Fusarium* genus and its mycotoxins in wheat harvested in 2009.

Materials and Methods

Mycological analysis. A total of 20 wheat grain samples (1 kg minimum) were collected in the production plots of the Institute for Animal Husbandry in Belgrade-Zemun. Samples were collected successively during the wheat harvest period (June-July, 2009). The average grain moisture content was 14%. Subsamples of each of wheat samples were first rinsed under running water for 2 hours, then surface-disinfected using a commercial solution of sodium hypochlorite (1%) for 1 min, rinsed twice with sterile distilled water, and dried in a laminar flow cabinet. A total of 2000 wheat grains, 10 grains per plate, were arranged on the 2% agar medium in 10-cm Petri dishes. The plates were incubated at room temperature for 7–10 days. Based on macroscopic and microscopic morphological traits the identification of *Fusarium* species was done according to *Nelson et al. (1983)* and *Burgess et al. (1994)*, while the remaining fungal genera were determined according to *Ellis (1971)* and *Watanabe et al. (1994)*.

Mycotoxin analysis. The mycotoxin content in the wheat grains was determined by a direct competitive Enzyme linked immunosorbent assay (ELISA). Five grams of the ground wheat sample were extracted with 25 ml solution of methanol:water (70/30 v/v) for ZON and distilled water for DON. Furthermore, 1 g NaCl was added with the solvent. The samples were blended for 3 minutes, then filtrated through Whatman filter paper 1 and filtrates were collected. Dilution of filtrates was carried out according to the manufacturer's instructions and differed depending on the concentration of toxin in the sample. The ELISA procedure was performed by following the manufacturer's recommendations (Tecna S. r. l., Trieste, Italy). Absorbance was determined using the spectrophotometer Elisa reader BioTek EL x 800TM (Absorbance Microplate Reader) at 450 nm.

Statistical analysis. Data obtained for the frequency of *Fusarium* species and concentrations of mycotoxins in 20 investigated wheat grain samples were used for the calculation of Pearson's coefficient of correlation. Significance of interrelations of these two factors was tested at the level of $P_{0.05}$ and $P_{0.01}$.

Results and Discussion

Out of 2000 observed wheat grains within the samples collected immediately after harvest in June-July, 2009, 100% grains were contaminated with various fungal species. The mycobiota associated with wheat grains are shown in Table 1. Based on the fungal frequency 12 filamentous genera were obtained: *Acremoniella*, *Acremonium*, *Alternaria*, *Arthrinium*, *Aspergillus*, *Bipolaris*, *Chaetomium*, *Fusarium*, *Nigrospora*, *Penicillium*, *Ramichloridium* and *Rhizopus*. Species of the genera *Alternaria* and *Fusarium* were predominant (81.55% and 12%, respectively). The presence of species of the following genera was recorded to a smaller extent: *Rhizopus* (3.75%), *Acremoniella* (1.15%), *Ramichloridium* (0.5%), *Acremonium* (0.45%), *Chaetomium* (0.25%), and *Penicillium* (0.15%). Species of the genera *Arthrinium*, *Aspergillus*, *Bipolaris* and *Nigrospora* were present in the smallest percentage (0.05%).

Table 1. Frequency of fungal genera in winter wheat grains

Fungal genera	Frequency (%)
<i>Acremoniella</i>	1.15
<i>Acremonium</i>	0.45
<i>Alternaria</i>	81.55
<i>Arthrinium</i>	0.05
<i>Aspergillus</i>	0.05
<i>Bipolaris</i>	0.05
<i>Chaetomium</i>	0.25
<i>Fusarium</i>	12.00
<i>Nigrospora</i>	0.05
<i>Penicillium</i>	0.15
<i>Ramichloridium</i>	0.50
<i>Rhizopus</i>	3.75

Among species of the genus *Fusarium*, the species *F. graminearum* (82.5%) was predominant, and followed by 9.17% of species from section *Liseola* (4.17% *F. proliferatum* (Matsushima) Nirenberg, 4.17% *F. subglutinans* (Wollenw. & Reink.) Nelson, Toussoun & Marasas comb. nov. and 0.83% *F. verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* (Sacc.) Nirenberg), *F. sporotrichioides* Sherb. (5.42%), *F. poae* (Peck) Wollen. (1.66%) and *F. semitectum* Berkeley and Ravenel (1.25%) (Table 2).

Table 2. Frequency of *Fusarium* species in winter wheat grains

<i>Fusarium</i> spp.	Frequency (%)
<i>F. graminearum</i>	82.50
<i>F. poae</i>	1.66
<i>F. proliferatum</i>	4.17
<i>F. sporotrichioides</i>	5.42
<i>F. semitectum</i>	1.25
<i>F. subglutinans</i>	4.17
<i>F. verticillioides</i>	0.83

DON and ZON were detected in 100% and 10% wheat samples, respectively. Co-occurrence of DON and ZON was found in 10% of the samples analysed. The DON mycotoxin analysis determined the average concentration of 490 $\mu\text{g kg}^{-1}$ with the limit values of 110 to 1200 $\mu\text{g kg}^{-1}$. ZON was present in the lowest average concentration of 70 $\mu\text{g kg}^{-1}$ with limits ranging from 60 to 80 $\mu\text{g kg}^{-1}$ (Table 3).

Table 3. Concentrations of *Fusarium* mycotoxins in winter wheat grains

Mycotoxins	Frequency (%)	Mycotoxin concentration ($\mu\text{g kg}^{-1}$)	
		Average	Rang
DON	100	490	110–1200
ZON	10	70	60–80

Statistically significant positive correlations were established between the ZON concentration and the frequency of *F. graminearum*/*Fusarium* spp. ($r = 0.63^{**}$, $r = 0.58^{**}$, respectively). A negative insignificant correlation was determined between DON and the percentage of the present *Fusarium* species.

The obtained results of microbiological analyses of wheat grain samples are in accordance with previously published results (Bočarov-Stančić, 1996; Bočarov-Stančić, 1998; Bočarov-Stančić et al., 2000; Balaž et al., 2003; Bagi et al., 2005; Stanković et al., 2007; Krnjaja et al., 2008; Lević et al., 2008b, 2009) or results of similar studies carried out in Serbia.

According to results achieved by Bočarov-Stančić (1996, 1998) and Bočarov-Stančić et al. (2000) out of *Fusarium* species determined on observed wheat grain, the species *F. graminearum* had the highest frequency. In accordance with the two-year studies (2001–2002) on the mycopopulation of durum wheat seed samples, the presence of the species of the genus *Alternaria* ranged from 25.1% (2001) to 69.3% (2002), while species of the genus *Fusarium* were less present and their presence varied from 7.4% (2001) to 20.6% (2002) (Balaž et al., 2003). Bagi et al. (2005) performed a three-year study (2002–2004) on mycopopulations on the durum wheat seeds and also established more significant presence of species of the

genus *Alternaria* (12.3% in 2004 to 28.8% in 2003) in relation to species of the genus *Fusarium* whose presence varied from 7.4% (2002) to 10.8% (2003). According to *Stanković et al. (2007)* two-year results showed that the greatest number of wheat grain samples was infected with species of the genera *Fusarium* (81.8 and 65.0%), and *Alternaria* (36.3 and 17.5%) with the intensity ranging from 9.4 to 84.0% in 2005 and from 23.4 to 80.6% in 2006. Out of 13 identified species belonging to the genus *Fusarium*, *F. graminearum* had the highest frequency (35.2 and 12.5%) and the intensity up to 67.2%, and 21.9%, in 2005 and 2006, respectively, followed by *F. poae*, but only in 2005 (20.4%), and *F. proliferatum* in 2006 (19.7%) (*Stanković et al., 2007*). Agroecological conditions in Serbia appeared favourable for pathogenic and toxigenic *Fusarium* species of a wider scale, which in some years, as was the case in 2005, could cause the decrease in the grain yield by 38% and the increase in mycotoxin contamination of wheat grain by 50% (*Lević et al., 2008a*). Under such conditions, in the last decade, *F. graminearum* and *F. poae* (>16%), and *F. proliferatum*, *F. verticillioides* and *F. subglutinans* (6–15%) were species most often determined in wheat (*Lević et al., 2009*).

According to data of the Republic Hydrometeorological Service of Serbia, during May of 2009, when wheat was in a pheno-phase susceptible to fusarioses (especially in the first decade of May), mean daily temperature was, on the average, 19.6°C with small precipitation sums (35 mm on the average). Cooler days in the beginning and at the end of this month, as well as, a very small precipitation sum did not favoured the development of *Fusarium* species under field conditions.

Although DON is probably the most distributed mycotoxin in food and feed all over the world, data on the presence of this mycotoxin in wheat in our country are very scarce. According to data obtained by *Jurić et al. (2007)*, studies carried out on wheat samples in Vojvodina during 2004 and 2005 showed that 41.6% of samples were contaminated with DON, whose concentrations varied from 57 to 1840 µg kg⁻¹. Studies mycotoxin contamination in wheat grains in Serbia showed the presence of DON in 33.3–50.0% of the samples, and the concentration of mycotoxins on average ranged from 124 to 1235 µg kg⁻¹, depending on the conditions in the year of the study (*Jajić et al., 2008*). According to *Stanković et al. (2008)* Serbian isolates of *F. graminearum* have high potential for DON production (up to 45,260 µg kg⁻¹). In Serbia, ZON is one of the most important fusariotoxins that caused mycotoxicoses of domestic animals and is one of the most frequent contaminant of feed mixtures and their components (*Bočarov-Stančić et al., 1995; Lević et al., 2004*). *Bočarov-Stančić (1996)* recorded a low level of ZON in observed food samples made of wheat, while the level of ZON in feed samples made of wheat amounted to 800 µg kg⁻¹ (*Bočarov-Stančić et al., 2000*). *Stojanović et al. (2005a)* performed mycotoxological studies on four different wheat grain fractions (healthy, dark, slightly *Fusarium*-infected and considerable *Fusarium*-

infected) of three wheat varieties originating from two locations and established that the ZON concentration varied from 0 to 500 $\mu\text{g kg}^{-1}$. By studying grain samples of six wheat varieties (location 1) and five varieties (location 2), which were grouped into considerably to slightly *Fusarium*-infected grains, it was established that 77% of samples was contaminated with ZON in the concentrations of 150 to 1600 $\mu\text{g kg}^{-1}$ (Stojanović *et al.*, 2005b). According to Pancladi *et al.* (2004) in grain samples from three studied cultivars of durum wheat inoculated in the field with *F. graminearum* and *F. culmorum* and untreated by fungicides, the DON concentration ranged from 0.500 to 1.040 mg kg^{-1} .

Reported correlations between FHB severity, *Fusarium* frequency in grain and mycotoxin concentrations are variably. Cromey *et al.* (2002) established that the incidence of FHB, levels of *Fusarium* infection and mycotoxins were very closely related in samples from a particular crop, but there were differences between crops. The frequency of visibly infected grains, FHB, grains contaminated with *Fusarium*, and mycotoxin levels also differed markedly between cultivars in the 1999/2000 survey in New Zealand. In investigated samples per particular crop, levels of DON mycotoxin were higher compared to levels of fusariotoxin nivalenol (NIV), however this was not constant in samples of different crops (Cromey *et al.*, 2002). A high correlation was found between the percentage of *Fusarium* damaged kernels and amounts of DON in wheat grain samples in consecutive years 1986-1989 in Poland. Taking into consideration their frequency and concentration, the most important mycotoxin detected in field samples of wheat grains was DON, occurring in 60-65% of samples in 1998 and 89-95% of samples in 1999 (Tomczak *et al.*, 2002).

In our studies a negative correlation between DON mycotoxin and the incidence of *Fusarium* species was established, which is similar to results of De Nijs *et al.* (1996) who established that there was no relationship between the counts of total fungi and high levels of the mycotoxins DON and ZON.

Conclusion

In agroecological conditions of Serbia, toxigenic *Fusarium* species and their mycotoxins are special hazard in the food chain. Results presented in this research impose constant need for monitoring the incidence of *Fusarium* species in the field and also later during storage and processing of grain. Mycotoxicological analyses are also one of the basic methods in the assessment of grain quality, as well as, of the quality of grain products. A regular application of microbiological and mycotoxicological methods, as well as, their improvement and development, are prerequisites for defining adequate prevention measures, i.e. the improvement of quality of life, primarily of human and animal health.

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Prisustvo toksigenih *Fusarium* vrsta i fuzariotoksina deoksinivalenola i zearalenona u ozimoj pšenici

V. Krnjaja, S. Stanković, J. Lević

Rezime

Učestalost gljiva i koncentracija mikotoksina deoksinivalenola (DON) i zearalenona (ZON) je proučavana u zrnu ozime pšenice požnjevenom 2009. godine. Najčešće izolovane vrste gljiva pripadale su rodovima *Alternaria* (81,55%) i *Fusarium* (12%), a zatim su sledili *Rhizopus* spp. (3,75%), *Acremoniella* spp. (1,15%) i druge gljive (*Acremonium* spp., *Arthrinium* spp., *Aspergillus* spp., *Bipolaris* spp., *Chaetomium* spp., *Nigrospora* spp., *Penicillium* spp. i *Ramichloridium* spp.) izolovane u manje od 1%. Unutar roda *Fusarium* identifikovane su sledeće vrste: *F. graminearum* (82,50%), *F. sporotrichioides* (5,42%), *F. proliferatum* (4,17%), *F. subglutinans* (4,17%), *F. poae* (1,66%), *F. semitectum* (1,25%) i *F. verticillioides* (0,83%). U 100% uzoraka zrna pšenice DON je bio detektovan u koncentracijama od 110 do 1200 $\mu\text{g kg}^{-1}$, sa prosečnom koncentracijom od 490 $\mu\text{g kg}^{-1}$. ZON je bio detektovan u 10% uzoraka u koncentracijama od 60 do 80 $\mu\text{g kg}^{-1}$, sa prosečnom koncentracijom od 70 $\mu\text{g kg}^{-1}$. Statistički značajna pozitivna korelacija utvrđena je između koncentracije ZON i frekvencije *Fusarium* spp. ($r = 0.58^{**}$) i frekvencije *F. graminearum* ($r = 0.63^{**}$). Negativna korelacija, statistički nesignifikantna, utvrđena je između nivoa DON-a i procentualne zastupljenosti *Fusarium* vrsta.

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