Pestic. Phytomed. (Belgrade), 36(1), 2021, 1–13
DOI: https://doi.org/10.2298/PIF2101001O

UDC 632.4:632.122:633.11+633.15+633.16(497.11)
Original scientific paper

Aggressiveness and trichothecene production of *Fusarium graminearum* isolates from cereals in Serbia

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Received: 8 February 2021
Accepted: 8 March 2021

SUMMARY

The aim of this study was to assess variations in aggressiveness and trichothecene production of *F. graminearum* isolates originating from maize, wheat and barley in Serbia. Analyzing *F. graminearum* isolates (98) obtained from various agroecological conditions of Serbia over the period from 1993 to 2010, using the HPLC method, the following two chemotypes were observed: 3-acetyl-deoxinivalenol (3ADON) and 15-acetyl-deoxinivalenol (15ADON). A great diversity in the production of deoxinivalenol (DON) derivatives was observed. A majority of *F. graminearum* isolates, regardless of their origin (maize, wheat or barley) belonged to the 15ADON chemotype. The 3ADON chemotype was also detected, but in a significantly smaller number (13/98) samples, compared to the 15ADON chemotype (85/98). None of the tested isolates belonged to the NIV chemotype. The examined isolates showed different pathogenicity on barley leaf, wheat class and maize ears. The average pathogenicity of the tested isolates was the highest on barley leaf. It was observed that isolates originating from wheat had the highest average daily increase in mycelium growth rate (27.37 mm). Statistical analysis of the obtained results for mycotoxins synthesis showed that there was a highly significant statistical correlation between the production potentials of total DON, 3ADON and 15ADON in *F. graminearum* isolates belonging to various chemotypes. However, there was no statistically significant correlation between the aggressiveness of isolates and the production of total DON in isolates belonging to 3ADON and 15ADON chemotypes.

Keywords: cereals, *Fusarium graminearum*, mycotoxins, chemotypes, trichotecene, pathogenicity

INTRODUCTION

Small grains and maize predominate in total crop production in Serbia regarding both per unit area and total yield. However, cereal production is endangered by a large number of pathogens, particularly the species *Fusarium graminearum*. It is considered to be one of the most destructive and widespread pathogens of cereals and industrial crops world-wide. The importance of this species is that it does not only reduce yield, but grain quality also, due to its ability to synthesise mycotoxins in infected plants, and it has an adverse effect on human

and animal health. Damage that it causes is further increased by its ability to synthesise probably more than 17 mycotoxins, of which trichothecenes, such as deoxynivalenol (DON), are the most widespread and significant (Logrieco et al., 2002; Moretti et al., 2014).

F. graminearum is a species within the Fg complex (*Fusarium graminearum* Species Complex-FGSC), named *Fusarium graminearum sensu lato,* and it contains at least 15 different phylogenetic species (O'Donnell et al., 2000, 2004, 2008; Starkey et al., 2007; Sarver et al., 2011). Species of this complex synthesise various metabolites, including deoxynivalenol (DON) and its derivatives (3-acetyl deoxynivalenol [3ADON] and 15-acetyl deoxynivalenol [15ADON]) and nivalenol (NIV) (O'Donnell et al., 2000). Based on the mycotoxicological profile of synthesised trichothecenes, *F. graminearum* isolates can be grouped into one of three chemotypes (3ADON, 15ADON and NIV) (O'Donnell et al., 2004). As belonging to a particular trichothecene chemotype plays a significant role in the aetiology of cereal diseases, knowledge about the toxicological profile of *F. graminearum* is essential for agriculture, as well as the food industry of any country. Previous studies have shown that different mycotoxins had different toxicological properties: the toxicity of NIV to humans and animals is almost 10-fold higher than that of DON (Lee et al., 2015). Moreover, the phenotypic analyses of *F. Asiaticum,* which produces 3ADON, showed major advantages over *F. asiaticum* which produces NIV with regard to pathogenicity, growth rate, fecundity, conidial length, trichothecene accumulation and resistance to benzimidazole (Ward et al., 2008; Zhang et al., 2012). Variations in mycotoxin synthesis and affiliation to a certain chemotype (3ADON, 15ADON or NIV) may have important implications for toxicity (Luongo et al., 2010). Therefore, chemotype identification is an important information for establishing a risk assessment strategy in order to protect human and animal health.

Biogeographical studies of trichothecene chemotypes within the Fg complex have been carried out in many countries worldwide over the past two decades. According to literature data, all three chemotypes are widespread in Asia. It has been determined that *F. graminearum* isolates in wheat and barley predominantly produced 15ADON, in contrast to *F. asiaticum* isolates, which mainly produced 3ADON or NIV. Previous studies have shown that isolates of the 15ADON chemotype within the FGSC are dominant in the United States (Abramson et al., 2001; Miller et al., 1991), while the 3ADON chemotype has been observed in a much smaller percentage. However, an increase in the presence of 3ADON chemotype has been observed in the United States (Abramson et al., 2001; Gale et al., 2007) and Canada (Ward et al., 2008; Guo et al., 2008). *F. graminearum* is a cosmopolitan (O'Donnell et al., 2000; Backhouse, 2014) and a dominant member of the FGSC in Europe (O'Donnell et al., 2004; Toth et al., 2005; Yli-Mattila et al., 2009). In fact, *F. graminearum* was the only member of the FGSC in Europe, with the exception of a small number of isolates of *F. boothii* and *F. vorosii* in Hungary (Toth et al., 2005; Starkey et al., 2007), and *F. cortaderiae* in France and Italy (Boutigny et al., 2014; Somma et al., 2014). Data on the distribution of chemotypes in Europe show that the 15ADON chemotype is dominant in a majority of countries, in contrast to 3ADON and NIV, which have been detected at significantly lower frequency. In Europe, 15ADON has been observed in southern and central Europe (Toth et al., 2005; Boutigny et al., 2014; Jennings et al., 2004), while 3ADON is dominant in northwestern parts of Europe (Yli-Mattila et al., 2009; Fredlund et al., 2013; Nielsen et al., 2012). In Serbia, there is a lack of data on the distribution and aggressiveness, as well as the synthesis of 3ADON, 15ADON and NIV chemotypes by *F. graminearum*. The results of preliminary studies performed by Obradović et al. (2017) indicated that the 15ADON chemotype was dominant in Serbia.

Data found in literature so far indicate that isolates belonging to the 3ADON chemotype cause a more intense fusariosis and produce higher amounts of DON than isolates belonging to the 15ADON chemotype (Puri & Zhong, 2010). According to studies performed in Norway, 3ADON and 15ADON isolates have shown significant differences in the average growth rate of mycelia under *in vitro* conditions. A difference was also found regarding the aggressiveness of these two populations in wheat (Aamot et al., 2015). Studies conducted in Canada and China have shown that there were differences in phenotypic traits between 3ADON and 15ADON populations, which may explain the rapid change and spread of populations with the 3ADON chemotype. In Canada, Ward et al. (2008) noticed that 3ADON isolates produce more colonies and longer conidia, and have higher rate of mycelial growth, as well as greater synthesis of DON in comparison to the 15ADON population. In China, it has been observed that isolates of *F. asiaticum* belonging to the 3ADON chemotype rapidly replaced populations with the NIV chemotype because they were more aggressive in wheat and were more toxigenic (Zhang et al., 2010, 2012).

3ADON isolates are more aggressive than 15ADON populations regarding the rate of mycelial growth and DON synthesis *in vitro* (Ward et al., 2008). However, little data are available on the aggressiveness and potential of DON synthesis in 3ADON and 15ADON isolates of *F. graminearum*. The aim of this study was to assess variations in the aggressiveness and trichothecene production of *F. graminearum* isolates from maize, wheat and barley in Serbia.

MATERIALS AND METHODS

Isolate collection

This study encompasses 98 *F. graminearum* isolates from the collection of the Maize Research Institute at Zemun Polje, Belgrade, Serbia, which had been collected from grains with FHB symptoms in various locations in Serbia over the period from 1993 to 2010. The isolates were obtained from maize, wheat and barley grain (Table 1).

Table 1. List of tested *Fusarium graminearum* isolates

Isolates from wheat	Year	Chemotype	Isolates from maize	Year	Chemotype	Isolates from barley	Year	Chemotype
203	2003	15ADON	257	2004	15ADON	654	2005	15ADON
618	2005	15ADON	581	2005	3ADON	770/2	2005	15ADON
670	2005	15ADON	656	2005	3ADON	798	2005	15ADON
677	2005	15ADON	699	2005	15ADON	805	2005	15ADON
681	2005	15ADON	762	2005	15ADON	891/2	2006	15ADON
687/2	2005	15ADON	880	2006	15ADON	1217/2	2006	15ADON
744	2005	15ADON	914	1996	3ADON	1493	2007	15ADON
749	2005	15ADON	943/2	2006	3ADON	1517	2007	15ADON
763	2005	15ADON	971	2006	15ADON	1526	2007	15ADON
764	2005	15ADON	1010	2006	3ADON	1528	2007	3ADON
766	2005	15ADON	1030	1999	15ADON	1534	2007	15ADON
767	2005	15ADON	1133	2006	15ADON	1772	2008	15ADON
779/2	2005	15ADON	1165	2006	3ADON	1800	2008	15ADON
789	2005	15ADON	1195	2006	3ADON	1801	2008	15ADON
795	2005	15ADON	1199	2006	15ADON	1812	2008	15ADON
800	2005	3ADON	1211	2006	15ADON	1839	2009	15ADON
825	2005	15ADON	1249	2006	3ADON	2045	2009	15ADON
831	2005	15ADON	1255	2006	15ADON	2078	2009	15ADON
836	2005	15ADON	1268	2006	15ADON	2254	2009	3ADON
864	2005	15ADON	1282/2	2006	15ADON	2630	2010	15ADON
866	2005	15ADON	1368	2007	3ADON	2672	2010	15ADON
870	2006	15ADON	1408	2007	15ADON	2627	2010	15ADON
892	2006	15ADON	1419	2007	15ADON			
1012	2006	15ADON	1482/2	2007	15ADON			
1337	2006	15ADON	1495	2007	15ADON			
1343	2006	15ADON	1554/2	2007	15ADON			
1348	2006	15ADON	1649	2007	15ADON			
1351/2	2007	15ADON	1673	2008	15ADON			
1370	2007	3ADON	1696	2008	15ADON			
1443	2007	15ADON	1751	1994	15ADON			
1485	2007	15ADON	2533	2010	15ADON			
1486/2	2007	15ADON	2624	2010	15ADON			
1490/2	2007	15ADON	2811	1993	15ADON			
1746	2010	15ADON	2812	1998	15ADON			
2621	2010	15ADON	2813	1999	15ADON			
2625	2010	15ADON	2815	1999	15ADON			
2635	2010	15ADON						
2818	2002	15ADON						
2820	1997	15ADON						
2823	2002	15ADON						

In vitro fungal growth

Mycelial growth rate was evaluated by measuring the diameter of colonies after their subculturing on PDA at 25°C. The isolates were subculutred in 9 cm Petri dishes using 5 mm plugs of actively growing mycelium. Colony diameter was measured on the second and fourth day after subculturing. Within each of three replicate tests, the growth rate of each fungal isolate was estimated as a mean radial mycelial growth rate per day within the time period between two measurements of growth.

Aggressiveness evaluation

Variations in pathogenic properties of the 98 *F. graminearum* isolates examined (Table 1) were studied by applying artificial inoculation of plants in three pathogenicity tests: pathogenicity test on barley leaves, pathogenicity test on maize ears and pathogenicity test on wheat spikes.

Pathogenicity test on barley leaves. In order to test the pathogenicity of isolates, a method described by Imathiu et al. (2009) was used. According to this method, barley leaves were inoculated with spore suspensions of *F. graminearum* isolates. Eight leaves were placed in each Petri dish (Ø 150 mm) with filter paper soaked in distilled water. The central part of each leaf was inoculated with 5 µl of conidial suspension of the fungus *F. graminearum*. The inoculum was prepared from 14-days old isolates of cultures grown on synthetic nutrition agar (SNA) (Burgess et al., 1994) under a combination of fluorescent and ultraviolet light at 20°C. The concentration was adjusted using a hemocytometer to approximately $1x10^5$ conidia/ml. Leaves inoculated with sterile distilled water were used as a negative control. Incubation of inoculated leaves was performed at the temperature of 25°C, and the length of spots was measured after five days.

Pathogenicity test on maize ears. The pathogenicity test of *F. graminearum* isolates on maize was conducted under field conditions by artificial inoculation according to the method described by Reid et al. (1996). Artificial inoculation was completed three days after silking of plants by injecting 2 ml of conidial suspension with concentration of approximately $1x10^5$ conidia/ml into the maize silk channel. The inoculum was prepared in the same way as for the pathogenicity test performed on barley leaves. The same procedure was applied to control plants, and sterile water was used instead of the inoculum. The intensity of fusariosis on maize ears was evaluated on a 1-7 scale at the harvest maturity stage (Reid et al., 1996).

Pathogenicity test on wheat spikes. In order to test the pathogenicity of *F. graminearum* isolates in wheat under field conditions, artificial inoculation of wheat spikes was done according to the method presented by Mesterházy et al. (1999). Artificial inoculation of spikes was performed at the wheat flowering stage with a spore suspension of approximately $1x10^5$ conidia/ml. Thirty plants per isolate were inoculated in three replications. The amount of inoculum used per replicate was 30 ml, while control plants were treated with the same amount of distilled water. After inoculation, spikes were isolated with water-moistened PVC bags, which were removed 48 h later. The degree of infection was evaluated on the 1-7 scale two weeks after inoculation, based on the presences of fusariosis symptoms on spikes (Blandino et al., 2012).

Mycotoxin analyses

Samples for the trichothecene analysis were prepared on sterile maize kernels according to a method described by Logrieco et al. (1995). A total of 50 g of maize kernels (45% moisture) were poured into Erlenmeyer flasks, which were then inoculated with three sections of fungal colony (0.5x0.5 cm) developed on PDA. Following inoculation, Erlenmeyer flasks were sealed and covered with aluminium foil and then stored at 25°C. After incubation, inoculated maize kernels were transferred to aluminium dishes and dried at the temperature of 50°C, and then they were ground using the analytical mill (A11 IKA, Germany).

HPLC method (High Performance Liquid Chromatography)

This method was used to determine the affiliation of trichothecenes to a certain chemotype, as well as to qualitatively and quantitatively determine mycotoxin concentrations. To a 5 g sample, 25 ml of a mixture of acetonitrile and water (84:16, v/v) was added, and then the mixture was homogenised in a blender for 60 seconds. After filtration through Whatman filter paper no. 41, the filtrate was divided into two segments. The first segment of the filtrate was passed through MycoSep 113 Trich (Romer Labs, USA) and the second one through MycoSep 230 Niv (Romer Labs, USA). Once passed through the appropriate MycoSep column, extracts were filtered (17 mm, PTFE membrane 0.45 µm) and injected by the autosampler (WPS-300SL) into the Dionex Ultimate 3000 liquid chromatographic system with the DAD-3000 detector (Thermo Scientific, Germany). The chromatographic separation of 3ADON, 15ADON and NIV was performed on the analytical column Acclaim Polar Advantage II, C18 (150 × 4.6 mm, 3 µm) at 25°C.

A mixture of water and acetonitrile (90:10; v/v) at a linear flow rate of 1 ml/min for 15 minutes was used as a mobile phase for the separation of 3ADON and 15ADON. Chromatograms were generated at 221 nm. On the other hand, a mixture of water, acetonitrile and methanol (90:5:5; v/v) was used at a linear flow rate of 0.8 ml/min for 15 minutes as a mobile phase for NIV separation. Chromatograms were generated at 218 nm.

Data analysis

The Pearson correlation method was used to determine interdependence between the production potential of total DON of each chemotype and aggressiveness on maize, wheat and barley.

RESULTS

Growth rate

The average daily growth of *F. graminearum* colonies did not differ significantly among the tested isolates in relation to their origin. The lowest daily mycelial growth was observed in isolates obtained from barley. Variations in daily growth of mycelia were most pronounced in the isolates obtained from maize, with a range of variation from 13.16 mm to 32.00 mm. Daily mycelial growth of the isolates obtained from barley ranged from 14.83 mm to 30.16 mm. The lowest variation in daily mycelial growth was observed in isolates derived from wheat and it ranged from 19.5 mm to 30.83 mm (Tables 2-4).

Table 2. Production of deoxynivalenol (DON), 3-acetyl-deoxinivalenol (3ADON) and 15-acetyl-deoxinivalenol (15ADON) (μg/g), aggressiveness and mycelial growth rate of *Fusarium graminearum* obtained from maize

Isolate	Chemotype	DON	15ADON	3ADON	Aggress. maize	Aggress. wheat	Aggress. barley	Mycelial growth (mm)
581	3ADON	64.97	20.92	41.11	2.06	3.18	3.44	26.83
656	3ADON	29.68	10.78	14.78	2.67	2.92	19.19	30.83
914	3ADON	35.68	12.37	14.57	3.10	2.62	21.75	27.66
943/2	3ADON	3.18	0.63	1.03	3.38	3.68	15.75	30.33
1010	3ADON	6.38	0.71	3.01	2.07	3.33	4.94	26.66
1165	3ADON	16.18	1.77	10.65	2.67	2.72	17.94	24.33
1195	3ADON	97.41	24.72	59.60	2.88	2.87	21.56	27.66
1249	3ADON	159.25	72.47	72.85	2.58	2.67	12.94	31.33
1368	3ADON	10.55	ND	3.42	2.16	2.05	7.56	27.16
Average		47.03	18.05	24.56	2.62	2.89	13.89	28.08
257	15ADON	31.88	18.57	5.85	3.03	2.96	22.25	29.33
699	15ADON	54.75	41.32	6.68	4.47	4.21	29.30	29.66
762	15ADON	27.32	14.66	4.39	3.00	3.42	16.00	29.33
880	15ADON	64.96	36.73	22.64	4.95	5.02	31.44	28.00
971	15ADON	27.49	22.66	1.73	4.82	5.07	33.88	25.50
1030	15ADON	24.63	18.67	1.58	3.15	2.89	25.13	29.00
1133	15ADON	154.97	125.97	6.33	3.45	3.66	11.56	26.00
1199	15ADON	94.67	57.59	25.51	1.92	2.18	4.88	26.50
1211	15ADON	56.97	46.71	1.46	2.96	3.68	15.81	27.50
1255	15ADON	43.19	30.57	5.69	2.97	2.00	25.38	30.50
1268	15ADON	107.31	54.67	42.20	4.57	4.85	27.25	31.00
1282/2	15ADON	42.18	25.12	5.18	2.31	2.67	12.06	22.00
1408	15ADON	39.63	26.62	5.32	1.75	1.86	2.06	20.16
1419	15ADON	40.38	20.79	13.97	2.96	3.41	16.44	15.50
1482/2	15ADON	42.67	32.14	2.52	4.58	2.39	33.31	24.66
1495	15ADON	17.69	12.12	1.67	2.42	3.93	23.75	25.66
1554/2	15ADON	94.66	65.02	21.04	2.36	2.57	17.75	23.00
1649	15ADON	19.95	13.41	2.21	5.11	4.67	29.56	28.50
1673	15ADON	14.57	9.75	ND	3.47	5.42	14.50	26.50
1696	15ADON	31.62	25.36	0.19	2.34	2.82	5.88	27.16
1751	15ADON	22.23	15.36	1.96	4.43	3.83	26.13	27.50
2533	15ADON	19.22	11.40	0.59	2.26	4.68	12.44	27.00
2624	15ADON	78.47	55.61	20.10	2.42	2.95	24.31	29.50
2811	15ADON	135.67	114.68	7.74	2.92	2.12	13.19	27.33
2812	15ADON	8.97	5.47	0.89	2.95	3.50	21.00	30.16
2813	15ADON	45.58	33.58	6.40	2.90	2.26	21.63	13.16
2815	15ADON	100.97	93.07	0.82	2.57	2.20	4.63	32.00
Average		53.43	38.06	8.26	3.22	3.38	19.31	26.37
Total average		51.83	33.48	12.45	3.07	3.26	17.96	26.80

In this study, the 3ADON chemotype of *F. graminearum* isolates had generally higher daily growth rates of colonies compared to the isolates of the 15ADON chemotype. On average, mycelial growth rate was higher in 3ADON (28.08 mm) than in 15ADON (26.37 mm) isolates from maize. On the other hand, similar average values of mycelial growth rate were measured in 3ADON and 15ADON isolates obtained from wheat and barley (Tables 2-4).

Pathogenicity test on barley leaves

Pathogenicity tests showed that all isolates (98) caused necrotic spots with yellow halos on the fifth day after inoculation. In the majority of tested isolates, complete necrosis and leaf decay occurred seven days after inoculation. According to the obtained results, the tested isolates showed different pathogenicity on barley leaves.

Tabela 3. Production of deoxynivalenol (DON), 3-acetyl-deoxinivalenol (3ADON) and 15-acetyl-deoxinivalenol (15ADON) (µg/g), aggressiveness and mycelial growth rate of *Fusarium graminearum* obtained from wheat

Isolate	Chemotype	DON	15ADON	3ADON	Aggress. maize	Aggress. wheat	Aggress. barley	Mycelial growth (mm)
800	3ADON	12.31	2.73	3.73	3.6	4.07	20.31	27.00
1370	3ADON	64.36	18.99	36.51	2.43	2.65	14.88	27.83
Average		38.33	10.86	20.12	3.01	3.36	17.59	27.41
203	15ADON	38.15	29.11	2.50	2.56	2.27	5.31	28.33
618	15ADON	57.68	46.81	6.66	2.21	3.42	18.31	29.83
670	15ADON	32.19	26.94	ND	2.91	2.61	16.25	28.16
677	15ADON	103.59	69.97	26.82	2.65	2.65	13.38	27.00
681	15ADON	71.26	58.76	3.90	3.16	3.93	26.56	20.00
687/2	15ADON	39.63	31.88	2.36	2.38	2.35	17.81	30.33
744	15ADON	26.96	16.75	2.85	2.28	2.31	27.75	29.16
749	15ADON	95.95	78.14	9.77	2.46	4.07	12.75	26.00
763	15ADON	9.18	5.68	ND	2.29	2.17	4.30	26.33
764	15ADON	40.36	23.31	9.52	2.84	2.97	15.75	25.33
766	15ADON	6.09	3.18	0.93	3.02	4.63	22.10	30.16
767	15ADON	19.85	12.38	1.65	2.25	2.33	14.56	19.50
779/2	15ADON	72.22	46.58	17.81	2.7	4.05	17.56	30.33
789	15ADON	43.47	27.19	12.37	2.77	2.87	21.69	27.33
795	15ADON	18.56	11.69	1.61	2.11	2.16	4.44	29.33
825	15ADON	21.59	16.07	1.76	2.68	2.95	23.19	28.83
831	15ADON	9.13	6.67	0.43	2.01	2.07	6.31	30.00
836	15ADON	24.56	15.40	2.39	2.67	3.16	11.88	28.16
864	15ADON	28.95	22.07	2.04	2.86	2.97	29.38	30.00
866	15ADON	5.27	1.69	1.08	2.47	2.32	12.45	27.66
870	15ADON	37.86	24.77	5.29	2.82	2.26	11.56	26.83
892	15ADON	11.20	6.91	0.59	2.78	2.75	22.00	26.83
1012	15ADON	16.28	10.05	1.84	2.37	3.32	25.31	27.50
1337	15ADON	39.63	26.46	7.09	2.69	2.5	24.50	27.00
1343	15ADON	46.38	24.69	16.41	2.89	3.06	18.25	27.83
1348	15ADON	75.69	46.84	22.79	4.22	5.06	30.94	26.83
1351/2	15ADON	101.97	90.97	5.91	2.82	2.95	9.00	28.50
1443	15ADON	52.97	35.66	9.06	2.87	2.93	23.07	19.66
1485	15ADON	59.25	50.05	1.49	4.99	5.00	34.88	22.33
1486/2	15ADON	14.69	9.58	0.38	2.46	3.85	20.25	28.16
1490/2	15ADON	46.74	40.66	1.89	2.58	2.66	13.75	28.00
1746	15ADON	41.78	32.15	4.25	2.91	3.38	27.44	27.16
2621	15ADON	46.32	37.75	1.79	4.48	2.75	32.69	27.16
2625	15ADON	19.82	14.37	0.85	2.62	3.05	17.13	24.83
2635	15ADON	45.69	34.60	1.96	3.53	4.17	13.00	30.50
2818	15ADON	37.39	25.13	2.18	3.14	3.43	18.19	29.16
2820	15ADON	57.36	48.85	0.99	2.9	2.91	13.13	30.83
2823	15ADON	94.98	75.12	12.35	4.57	5.37	31.44	29.00
Average		42.38	31.18	5.65	2.86	3.15	18.64	27.36
Total average		42.18	30.19	6.39	2.87	3.16	18.58	27.37

No symptoms of disease appeared on leaves inoculated with sterile distilled water as a negative control.

The obtained results showed that 15ADON isolates expressed stronger pathogenicity under laboratory conditions than 3ADON isolates. The difference in pathogenicity between 3ADON and 15ADON isolates was more pronounced in isolates obtained from barley (3ADON-12.43 mm, 15ADON-18.28 mm) than in isolates from maize (3ADON-13.89 mm, 15ADON-19.32 mm). On the other hand, in isolates obtained from wheat the observed variations in isolate pathogenicity between 3ADON and 15ADON chemotypes were low (3ADON-17.59 mm, 15ADON-18.64 mm). Furthermore, 3ADON isolates originating from wheat (17.59 mm) were more aggressive than 3ADON isolates obtained from maize (13.89 mm) and barley (12.43 mm) (Table 2-4).

Pathogenicity test on maize ears

Pathogenicity of *F. graminearum* isolates in the field was confirmed after artificial inoculation of maize ears. The results indicated that all observed isolates were pathogenic as a characteristic symptom of red-pinkish rot appeared on all inoculated ears. Depending on isolate aggressiveness, ears were completely or partially affected by fungus mycelia. No disease symptoms occurred on ears in the negative control inoculated with sterile distilled water. The results indicated different degrees of aggressiveness that was evaluated based on the degree of infection. The 15ADON chemotypes were more aggressive than the 3ADON chemotypes isolated from maize and barley. The mean pathogenicity evaluation of 3ADON chemotype isolates ranged from 2.62 to 3.02 in isolates from maize and wheat, respectively, while the average evaluation of pathogenicity of the 15ADON chemotype ranged from 2.85 in isolates from wheat to 3.22 in isolates from maize. In contrast to isolates from maize and barley, those of the 3ADON chemotype from wheat showed greater aggressiveness (3.02) compared to the 15ADON isolates (2.85). Considering average values for the isolates obtained from maize, 15ADON isolates demonstrated higher aggressiveness (3.22) than the 3ADON isolates (2.62) (Table 2).

Table 4. Production of deoxynivalenol (DON), 3-acetyl-deoxinivalenol (3ADON) and 15-acetyl-deoxinivalenol (15ADON) (µg/g), aggressiveness and mycelial growth rate of *Fusarium graminearum* obtained from barley

Isolate	Chemotype	DON	15ADON	3ADON	Aggress. maize	Aggress. wheat	Aggress. barley	Mycelial growth (mm)
1528	3ADON	4.12	1.07	1.19	3.27	3.07	22.81	27.00
2254	3ADON	11.09	2.33	3.28	2.46	2.93	2.06	26.16
Average		7.60	1.70	2.23	2.86	3.00	12.43	26.58
654	15ADON	61.23	26.71	26.63	2.63	3.12	18.44	27.66
770/2	15ADON	14.21	6.21	1.35	3.03	2.80	12.50	28.66
798	15ADON	12.83	7.20	0.22	2.62	3.88	7.94	30.16
805	15ADON	46.41	32.81	4.85	4.33	4.06	24.69	18.16
891/2	15ADON	50.12	31.23	10.17	2.78	2.60	14.25	28.66
1217/2	15ADON	75.18	62.26	3.61	2.90	4.00	25.38	28.16
1493	15ADON	21.14	14.19	0.10	5.26	3.93	37.19	28.16
1517	15ADON	15.36	10.34	2.49	2.44	2.95	9.63	28.00
1526	15ADON	41.87	25.89	8.87	2.60	3.21	16.25	26.50
1534	15ADON	8.74	6.23	0.68	5.12	3.43	34.88	28.66
1772	15ADON	58.36	49.84	5.33	2.99	4.61	17.31	26.33
1800	15ADON	8.32	4.49	0.92	3.33	3.15	27.10	29.00
1801	15ADON	104.98	95.07	3.10	2.78	2.87	7.00	14.83
1812	15ADON	31.69	23.68	3.25	2.71	2.51	22.56	22.83
1839	15ADON	17.38	10.59	0.11	1.92	2.00	3.56	26.50
2045	15ADON	19.61	9.86	3.71	2.66	3.19	14.19	26.33
2078	15ADON	29.97	21.10	4.61	2.66	3.80	26.94	27.33
2627	15ADON	42.55	19.98	15.08	3.27	3.53	19.31	29.00
2630	15ADON	22.43	13.44	0.67	3.72	4.06	16.44	24.83
2672	15ADON	102.32	71.05	26.76	2.92	3.05	16.44	26.66
Average		39.23	27.11	6.12	3.13	3.34	18.60	26.32
Total average		36.36	24.79	5.77	3.11	3.31	18.04	26.34

Pathogenicity test on wheat spikes

The aggressiveness of *F. graminearum* isolates on wheat spikes was also tested under field conditions after artificial inoculation. Symptoms on wheat spikes occurred during the formation and maturation of grain. *F. graminearum* isolates differed in the expression of pathogenicity on wheat spikes in the field. No disease symptoms appeared on spikes inoculated with sterile distilled water that served as a negative control. The tested *F. graminearum* isolates expressed variations in aggressiveness on wheat spikes in the field. On average, the degree of aggressiveness ranged from 2.89 to 3.36 in 3ADON isolates, and from 3.15 to 3.38 in 15ADON isolates. The pathogenicity test of the isolates obtained from wheat spikes showed similar results to pathogenicity on maize ears, and isolates of the 15ADON chemotype were on average more aggressive than those of the 3ADON chemotype obtained from maize and barley, while 3ADON isolates obtained from wheat were more aggressive than isolates of the 15ADON chemotype (Tables 2-4).

HPLC method

Using the HPLC method to test *F. graminearum* isolates (98), the following two chemotypes were observed: 3ADON and 15ADON. According to the results, a great diversity was observed in the production of DON derivatives. The majority of *F. graminearum* isolates, regardless of their origin (maize, wheat or barley) belonged to the 15ADON chemotype. The 3ADON chemotype was also detected but a significantly smaller number of isolates (13/98) was found, compared to the 15ADON chemotype (85/98), while none of the tested isolates belonged to the NIV chemotype. The greatest number of isolates obtained from wheat belonged to the 15ADON chemotype (38/40), followed by isolates derived from barley (20/22), while the lowest number of isolates obtained from maize (27/36) belonged to the 15ADON chemotype (Tables 2-4).

The highest level of variation in 15ADON concentrations was observed in isolates derived from maize (5.47-125.97 μg/g) (Table 2), while the lowest variation in concentrations was observed in isolates obtained from barley (1.07-71.05 μg/g) (Table 4). Furthermore, isolates derived from maize also had the greatest range of variation in 3ADON concentrations (1.03-72.85 μg/g), while the lowest variation of this mycotoxin was observed in isolates derived from barley (0.10-26.76 μg/g) (Tables 2 and 4). There was no statistically significant correlation between the aggressiveness and production of total DON of 3ADON and 15ADON isolates (Table 5).

There was no statistically significant correlation between aggressiveness and the production of total DON by 3ADON and 15ADON isolates. The tested isolates originating from maize synthesised the highest average concentration of total DON (51.83 μg/g) (Table 2), followed by isolates from wheat (42.18 μg/g) (Table 3) and barley (36.36 μg/g) (Table 4). Moreover, a comparison of average concentrations of total DON between isolates of the 3ADON and 15ADON chemotypes indicated that 15ADON isolates

Table 5. Correlation coefficient (r) between the production potential of total DON of each chemotype and aggressiveness on maize, wheat and barley

Evaluation of aggressiveness	Total DON (μg/g)	
	Chemotype 15ADON	Chemotype 3 ADON
maize	0.07^{ns}	-0.19^{ns}
wheat	0.06^{ns}	-0.28^{ns}
barley	-0.08^{ns}	0.04^{ns}

ns - not statistically significant

Table 6. Corellation coefficient (r) between two chemotypes in production potential of total DON, 3ADON and 15ADON

	Chemotype 15ADON		Chemotype 3ADON	
	Total DON	15ADON	Total DON	15ADON
15ADON	0.968**		0.966**	
3ADON	0.583**	0.373**	0.979**	0.899**

** Statistically highly significant difference (P ≤ 0,01)

synthesised higher concentrations of total DON. The highest differences in the synthesis of total DON between isolates of the 3ADON chemotype (7.6 μg/g) and 15ADON chemotype (39.2 μg/g) were found in isolates obtained from barely, while these differences were smaller in isolates derived from wheat and maize (Table 2-4). There was a highly significant statistical correlation between the production potentials of total DON, 3ADON and 15ADON in *F. graminearum* isolates belonging to different chemotypes (Table 6).

DISCUSSION

Previous data reported from around the world had shown that the 15ADON chemotype was dominant, while 3ADON chemotype was observed at a much smaller percentage (Gale et al., 2007; Ward et al., 2008; Prodi et al., 2011; Boutigny et al., 2014; Somma et al., 2014: Bozac et al., 2016). Recently, the presence of 3ADON chemotype has increased in the USA and Canada (Gale et al., 2007; Kuhnem et al., 2015), while in Europe it has been detected in Norway (Aamot et al., 2015), Sweden and Finland (Fredlund et al., 2013; Lindblad et al., 2013; Kuhnem et al., 2015), France (Boutigny et al., 2014) and Italy (Somma et al., 2014). The present study shows that the 15ADON chemotype of *F. graminearum* isolates is dominant in Serbia, while the NIV chemotype was not detected. The 3ADON chemotype was observed in a significantly lower percentage than the 15ADON chemotype (13.28% vs. 86.73%, respectively). The highest percentage (25%) of 3ADON chemotype was detected in isolates obtained from maize, followed by barley (9.09%) and wheat (5%). Previous results had shown that 15ADON chemotype produced both 3ADON and NIV but in much lower concentrations (Ward et al., 2008; Puri & Zhong, 2010).

Furthermore, this study shows that the examined *F. graminearum* isolates differed regarding their average concentrations of total DON. The highest average concentrations of DON were determined in isolates collected from maize grain (51.83 μg/g), then from wheat grain (42.18 μg/g) and finally from barley grain (36.36 μg/g). Contrary to our results, previous studies had shown that *F. graminearum* isolates obtained from wheat grain had synthesised higher average concentrations of DON than those derived from maize grain (Tančić et al., 2015). Moreover, Kuhnem et al. (2015) observed significant differences in DON production between isolates from different sources. Isolates derived from wheat produced 73.96 μg/g of DON, which was 30 μg/g higher on average than DON from isolates obtained from maize (44.57 μg/g).

The results indicate that 15ADON isolates of *F. graminearum* synthesised higher total amounts of DON than 3ADON isolates. However, in Canada, Ward et al. (2008) observed that 3ADON isolates of *F. graminearum* had significantly higher production of trichothecenes compared to 15ADON isolates. Similar to these results, in North America, Puri and Zhong (2010) found that 3ADON isolates of *F. graminearum* synthesised 1.5 and 86 times higher total amounts of DON and 3ADON, respectively, than 15ADON isolates. Likewise, 3ADON isolates synthesised six times less 15ADON than 15ADON isolates. In that study, higher concentrations of 15ADON (up to 125.97 μg/g) were observed in 15ADON isolates than in 3ADON isolates (up to 72,85 μg/g). In contrast to the findings in the United States reported by Ward et al. (2008) and Puri and Zhong (2010), the largest differences in the synthesis of total DON in our studies were between 3ADON and 15ADON isolates from barley (five times higher concentrations of total DON in 15ADON isolates). However, Kuhnem et al. (2015) observed that total DON production in wheat did not differ between 3ADON (53.1 μg/g) and 15ADON (47.6 μg/g) isolates.

In the present study, a connection between the pathogenicity of isolates and their origin was not established. These results are in accordance with data reported by Lee et al. (2016), who studied the pathogenicity of *F. graminearum* isolates originating from different cereals and found that it did not depend on the origin of isolates. Namely, isolates obtained from maize were less aggressive than those derived from barley and rice. Similarly, Kuhnem et al. (2015) studied the pathogenicity of *F. graminearum* isolates originating from maize and wheat, and concluded that there was no connection between the pathogenicity and origin of isolates. Nevertheless, when observing variations in pathogenic properties of *Fusarium* spp. obtained from maize and wheat grain, Tančić et al. (2015) detected that intraspecies variation in aggressiveness was the most pronounced in *F. graminearum* isolates. Also, these authors found that *F. graminearum* isolates originating from maize grain were more pathogenic than isolates originating from wheat grain, when pathogenicity was tested on maize.

In previous studies, researchers paid attention to differences in colony growth rate of different chemotypes within the Fg complex (Aamot et al., 2015; Ward et al., 2008; Puri & Zhong, 2010). Conducting the current study, we observed that the average daily growth of mycelium was higher in 3ADON isolates. Similarly, Ward et al. (2008), studying the spread of Fg populations in North America, observed that *F. graminearum* isolates

with the 3ADON chemotype had a significantly higher colony growth on PDA than isolates with the 15ADON chemotype. The present study shows that the greatest variation in daily growth rate between isolates with the 3ADON and 15ADON chemotypes was observed in isolates obtained from maize, compared to those derived from wheat and barley. The results showed that a large variation in mycelial growth was observed among the studied *F. graminearum* isolates, but the difference between isolates of various trichothecene chemotypes (3ADON and 15ADON) was not significant. Furthermore, certain authors had determined that belonging to a chemotype did not affect the mycelial growth rate of isolates. Puri and Zhong (2010), studying *F. graminearum* isolates, observed that there were no significant differences in the growth rate of colonies between 3ADON and 15ADON isolates. Moreover, no significant correlation was observed between the degree of aggressiveness in wheat and the mycelial growth rate of 20 studied *F. graminearum* isolates on PDA (Aamot et al., 2015). There is a large number of different literature data on the association between pathogenicity of isolates and affiliation to trichothecene chemotypes (Ward et al., 2008; Zhang et al., 2012; Puri & Zhong 2010; Aamot et al., 2015; Kuhnem et al., 2015; Carter et al., 2002; Von der Ohe et al., 2010; Li et al., 2010). Trichothecene production can increase the pathogenicity of some species of the genus *Fusarium* depending on their host species (Villafana et al., 2019). Studying the pathogenicity of *F. graminearum* isolates, Carter et al. (2002) proved that different chemotypes were equally pathogenic to wheat, while the NIV chemotype was more pathogenic to maize. Zhang et al. (2012) studied *F. graminearum* isolates obtained from wheat and observed that the pathogenicity of 3ADON and 15ADON chemotypes was significantly higher than the pathogenicity of NIV chemotypes in wheat. Similar results were reported by Li et al. (2010), who also observed that isolates with the 3ADON chemotype were more virulent than NIV populations in China. Furthermore, Gale et al. (2011) established that isolates that synthesised DON caused disease symptoms that spread significantly faster on wheat spikes than those that synthesised NIV. However, Goswami and Kistler (2005) analysed isolates of the Fg complex derived from various hosts, and observed a great variation in the pathogenicity of isolates that did not depend on the type of produced mycotoxin. A statistically significant correlation was observed between the concentration of trichothecene produced by each species and the degree of aggressiveness in wheat.

The obtained results show that isolates with the 15ADON chemotype were more aggressive than those with 3ADON in the pathogenicity test on barley leaves. Moreover, pathogenicity tests on maize and wheat showed that 15ADON isolates derived from maize and barley were more aggressive than isolates with the 3ADON chemotype. The same conclusion was drawn by Aamot et al. (2015), who observed differences in aggressiveness of certain isolates of *F. graminearum* in Norway, and it was found that the aggressiveness of isolates in wheat was associated with trichothecene chemotypes, i.e. the authors detected a significantly higher average aggressiveness in 15ADON isolates than in 3ADON isolates. According to the obtained results on the pathogenicity of isolates derived from maize and wheat, 3ADON isolates obtained from wheat were more aggressive than 15ADON isolates. Kuhnem et al. (2015) established a statistically significant positive correlation between total accumulation of trichothecene (deoxynivalenol [DON] and its acetyl derivatives) and pathogenicity in wheat and maize. One tested isolate produced neither DON nor ADON in wheat and maize grain, but was aggressive to both hosts. Similar results were reported by Puri and Zhong (2010), who observed that 3ADON isolates were more aggressive and caused significantly higher disease intensity in wheat than 15ADON isolates. However, Ward et al. (2008) and Von der Ohe et al. (2010) compared the aggressiveness and production of deoxynivalenol between 3ADON and 15ADON chemotypes and established that there was no significant difference in pathogenicity between them. Similar to our present results, zero or negative correlation between the aggressiveness and production of DON in grain were also revealed by Atanassov et al. (1994) and McCormick (2003).

Global studies on trihotecene synthesis within the Fg complex in cereals are important so as to establish a genotype database which will enable that each shift in populations can be traced in the future (Starkey et al., 2007; Ward et al., 2008; Gale et al., 2007, 2011; Guo et al., 2008; Schmale et al., 2011). In recent years, global climate change has caused variations in agro-climatic conditions, which may stimulate the synthesis of higher concentrations of mycotoxins in cereal grain during the growing season and cause economic losses in the production, as well as increased risk to human and animal health (Moretti et al., 2018). The mentioned reasons indicate a need for permanent monitoring of these toxigenic species in the production of cereals. Knowledge about all factors that directly or indirectly affect disease development is a necessary prerequisite for successful prevention of damage caused by toxigenic and pathogenic fungal species.

ACKNOWLEDGMENT

The results obtained in the present study are part of the Project TR31023 that was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Agresivnost i sinteza trihotecena izolata *Fusarium graminearum* poreklom sa zrna žitarica u Srbiji

REZIME

Cilj ovog rada bio je da se utvrde razlike u agresivnosti i sintezi trihotecena kod izolata *F. graminearum* poreklom sa zrna kukuruza, pšenice i ječma u Srbiji. Proučavanjem izolata *F. graminearum* (98), izolovanih iz različitih agroekoloških uslova Srbije u periodu od 1993. do 2010. godine, primenom HPLC metode, identifikovana su dva trihotecen hemotipa: 3-acetil-deoksinivalenol (3ADON) i 15-acetil-deoksinivalenol (15ADON). Uočen je veliki diverzitet u sintezi deoksinivalenol (DON) derivata. Bez obzira na poreklo (kukuruz, pšenica ili ječam), većina ispitivanih izolata *F. graminearum*, pripadala je 15ADON hemotipu. Identifikovan je i 3ADON hemotip, ali u znatno manjem broju (13/98), u poređenju sa 15ADON hemotipom (85/98). Nijedan od ispitivanih izolata nije pripadao NIV hemotipu. Ispitivani izolati su pokazali različitu patogenost na listu ječma, klasu pšenice i klipu kukuruza. Prosečna patogenost ispitivanih izolata bila je najveća na listu ječma. Uočeno je da su izolati poreklom sa pšenice imali najveći prosečan dnevni porast micelije (27.37 mm). Utvrđena je statistički visoko značajna pozitivna korelacija između 3ADON i 15ADON izolata *F. graminearum* i sinteze ukupnog DON, 3ADON i 15ADON. Međutim, između stepena agresivnosti ispitivanih izolata i sinteze ukupnog DON nisu utvrđene statistički značajne korelacije.

Ključne reči: žitarice, *Fusarium graminearum*, mikotoksini, hemotipovi, trihoteceni