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Original scientific paper

TRICHOTHECENE CHEMOTYPE DIVERSITY OF *Fusarium graminearum* ISOLATED FROM WHEAT, MAIZE AND BARLEY IN SERBIA

Ana OBRADOVIĆ[1], Slavica STANKOVIĆ[1], Vesna KRNJAJA[2], Ana NIKOLIĆ[1], Dragana IGNJATOVIĆ-MICIĆ[1], Jelena STEPANOVIĆ[3] and Bojan DUDUK[3]

[1]Maze Research Institute Zemun Polje, Belgrade, Serbia
[2]Institute for Animal Husbandry, Belgrade, Serbia
[3]Institute of Pesticides and Environmental Protection, Belgrade, Serbia

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Diversity of trichothecene chemotypes of *Fusarium graminearum* isolated from kernels of wheat, barley and maize grown under various agro-ecological conditions on 13 locations was analysed. Sixteen strains were tested for the effective capability to produce 15-ADON, 3-ADON and NIV, by using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. Fourteen out of sixteen analyzed strains produced 15-ADON, while remaining two were of the 3-ADON chemotype. Multiplex PCR reaction with two sets of specific primers for *TRI3* and *TRI12* genes was applied to identify trichothecene chemotypes (3-ADON, 15-ADON and NIV). The expected sizes of amplified fragments for *TRI3* gene primer set are 840 bp (NIV), 610 bp (15-ADON) and 243 bp (3-ADON). The amplified fragments for *TRI12* gene primer set should be 840 bp (NIV), 670 bp (15-ADON) and 410 bp (3-ADON). All *F. graminearum* isolates were of the 15-ADON chemotype, i.e. their bands were 610 bp and 670 bp size for *TRI3* and *TRI12* genes, respectively. The results indicate that genotypic characterisation does not correspond to determined chemotypes and this is a reason why the analyses for the risk of mycotoxins contamination should not be based only on trichotecene genotype determination. Due to high temperature differences in cereal growing regions in Serbia, the presence of other chemotypes could be expected. In order to determine whether besides 15-ADON there are other F. *graminearum* chemotypes on wheat, barley and

Corresponding author: Slavica Stanković, Maize research institute, S.Bajica 1, 11185 Belgrade, Serbia, e-mail: sstojkov@mrizp.rs

maize kernels, further studies should include a large number of isolates from different agro-ecological conditions.

Key words: Fusarium graminearum, trichothecene chemotype, NIV, 15-ADON, 3-ADON

INTRODUCTION

Fusarium graminearum Schwabe, the anamorphic stage of the fungus *Giberella zeae* Schwein (Petch), causes stalk rot, maize ear mouldiness and head blight of wheat. This species has a broad range of hosts and is one of economically most important factors that jeopardise the crop production, causing the reduction in grain yield and quality. Moreover, the significance of the fungus is even greater due to its capability to synthesise mycotoxins in infected plants, which adversely affects human and animal health. During the past two decades, *F. graminearum* has become one of the most destructive pathogens of diseases in cereals, causing grain losses in the industry of several billion dollars (CLEAR and PATRICK, 2000; WINDELS, 2000). Understanding all the factors that directly or indirectly affect the development of diseases is a necessary prerequisite for success in preventing damages caused by this pathogenic species. Having information on toxicological profile of *F. graminearum* species is of essential importance for agriculture and food industry of any country, because trichothecene have a significant role in the aetiology of human and animal diseases. It was observed that different mycotoxins have different toxicological properties, hence nivalenol is more toxic than deoxynivalenol for humans and animals (LEE *et al*., 2015).

F. graminearum synthesises a great number of mycotoxins and the most important are type B trichothecene - deoxynivalenol (DON), its acetyl-ester derivatives (3-ADON and 15-ADON) and nivalenol (NIV). With regard to the synthesis of trichothecenes (chemical structures differing in the position of acetyl-ester derivatives), two chemotypes are described. Chemotype I synthesises deoxynivalenol and its acetyl-ester derivatives, while chemotype II synthesises nivalenol and fusarenon - X (MULÉ *et al*., 1998). The species synthesising 3AcDON, i.e. 15-ADON are chemotype Ia and Ib, respectively (WARD *et al*., 2002).

Previous phylogenetic studies on *F. graminearum* species point out to differences of various chemotypes of isolates originating from different geographic regions. *F. graminearum* isolates form Europe and America have greater potential to synthesise 15-ADON, while those from Asia synthesise more 3-ADON, while NIV chemotype prevails in New Zealand and Africa (STARKEY *et al*., 2007; WARD *et al*., 2008; GALE *et al*., 2007, 2011). Results obtained by GALE *et al*. (2002), ZHANG *et al*. (2007) and QU *et al*. (2008) indicate that the highest number of *F. graminaerum* isolates from China are of 3-ADON chemotype. The analysis of chemotypes in Japan pointed to the dominance of 3-ADON, but the percentage of the NIV chemotype was also high (KARUGIA *et al*., 2009). Geographic variation of chemotypes has been observed in Iran: 15-ADON chemotype prevailed in the western part of the country, while NIV chemotype abounded in eastern Iran (DAVARI *et al*., 2013).

However, it should be noted that, probably due to climatic changes, dominance of chemotypes may be altered within the same geographic region. Hence, in Canada the frequency of 3-ADON producers increased from the eastern towards the western part of the country (WARD *et al*., 2008; GUO *et al*., 2008). WARD *et al*. (2008) analysed 492 *F. graminearum* isolates originating from North America and detected approximately 25% of the 3-ADON chemotype, while remaining 75% were of the 15-ADON chemotype. The same authors observed that 100% of

samples from east were of the 3-ADON chemotype, while this percentage decreased across Canada and amounted to less than 10% in the west part of the country. It was determined that 15-ADON (approximately 95%) dominated in the central and western parts of the USA, while the detected percentage of the 3-ADON chemotype was low (GALE *et al*., 2007).

The studies carried out in Europe showed that 15-ADON chemotype predominated on wheat, barley and maize in France, while NIV chemotype was less present and found only on wheat and maize (BOUTIGNY *et al*., 2014). According to the studies performed in Italy and England the majority of isolates were 15-ADON producers (SOMMA *et al*., 2014; JENNINGS *et al.*, 2004).

Hitherto, there are no data on biodiversity and distribution of *F. graminearum* chemotypes in different agro-ecological conditions of Serbia. STANKOVIĆ *et al***.** (2008) analysed DON production by *F. graminearum* and *F. culmorum* isolated from wheat grains grown under different agroecological conditions in Serbia during two years (2005-2006). Fourteen isolates of *F. graminearum* produced considerable amounts of DON, up to 45.260 ppb. Data base on diversity and distribution of *F. graminearum* trichothecene chemotypes is of great importance for any country, because knowledge of their toxicological profiles enables efficient risk management. The objective of the study presented herein was to determine diversity of *F. graminearum* trichothecene chemotypes (trichothecene mycotoxicological profile) isolated from kernels of wheat, barley and maize, which were collected from different agro-ecological regions of Serbia.

MATERIAL AND METHODS

A total of 16 *F. graminearum* isolates from the collection of fungal cultures of Maize Research Institute, Zemun Polje (MRIZP) were analysed. These isolates were obtained from wheat, barley and maize kernels, collected from 13 different locations in Serbia (Table 1.).

Table 1. List of F. graminearum isolates analysed for trichocene chemotypes

Isolate Nº (MRIZP)	Location	Origin
681	Sombor	wheat
687	Kikinda	wheat
699	Zemun Polje	maize
762	Titel	wheat
763	Ruma	wheat
764	Novi Sad	wheat
1133	Senta	maize
1165	Zemun Polje	maize
1249	Loznica	maize
1255	Kovin	maize
1772	Sombor	barley
2254	Zemun Polje	barley
2812	Velika Plana	maize
2813	Sremska Mitrovica	maize
2822	Srbobran	maize
2823	Erdevik	wheat

Chemical detection of trichothecene chemotypes

Sixteen strains were tested for the effective capability to produce 15-ADON, 3-ADON and NIV, by using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. The strains were grown on plates containing potato dextrose agar (PDA) for seven days. Pieces of these cultures were added to 50 g of autoclaved maize kernels previously kept to 45% of moisture overnight. Negative control maize kernels were prepared in the same way, except that it was not inoculated. Cultures were incubated at 25° C in the dark for 4 weeks, grounded to a fine powder and used for chemical analyses. Five grams of each powdered sample was dissolved in 25 ml acetonitrile - distilled water solution (75/25, V/V). The sample homogenization was done in an Osterizer blender for 3 minutes. A homogenized mixture was filtered through Whatman 1 filter paper and filtrate was applied to MycoSep columns (113 Trich and 230 NIV, Romer Labs, USA).

Dionex UltiMate 3000 liquid chromatography system (Thermo Scientific, Germany) fitted with DAD detector, analytical column, Acclaim Polar Advantage II, C18 (150 × 4.6 mm, 3 μm) was used for chromatographic separation. Mixture of water-acetonitrile, (90:10 v/v) was used as mobile phase for separation of 3-ADON and 15-ADON **(**MATEO *et al*., 2011), while for separation of NIV mixture of water-acetonitrile-methanol, (90:5:5, v/v/v) was used (YUE *et al*., 2010). The flow rate was 1.2 mL/min, temperature of column was set at 25 ºC and the injection volume was 10 μL for both analyses. Chromatograms were generated at 218 nm and 220 nm. Tested mycotoxins were identified and quantified by comparing characteristic retention time of appropriate standards.

Molecular detection of trichothecene chemotypes

DNA was isolated from the mycelium of the fungus cultivated on PDA in the dark at 25ºC for 7 days. Commercial DNeasy Plant Mini Kit (Qiagen, Hilden, German) was used according to the manufacturer's instructions. Two primer sets for *TRI3* and *TRI12* genes (WARD *et al*., 2002) were used to detect chemotypes (Table 2).

Table 2. List of primers used to detect trichocene chemotypes of F. graminearum

Gene		Primer	Primer sequence 5'-3'	Fragment size (bp)
	common	3CON	TGGCAAAGACTGGTTCAC	-
TRI3	specific	3NA	GTGCACAGAATATACGAGC	840
		3D15A	ACTGACCCAAGCTGCCATC	610
		3D3A	CGCATTGGCTAACACATG	243
	common	12CON	CATGAGCATGGTGATGTC	-
TRI12	specific	12NF	TCTCCTCGTTGTATCTGG	840
		12-15F	TACAGCGGTCGCAACTTC	670
		12-3F	CTTTGGCAAGCCCGTGCA	410

Both multiplex PCR reactions were performed in 10 μl volumes with 1X GeneAmp PCR buffer, 2mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate, 0.2 μM

concentrations of each primer, 0.5 U of AmpliTaq DNA Polymerase (Thermo scientific) and 50 ng of genomic DNA. The reaction mixture with added sterile distilled water instead of the DNA sample was used as a negative control. The 25 cycles PCR reaction was carried out in a thermal cycler (Thermocycler T1, Biometra, Germany) under the following conditions: 30 sec (2 min and 30 sec for the first cycle) for denaturation step at 94ºC, 30 s for annealing at 52ºC and 1 min for extension at 72ºC.

Amplified fragments were electrophoretically separated on a 1% agarose gel in 1X TBE buffer, stained with ethidium bromide and visualized under UV light on transilluminator. The 100 bp DNA ladder was used as a marker to determine the fragment size (Fermentas, Lithuania).

RESULTS AND DISCUSSION

Chemical characterization of thrichotecene production showed that all tested strains of *F. graminearum* were DON producers (Table 3.). Total amount of the produced DON varied from 10.8 to 127.5 μg/g. Fourteen out of sixteen analyzed strains produced 15-ADON, while remaining two were of the 3-ADON chemotype (MRIZP 1165 and MRIZP 1240). However, *F. graminearum* isolates had the amplification products of 610bp (*TRI3*) and 670bp (*TRI12*) (Fig. 1). The results of molecular analysis point out that all isolates collected from different locations in Serbia from wheat, maize and barley kernels are 15-ADON producers. According to DESJARDINS *et al*. (2008), results of genetic and phenotypic characterisation of *F. graminearum* strains do not have to be congruent, and this is the reason why the analysis for the risk of mycotoxins contamination should not be base only on the trichotecene genotype determination. The chemical validation of molecular genotypes is often absent in reports, which can cause misleading data (SOMMA *et al*., 2014).

Table 3. Trichothecene production of 16 Fusarium graminearum strains isolated from cereals kernel

Strain (MRIZP)	Mycotoxins ($\mu g/g$)				Chemotype
	DON	15-ADON	3-ADON	NIV	
681	60.1	38.7	3.9	1.5	15-ADON
687	49.8	31.8	2.4	ND	15-ADON
762	35.7	14.6	4.4	0.3	15-ADON
763	23.8	5.7	ND	0.2	15-ADON
764	42.1	23.3	9.5	1.3	15-ADON
2823	88.7	55.1	12.3	1.3	15-ADON
699	64.4	41.3	6.7	0.4	15-ADON
2812	12.6	5.5	0.9	0.4	15-ADON
1133	27.7	12.9	6.3	0.7	15-ADON
1165	105.5	21.7	40.6	ND	3-ADON
1249	127.5	42.5	72.8	1.4	3-ADON
1255	59.4	30.6	5.7	ND	15-ADON
2813	53.7	33.6	6.4	0.4	15-ADON
2822	15.6	11.6	2.6	0.4	15-ADON
1772	79.2	49.8	5.3	0.4	15-ADON
2254	10.8	2.3	3.3	1.5	15-ADON
Control	ND	ND	ND	ND	

*ND-Not Detected

Previous studies indicated that 15-ADON was predominant in North and South America, 3-ADON in Asia, while NIV prevailed in Africa and Australia. According to studies carried out in Europe, the 15-ADON chemotype is dominant in Italy (SOMMA *et al*., 2014; PRODI *et al.* 2009, 2011), France (BOUTIGNY *et al*., 2014), Netherlands (WAALWIJK *et al*., 2003), England (JENNINGS *et al.*, 2004) and Turkey (YÖRÜK *et al*., 2014). Similar to these results, our research showed that all observed *F. graminearum* isolates possessed the 15-ADON chemotype. However, the distribution of chemotypes differs in the northern part of Europe from the rest of the continent. Results gained by YLI-MATTILA *et al*. (2009) pointed out that isolates recovered in Finland and northern Russia were exclusively 3-ADON producers. Some agro-ecological factors, such as crop rotation, climatic changes, and in some cases modifications of agricultural strategies of a country can affect the changes in the chemotype composition. The present study did not show the differences in mycotoxin production among isolates of various origins (host plants and locations).

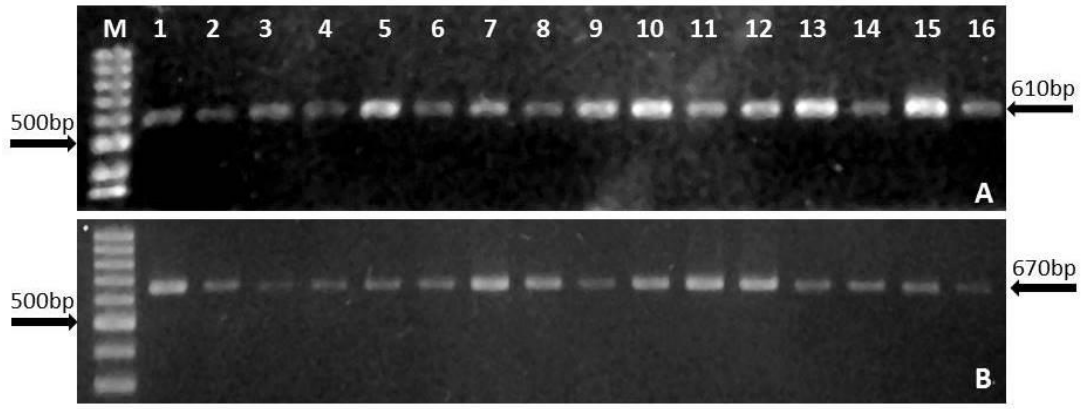

Fig. 1. Electrophoregram profiles obtained with multiplex primer sets for *TRI3* (A) and *TRI12* (B) trichotecene genes of *Fusarium graminearum*. A - amplification products of 610 bp characteristic for 15AcDON. B - amplification products of 670 bp characteristic for 15AcDON. M: 100 bp DNA marker. 1-16: different isolates of *Fusarium graminearum*.

Two isolates of the 3-ADON chemotype (MRIZP 1165 and MRIZP 1249), produced the highest DON quantities (105.5 and 127.5 μg/g, respectively) and it was two to ten times more than amount of the DON produced by 15-ADON chemotypes. This data agree with previous reports by PURI and ZHONG (2010), who evaluated randomly selected 3-ADON and 15-ADON isolates, showing that 3-ADON population caused a higher disease severity on the spring wheat genotypes and produced 1.5 time more DON than the 15-ADON population. WARD *et al*. (2008) noticed significantly lower trichotecene accumulation for the 15-ADON population than for the 3-ADON population *in vitro*. Molecular and chemical characterisation of chemotypes is important for establishing a strategy to assess the risk concerning the protection of human and animal health.

The results presented herein are the first preliminary results of the distribution of chemotypes on the territory of Serbia. Further studies should include a greater number of isolates from locations with various agro-ecological conditions, in order to determine whether there is any

other chemotype, besides15-ADON, on wheat, barley and maize. Considering great temperature differences in cereal growing regions in Serbia, the presence of other chemotypes might be expected, because the 15-ADON chemotype occurs at average annual temperatures below 15 ºC, while the 3-ADON chemotype mainly occurs at temperatures above 15ºC (SUGA *et al*., 2008).

The achieved results provide information on the distribution of chemotypes in Serbia. It is particularly important that these results are actually an introduction to the molecular characterization of the *F. graminearum* species and contamination by mycotoxins in infected plats, which adversely affect human and animal health. Research of variation in pathogen populations has also a crucial role in development of the strategic objectives in plant breeding, because knowledge in pathogen toxicity enables development of resistant cereal genotypes.

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DIVERZITET TRIHOTECEN HEMOTIPOVA VRSTE *Fusarium graminearum* IZOLOVANE IZ PŠENICE, KUKURUZA I JEČMA POREKLOM IZ SRBIJE

Ana OBRADOVIĆ[1], Slavica STANKOVIĆ[1], Vesna KRNJAJA[2], Ana NIKOLIĆ[1], Dragana IGNJATOVIĆ-MICIĆ[1], Jelena STEPANOVIĆ[3] and Bojan DUDUK[3]

[1]Institut za kukuruz Zemun Polje, Beograd, Srbija
[2]Institut za stočarstvo, Beograd, Srbija
[3]Institut za pesticide i zaštitu životne sredine, Beograd, Srbija

Izvod

U radu je ispitivan genetički diverzitet trihotecenskih hemotipova vrste *Fusarium graminearum* iz različitih agroekoloških uslova u Srbiji. Ukupno je analizirano 16 izolata *F. graminearum* poreklom sa zrna pšenice, ječma i kukuruza iz 13 različitih lokaliteta. Određivanje pripadnosti trihotecenskim hemotipovima (3-ADON, 15-ADON i NIV) obavljena je pomoću multipleks PCR reakcije sa dva seta specifičnih prajmera *TRI3* i *TRI12*. Očekivana veličina amplifikovanih produkata za prvi set prajmera *TRI3* je 840 bp (NIV), 610 bp (15-ADON) i 243 bp (3-ADON). Drugi set specifičnih prajmera *TRI12* ima očekivanu veličinu traka 840 bp (NIV), 670 bp (15-ADON) i 410 bp (3-ADON). Utvrđeno je da su svi ispitivani izolati *F. graminearum* bili 15-ADON hemotip, odnosno imali su očekivanu veličinu traka 610 bp za *TRI3* i 670 bp za *TRI12*. S obzirom na velike temperaturne razlike u područjima gajenja žitarica u Srbiji, mogla bi se očekivati prisutnost drugih hemotipova. Dalja istraživanja trebalo bi da uključe veći broj izolata iz različitih agroekoloških uslova, da bi se moglo utvrditi da li pored 15-ADON na zrnu pšenice, ječma i kukuruza postoji i drugi hemotip na teritoriji Srbije.

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