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FREQUENCY OF TOXIGENIC *FUSARIUM* SPECIES AND FUSARIOTOXINS IN WHEAT GRAIN IN SERBIA

ABSTRACT: A total of 88 and 40 wheat samples collected immediately prior to harvest in 2005 and 2006, respectively, under different agroecological conditions, were studied in respect to the occurrence of *Fusarium* spp. and the production of fusariotoxins. The greatest number of 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%). The natural occurrence of mycotoxins in positive samples varied from 37 to 331 ppb for zearalenone and from 31 to 125 ppb for diacetoxyscirpenol (DAS) and T-2 toxin. The concentration of mycotoxins amounted, on average, to 133.4, 61.0 and 45.7 ppb for zearelenone, DAS and T-2 toxin, respectively.

KEY WORDS: diacetoxyscirpenol (DAS), *Fusarium* spp., T-2 toxin, wheat, zearalenone

INTRODUCTION

The *Fusarium* species predominantly found associated with Fusarium head blight (FHB) in wheat and other cereals all over Europe are *F. graminearum*, *F. avenaceum* and *F. culmorum*. Among the less frequently encountered species are several others which are less pathogenic or opportunistic, but also toxigenic. These include *F. cerealis* (Cooke) Sacc., *F. equiseti* (Corda) Sacc., *F. sporotrichioides* Sherb., *F. tricinctum* (Corda) Sacc., *F. acuminatum* Ell. & Ev., *F. subglutinans* (Wollenw. & Reinking) Nelson, *F. solani* (Mart.) Appel & Wollenw. and *F. verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* Scheldon) (Bottalico and Perrone, 2002). However, *F. poae* (Peck)

Wollenw. and *F. subglutinans* prevail in the Netherlands, Belgium, Switzerland, France and Austria, whereas *F. tricinctum, F. equiseti* and *F. acuminatum* are significantly less frequently encountered species (Mauler-Machnik and Suty, 1997; Muler and Reiman, 1997; Parry et al., 1995; Waalwijk, 2002). *F. avenaceum*, *F. poae*, *F. tricinctum* and *F. graminearum* are prevalent in Norway, Sweden and Finland (Kosiak et al., 2003; Langseth et al., 1997).

According to the global studies, approximately 25% of cereals are contaminated with mycotoxins, and this percentage is even higher if certain mycotoxins such as deoxynivalenol and fumonisin, are taken into consideration, or if unidentified mycotoxins, whose presence has been established by biological tests, are also included (Logrieco et al., 1992; Bottalico, 1998). The most frequently encountered *Fusarium* mycotoxins in FHB have proved to be deoxynivalenol (DON) and zearalenone (ZEA), produced by *F. graminearum* and *F. culmorum*. The increased production of the T-2 toxin and diacetoxyscirpenol (DAS) is attributed to sporadic epidemics of *F. sporotrichioides* and *F. poae* (Bottalico and Perrone, 2002).

The prevalence of the genus *Fusarium* has been determined on wheat grain in Serbia too, but the composition and the intensity of occurrence of certain species have been varying over years (Balaž et al., 2003; Dopuđa and Lević, 2004). *F. graminearum* or *F. culmorum* have been prevailing on wheat grain in various periods since the 1960s, although *F. graminearum* was encountered each year in higher or lower intensity, which was not the case with *F. culmorum* (Lević et al., 2004).

Although species of the genus *Fusarium* have been mainly isolated from maize and wheat grain in Serbia, studies on the natural occurrence of fusariotoxins in feed have been much more numerous (Lević et al., 2004). According to these authors the data related to the natural occurrence of zearalenone (ZEA) and T-2 toxins are the most numerous, while there is a smaller number of data related to diacetoxyscirpenol (DAS). Little work has been done in studying the natural presence of deoxynivalenol (DON); there have not been any studies on its derivates and just a few on fumosions, which is not in accordance with the widespread distribution of producers and favourable conditions for biosynthesis of these mycotoxins during certain years.

With the aim to determine the intensity of the occurrence of species of the genus *Fusarium*, and the natural appearance of fusariotoxins in wheat grain, studies were carried out on the samples collected under different agroecological conditions in Serbia in 2005, the year that favoured FHB, and in 2006, the year that did not favour FHB.

MATERIAL AND METHODS

Fungal isolation and determination

Samples of wheat spikes of 12 varieties were collected immediately prior to harvest at 22 and 10 locations in 2005 and 2006, respectively. The samples

were drawn diagonally from each plot, each sample from an area of 50 x 50 cm (0.25 m^2). Thirty two kernels from each sample were analysed in four replications. Eight kernels, each surface sterilised with 1% of sodium hypochlorite and rinsed with distilled water were placed on the water agar (WA) in 10-cm Petri dishes, and incubated under indoor conditions for seven days. Resulting colonies were purified by the procedure of obtaining single-spored cultures that were then used for the identification of *Fusarium* spp. Single-spored cultures were subcultured on the potato dextrose agar (PDA), carnation sterilised leaf-fragment agar (CLA) and synthetic nutrient agar (SNA). Cultures on the PDA were incubated in the dark at 25 ± 1°C, while cultures on the CLA and SNA were incubated under fluorescent and near ultraviolet light for 12 hours at 25 ± 1°C, and in the dark for 12 hours at 20 ± 1°C. The identification of the obtained species was done according to Nelson et al. (1983) and Burgess et al. (1994). Fungal cultures not belonging to species of the genus *Fusarium* were also grown on the three stated media and were identified according to Ellis (1971) and Watanabe (1994).

Identification of fusariotoxins

Mycotoxicological analyses were performed on the wheat samples that had been ground to powder granulation. Zearalenone (ZEA) was determined by the multitoxin method developed by Balzer et al. (1978). The type-A trichothecene (T-2 toxin and diacetoxyscirpenol-DAS) were isolated and purified by the method of Romer et al. (1978), whereas thin layer chromatography of T-2 toxin and DAS was done according to Pepeljnjak and Babić (1991).

RESULTS AND DISCUSSION

Mycopopulations on wheat grain

The mycopopulation on wheat grain differed over years in both the composition and the frequency of certain species, as it was expected due to weather conditions in these two years. Temperatures and precipitation in the wheat flowering period in 2005 favoured the FHB development, while the same period in 2006 was characterized with a much lower amount of precipitation than the long-term mean.

Out of 12 identified fungal species, species belonging to the genus *Fusarium* were isolated from the greatest number of samples in both years, 2005 and 2006 (81% and 65%, respectively) and with a high frequency (67.2% and 21.9%, respectively) (Table 1).

Tab. 1 — Frequency (%) of fungal species originating in wheat grain collected at different locations in Serbia in 2005 and 2006

No.	Species	2005			2006		
		PS[a] (%)	Frequency (%)		PS[a] (%)	Frequency (%)	
			Min	Max		Min	Max
1.	*Acremonium* spp.	7.9	0.78	2.30	10.0	1.6	14.1
2.	*Alternaria* spp.	36.3	9.4	84.0	17.5	23.4	80.6
3.	*Aspergillus* spp.	nd	nd	nd	5.0	1.6	7.8
4.	*Bipolaris* spp.	10.2	0.78	1.56	2.5	0.8	0.8
5.	*Cladosporium* spp.	6.8	0.78	3.90	2.5	0.8	0.8
6.	*Chaetomium* spp.	12,5	1.5	20.30	5.0	0.8	20.3
7.	*Epicoccum* spp.	4.5	0.78	1.56	10.0	1.6	7.8
8.	*Fusarium* spp.	81.8	0.78	67.20	65.0	0.8	21.9
9.	*Mucor* spp.	nd	nd	nd	7.5	1.6	7.8
10.	*Penicillium* spp.	nd	nd	nd	12.5	2.5	3.1
11.	*Phoma* spp.	2.2	0.78	1.56	nd	nd	nd
12.	*Trichoderma* spp.	nd	nd	nd	2.5	3.9	3.9

[a] Positive sample (PS) — percentage was estimated on the basis of samples in which the fungus had been identified
nd — no data

Although percentage of species of the genus *Alternaria* was not high (36% and 17.5%), the frequency (84% and 80.65%) was. Other pathogenic and toxigenic fungal species, such as *Penicillium* spp. and *Aspergillus* spp., were isolated in 2006 in the amount of 12.5%, i.e. 5% of samples with the intensity of 3.1%, i.e. 7.8%. Besides the stated species, *Chaetomium* spp. and *Acremonium* spp. (up to 20.3% and 14.1%, respectively) prevailed in certain samples.

Previous studies (Milošević et al.; 1995; Dopuđa and Lević, 2004) show a similar frequency of all three fungal genera on wheat grain in Serbia. Balaž et al. (2003) state a significantly lower frequency of *Fusarium* spp. on wheat grain (11.3—20.6%), as well as of *Asspergillus* spp. (0—1.2%) and *Penicillium* spp. (0—8.9%). According to these authors species of the genus *Alternaria* spp. (up to 86.8%) prevail.

The occurrence of species of the genus Fusarium on wheat grain

F. graminearum was a prevalent species of the genus *Fusarium*, and was isolated from the greatest number of samples in 2005 and 2006 (35.2% and 12.5%, respectively) with a very high frequency (up to 67.2%) (Table 2). Bočarov-Stančić (1996) also states that this species was prevalent on wheat grain harvested in semi-humid year of 1991. In contrast to these results, Stojanović et al. (2002) stated that *F. oxysporum* (19.44—25%) was a prevalent species, although they determined the presence of *F. graminearum* in wheat at all observed locations with the frequency of 2.78—15.38%.

Tab. 2 — Frequency of fungal species originating in wheat grain collected at different locations in Serbia in 2005 and 2006

No.	Species	2005			2006		
		PS[a] (%)	Frequency (%)		PS[a] (%)	Frequency (%)	
			Min	Max		Min	Max
1.	*F. arthrosporioides*	1.1	0.8	0.8	7.5	0.8	5.5
2.	*F. avenaceum*	2.2	0.8	3.3	5.0	0.8	3.1
3.	*F. equiseti*	2.2	0.8	1.6	5.0	0.8	0.8
4.	*F. culmorum*	0.0	0.0	0.0	2.5	0.8	0.8
5.	*F. graminearum*	35.2	7.1	67.2	12.5	3.9	21.9
6.	*F. poae*	20.4	0.8	6.3	7.5	0.8	2.4
7.	*F. proliferatum*	3.4	0.8	7.8	20.0	0.3	19.7
8.	*F. semitectum*	2.2	0.8	0.8	nd	nd	nd
9.	*F. sporotrichioides*	2.2	0.8	0.8	5.0	0.8	0.8
10.	*F. subglutinans*	4.4	0.8	3.2	2.5	0.8	0.8
11.	*F. tricinctum*	1.1	2.3	2.3	2.5	2.3	2.3
12.	*F. verticillioides*	7.0	0.8	3.2	7.5	0.8	2.4
13.	*Fusarium* spp.	33.0	0.1	0.2	7.5	2.4	3.9

[a] Positive samples (PS) — percentage was estimated on the basis of samples in which the fungus had been identified
nd — no data

The species *F. poae* was isolated in a higher percentage of samples (20.4%) and with a higher frequency (up to 6.3%) in the first, than in the second year of the investigation.

If the occurrence of the remaining *Fusarium* spp. is observed, the difference over years is perceived (Table 2). The species *F. proliferatum* was isolated in the range of 0.8%—7.8%, i.e. 0.3—19.7% in 2005, i.e. 2006, respectively. The number of samples infected with *F. verticillioides* was approximately equal (7.0% and 7.5%) in both years, but the intensity was higher in 2005 than in 2006 (3.2% vs. 2.4%). The occurrence of *F. arthrosporioides, F. avenaceum, F. culmorum*, *F. equiseti, F. sporotrichioides, F. subglutinans* and *F. tricinctum* was determined in the range from 0.8% to 3.2%.

Our results are in accordance with the results obtained by D o p u đ a and L e v i ć (2004) who studied the mycobiot of wheat grain during 2002 and 2003 especially in regard to varying of certain species during the years of the investigation. These authors determined that species of the genus *Fusarium* prevalent in 2002 were *F. graminearum* (up to 61%) and *F. verticillioides* (up to 10%), and in 2003 were *F. poae* (up to 12%) and *F. prolifearatum* (5%), whereas the prevalence of remaining *Fusarium* spp. ranged from 4—5% (*F. sporotrichioides*) to 1—3% (*F. acuminatum, F. avenaceum, F. culmorum*, *F. equiseti, F. oxysporum*, *F. tricinctum*).

Natural occurrence of fusariotoxins in wheat grain

The natural occurrence of zearalenone and trichothecene (DAS, T-2 toxin) in wheat grain varied in dependence on a variety and agroecological conditions of locations at which wheat was grown in 2005 (Table 3).

Tab. 3 — Natural occurrence of fusariotoxins in grain of different wheat varieties grown under various agroecological conditions in Serbia in 2005

Sample code	Variety	Location	Mycotoxin contents (ppb)		
			ZEA	Trichothecenes	
				DAS	T-2
234	Dragana	Pirot	74	31	31
164	Evropa	Sombor	0	75	50
167	Evropa	Gredetin	0	0	0
216	Evropa	Krsmanovci	0	37	0
208	Evropa-90	Bač	147	0	0
172	Evropa-90	Kikinda	331	0	0
162	Evropa-90	Sombor	37	0	0
169	Evropa-90	Lipnički Šor	0	62	31
212	Evropa-90	Veliki Radinci	0	0	62
222	Kg-20	Zobnatica	0	0	0
207	Kg-20	Zemun	184	0	0
210	Mina	Bački Petrovac	258	0	0
151	Pahulja	Zemun Polje	74	0	0
219	Pesma	Maglić	0	0	0
223	Pesma	Zobnatica	37	0	0
170	Pobeda	Runjani	147	0	0
150	Pobeda	Zemun Polje	0	31	31
175	Pobeda	Kovin	184	0	0
213	Pobeda	Nova Pazova	0	0	31
214	Pobeda	Stari Banovci	74	0	0
215	Pobeda	Sremska Mitrovica	37	0	0
155	Renesansa	Zemun Polje	0	125	50
163	Renesansa	Sombor	147	0	0
174	Renesansa	Kikinda	138	0	25
209	Renesansa	Rimski Šančevi	110	0	31
211	Renesansa	Veliki Radinci	147	62	0
217	Renesansa	Zobnatica	37	0	0
220	Renesansa	Despotovac	110	0	31
165	Simonida	Loznica	0	125	125
153	Takovčanka	Zemun Polje	138	1	50
152	Žitka	Zemun Polje	257	0	0

Zearalenone was determined in 20 samples in the range from 37 to 331 ppb or in 64.52% of the samples with the average for positive samples of 133.4 ppb. The presence of zearalenone was determined in three most cultivated varieties in Serbia (Evropa-90, Pobeda and Renesansa), in the majority of studied locations with the average for positive samples of 171.67, 110.50 and 114.83 ppb. Contamination in a high number of wheat grain samples (78%) with ZEA (160—500 ppb) was also recorded by Stojanović et al. (2002),

who analysed the three varieties (Evropa 90, Kg56S and Nora) in two trial spots. Mesterhazy (1997) states that a plant genotype is of a great importance in the accumulation of toxins and that generally less total toxins, are produced in more resistant cultivars.

The presence of zearalenone is not surprising, as *Fusarium* spp. (Tabela 2) were isolated from the analysed samples. According to Marasas et al. (1984) these species can biosynthesise the same fusariotoxin under certain ecological conditions. This statement is also confirmed by our previous *in vitro* studies on the ZEA production in the *F. oxysporum* isolates from which grain contaminated with the same mycotoxin in the amount of 1540 ppb, Bočarov-Stančić et al. (2003).

Out of 31 wheat grain samples, DAS was determined in nine samples in the range from 31 to 125 ppb, or in 29.03% of the samples with the average of 61.0 ppb for positive samples (Table 3). The T-2 toxin was determined in the same range as DAS in 12 samples, but its presence was greater (38.72%) and while the average for positive samples was smaller (45.7 ppb). DAS and T-2 toxin were simultaneously identified in six samples (19.35%), and individually in two, i.e. six samples, respectively.

The presence of these trichothecenes is not surprising considering that the species *F. avenaceum, F. equiseti, F. culmorum, F. sporotrichioides* and *F. tricinctum* (Table 2) isolated from wheat, harvested in 2005 and 2006, can biosynthesise type-A trichothecenes under laboratory conditions, as it was concluded in our previous study Bocarov-Stančić et al. (1986).

Gained data on the concentrations of zearalenone and T-2 toxin in wheat grain in 2005 were lower than those found in the literature. Stojanović (1999) determined the natural occurrence of zerelenone in 87.5% of wheat grain samples and with the concentration up to 500 ppb, while Bočarov-Stančić et al. (1998) detected T-2 toxins in 33 of the samples with the concentration in the range from 500 to 750 ppb.

CONCLUSION

The two-year studies (2005—2006) on the mycobiot of wheat grain show that *Fusarium* spp. are widespread and prevalent fungi in Serbia (81.8—65.0%), but that species of the genus *Alternaria* (84.0—80.6%) are more frequent.

F. graminearum is predominant *Fusarium* species that prevailed in wheat grain in 2005 and 2006, not only by its distribution (35.2% and 12.5%, respectively), but also by its intensity (67.2% and 21.9%, respectively). *F. poae* was more frequent in 2005 (20.4%) than in 2006 (7.5%), but the intensity did not exceed 6.3%. The species *F. proliferatum* was isolated in the range from 0.8% to 7.8%, i.e. from 0.3 to 19.7%, in 2005, and 2006, respectively. *F. verticillioides* was equally distributed during the both years (7.0% and 7.5%), but the intensity was somewhat higher in 2005 than in 2006 (3.2% vs. 2.4%). The distribution and intensity of the occurrence of remaining 10 identified species of the genus *Fusarium* varied during the years of investigation.

The analysis of 31 wheat samples, collected in 2005, shows that the natural occurrence of zearalenone, DAS and T-2 toxin was determined in 20 (64.52%), 9 (29.03%) and 12 samples (38.72%), respectively. The greatest range and concentration (37—331 ppb) of determined mycotoxins were detected in zearalenone (133.4 ppb on average). This is in accordance with the distribution and the intensity of the occurrence of *F. graminearum*, which is one of the most important producers of this mycotoxin. Although 11 species of the genus *Fusarium*, synthetising the type-A trichothecenes (DAS and T-2 toxin), were identified in this study (in 2005), the natural occurrence of these mycotoxins varied from 31 to 125 ppb. These results point out that the species of the genus *Fusarium* originating in wheat from Serbia have a low potential for synthesis of these fusariotoxins.

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УЧЕСТАЛОСТ ТОКСИГЕНИХ ВРСТА *FUSARIUM* И ФУЗАРИОТОКСИНА У ЗРНУ ПШЕНИЦЕ У СРБИЈИ

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Резиме

Осамдесет осам и 40 узорака пшенице, прикупљених непосредно пред жетву у 2005. и 2006. години у различитим агроеколошким условима у Србији, проучавани су ради праћења појаве *Fusarium* spp. и стварања фузариотоксина. Највећи број узорака био је заражен врстама рода *Fusarium* (81,8 и 65,0%), а затим са *Alternaria* spp. (36,3 и 17,5%) са интензитетом 9,4—84,0% у 2005. и 23,4—80,6% у 2006. години. Од укупно 13 идентификованих врста из рода *Fusarium* најучесталија је била *F. graminearum* (35,2 и 12,5%) са интензитетом до 67,2% у 2005. и до 21,9% у 2006. години, а затим *F. poae*, али само у 2005. години (20,4%), и *F. proliferatum* у 2006. години (19.7%). Природна појава микотоксина у позитивним узорцима је варирала од 37 до 331 ppb за зеараленон и од 31 до 125 ppb за диацетоксисцирпенол (ДАС) и Т-2 токсин. У просеку, концентрација микотоксина је била 133,4 ppb за зеарелонон, 61,0 ppb за ДАС и 45,7 ppb за Т-2 токсин.