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Species composition and molecular assessment of the toxigenic potential in the population of *Fusarium* spp. isolated from ears of winter wheat in southern Poland

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Summary

The aim of this study was to identify the species composition and to evaluate the prevalence of genes involved in the synthesis of the following mycotoxins: deoxynivalenol, nivalenol and fumonisins in the population of *Fusarium* spp. isolated from ears of winter wheat in southern Poland. All fungal isolates were identified by the species-specific PCR or sequencing of the translation elongation factor. Significant differences were observed in both abundance and species composition of the collected strains between two years of studies. A total of 304 ear samples were processed and 107 *Fusarium* strains belonging to 10 species: *F. graminearum*, *F. culmorum*, *F. sporotrichioides*, *F. poae*, *F. avenaceum*, *F. oxysporum*, *F. verticillioides*, *F. equiseti*, *F. tricinctum* and *F. cerealis* were isolated in 2012 and 2013. Numerous presence of mycotoxin-biosynthesis pathway genes was detected in the examined material, which evidences the potential toxicity of the analyzed *Fusarium* fungi.

Introduction

Fungi from the genus *Fusarium* are extremely diverse group of microorganisms, both in terms of physiological and morphological characteristics. Their adaptability caused that they are currently considered as ones of the most important pathogens worldwide. Plant diseases caused by *Fusarium* spp. are global and contribute to measurable economic loss. *Fusarium* spp. cause, among others, seedling blight, root rot, leaf spot and *Fusarium* ear blight (Leslie and Summerell, 2006). Another extremely important aspect is the negative effect of secondary metabolites produced by *Fusarium* spp. on human and animal health (Wolny-Koładka, 2014).

Apart from *Aspergillus* spp. and *Penicillium* spp., fungi from the genus *Fusarium* are considered as ones of the most toxinogenic microorganisms worldwide. Mycotoxins produced by *Fusarium* spp. exhibit phytotoxic, zootoxic and antibiotic activity (Chełkowski, 1985; Tate, 2000). Secondary metabolites of *Fusarium* spp. have mutagenic, teratogenic and estrogenic properties, and while accumulating in the organism they become the cause of chronic multi-organ diseases (Pławińska-Czarnak and Zarzyńska, 2010).

Fungi from the genus *Fusarium* produce mycotoxins not only during growing season, but also during harvesting, transport and storage (Creepy, 2002). The ability to produce mycytoxins is a strain-specific feature and is not characteristic for the entire species (Tan and Niessen, 2003). Among the most frequently produced mycotoxins by the genus *Fusarium* there are deoxynivalenol (DON), nivalenol (NIV), zearalenon (ZEA) and fumonisins (FUM). Numerous genes are involved in the mycotoxin synthesis, among which the following systems were distinguished: TRI (deoxynivalenol, nivalenol), PKS (zearalenone) and FUM (fumonisins) (Alexander et al., 1999; Ward et al., 2002; Alexander et al., 2004; Fanelli et al., 2013). The potential toxicity of fungi can be inferred from the detection of particular genes involved in the biosynthetic pathway of mycotoxins. Currently, the most relevant methods in the detection of toxinogenic strains include the molecular biology techniques. These methods are based on polymerase chain reactions with the use of specific primers and allow both rapid identification of strain species as well as their potential to produce mycotoxins (Niessen, 2007).

The aim of this study was the molecular identification of fungi from the genus *Fusarium* isolated from winter wheat ears in southern Poland in 2012 and 2013. Moreover, the presence of the key genes involved in the biosynthetic pathways of deoxynivalenol, nivalenol and fumonisins was assessed in the studied *Fusarium* spp. strains, in order to indicate the potential ability for the production of these mycotoxins.

Materials and methods

Fungal isolation from winter wheat ears was conducted during two growing seasons of 2012 and 2013. The ears were randomly collected into sterile bags from the same fields in both years in southern Poland and after collection transported to the laboratory, in order to isolate the *Fusarium* strains. Eight ears were collected per each field and one ear represented a single sample. A total of 304 ear samples were analyzed, 152 each year. The material was surface sterilized with 1 % sodium hypochlorite and then rinsed in sterile distilled water and placed onto potato-dextrose agar (PDA). The plates were incubated at 24 ºC for 7 days and then mycelium fragments were subcultured onto subsequent Petri dishes with PDA until homogeneous cultures were obtained (Kowalska, 2011). The initial identification of fungi was conducted using the diagnostic manuals: Gilman (1957), Domsch et al. (1980), Marcinowska (2003). Macroscopic evaluation of the cultures was also conducted, taking into account the structure and pigmentation of the mycelium. Subsequently all isolates qualified as the genus *Fusarium* were counted.

Prior to DNA extraction *Fusarium* spp. isolates were cultured on PDA for 7 days. Afterwards, mycelia were scraped into Eppendorf tubes with a sterile spatula. The mycelium was ground with 2 mm wolfram beads using Retsch MM 400 Mixer Mill (30 Hz, 3 min). Total DNA was extracted from mycelium of each isolate (about 100 mg wet weight) using Genomic Mini AX Plant DNA extraction kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The quality and quantity of DNA obtained was assessed using NanoDrop spectrophotometer (Thermo Scientific, USA).

Molecular identification of the collected *Fusarium* spp. isolates was conducted based on the species-specific PCR assay using previously published primer pairs for *F. poae*, *F. oxysporum*, *F. graminearum*, *F. sporotrichioides*, *F. culmorum*, *F. proliferatum* and *F. verticillioides* (Tab. 1). PCR reactions were performed for all isolates and sterile deionized water served as negative control. The reaction mixtures of a total volume of 25 µl contained 10×PCR buffer, 1.5 mM of $MgCl_2$, 0.3 µM of each primer, 0.2 mM of dNTPs, 1 U of Taq DNA Polymerase (Thermo Scientific - Fermentas, Canada) and approximately 25 ng of fungal template DNA. PCR amplification was carried out in the BioRad T100 Thermal Cycler (BioRad, USA) using temperature profiles described by Nicholson et al. (1998), Mishra et al. (2003), Mulè et al. (2004a), Demeke et al. (2005), Koncz et al. (2008), and Rahjoo et al. (2008). The PCR products were visualized

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by 1×TBE electrophoresis in ethidium bromide-stained 1% agarose gel. The strains which did not give any positive result in the PCRs, were subjected to sequencing of EF-1α gene. This enabled the unequivocal identification of all doubtful strains as it provided the exact information about one of the most frequently used *Fusarium* barcode sequence. A standard PCR reaction was used to amplify the EF-1α gene of 62 samples whose identification based on species-specific PCR was unsuccessful or gave uncertain results, for instance lack of band, very weak/not sharp band or multiple weak bands. The primer pair EF1 and EF2 (Tab. 1) was used in PCR reactions performed as given earlier using the temperature profile described by Amatulli et al. (2010). The PCR products were visualized by 1×TBE electrophoresis in ethidium-bromide-stained, 1% agarose gel and then purified using Clean Up purification kit (A&A Biotechnology, Poland), according to the manufacturer's instructions and sequenced in 3500 series sequencer (Applied Biosystems, USA). Sequencing data were edited with Chromas (Technelysium, Australia) and used as a query against National Centre for Biotechnology Information (NCBI) using BLAST network services and a Fusarium-ID database (http:// isolate.fusariumdb.org/).

The potential of *Fusarium* spp. isolates to produce fumonisin and trichothecenes was determined by the PCR-based molecular analyses using the Tri7F and Tri7DONR, Tri13NIVF and Tri13R, and FUM1F and FUM1R specific primer pairs (Tab. 1) which target the mycotoxin-synthesis pathway genes: *Tri7DON* (deoxynivalenol), *Tri13NIV* (nivalenol) and *fum1* (fumonisin), respectively. The PCRs were performed after completed species identification for all isolates belonging to the species that could be the potential producers of given mycotoxins. The reaction mixtures of a total volume of 25 µl contained 10×PCR buffer, 2 mM of $MgCl_2$, 0.4 µM of each primer, 0.2 mM of dNTPs, 0.75 U of Taq DNA Polymerase (Thermo Scientific - Fermentas, Canada) and approximately 50 ng of fungal template DNA. Sterile deionized water served as negative control. PCR amplification was carried out in the T100 Thermal Cycler (BioRad, US) according to temperature profiles described by Lenc et al. (2008) and Yazeed et al. (2011). The PCR products were visualized by 1×TBE electrophoresis in ethidium-bromide-stained, 1% agarose gel.

Results

In total 107 strains of *Fusarium* spp. were isolated during two years of studies. The frequency of isolation of *Fusarium* spp. from winter wheat ears was found at the level of 27.63% in 2012 and 42.76% in 2013. The two-stage identification with the use of species-specific PCR and EF-1α region sequencing allowed to determine the systematic position of all isolates. The obtained nucleotide sequences were submitted in the GenBank database with the following accession numbers: KJ947314 – KJ947343; KM025393 – KM025423 and KM052630 – KM052646. There were significant differences in the species composition and the prevalence of strains between the two seasons of the study, as presented in Tab. 2.

Based on the results of species-specific PCR and EF-1α region sequencing it can be stated that *F. graminearum* (Fig. 1) was the predominant species in 2012, while in 2013 *F. poae* was the predominant one.

The prevalence of *Tri7DON*, *Tri13NIV* and *fum1* genes also varied over the analyzed period (Tab. 2). 32 out of 107 identified *Fusarium* isolates were capable of the production of the tested mycotoxins. In 2012, 20 out of 40 isolates (50%) possessed the examined genes, while in 2013 the 381-445 bp-long band was observed in 12 out of 64 isolates (18.75%), which evidenced the presence of *Tri7DON* only.

Discussion

The conducted studies included 107 strains from the genus *Fusarium* isolated from the ears of winter wheat growing in southern Poland. The systematic position of all species was detected using species-specific PCR and sequencing of EF-1α region. Additionally, the

Tab. 1: List of primer sequences, expected DNA fragment length and sources of primers.

Primer	5' – 3' sequence	Product length (bp)	Target sequence	Reference
Fspo-R	CAGCGCACCCCTCAGAGC	~ 400	*F. poae*	Jurado et al., 2005
Fsp-F	CGCACGTATAGATGGACAAG		*F. poae*, *F. sporotrichioides*	
Fsp-R	GTCAGAAGAGACGCATCCGCC	~ 400	*F. sporotrichioides*	Jurado et al., 2005
Fgr-F	GTTGATGGGTAAAAGTGTG	~ 500	*F. graminearum*	Jurado et al., 2005
Fgr-R	CTCTCATATACCCTCCG			
VER1	CTTCCTGCGATGTTTCTCC	578	*F. verticillioides*	Mulè et al., 2004b
VER2	AATTGGCCATTGGTATTATATATCTA			
FOF1	ACATACCACTTGTTGCCTCG	~ 340	*F. oxysporum*	Mishra et al., 2003
FOR1	CGCCAATCAATTTGAGGAACG			
C51F	ATGGTGAACTCGTCGTGGC	~ 570	*F. culmorum*	Nicholson et al., 1998
C51R	CCCTTCTTACGCCAATCTCG			
EF1	ATGGGTAAGGA(A/G)GACAAGAC	~ 700	EF-1 α gene	O'Donnell et al., 1998
EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT			
FUM1 F	CCATCACAGTGGGACACAGT	183	fum1 gene	Bluhm et al., 2004
FUM1 R	CGTATCGTCAGCATGATGTA			
Tri7F	TGCGTGGCAATATCTTCTTCTA	381-445	Tri7DON gene	Chandler et al., 2003
Tri7DONR	GTGCTAATATTGTGCTAATATTGTGC			
Tri13NIVF	CCAAATCCGAAAACCGCA	312	Tri13NIV gene	Chandler et al., 2003
Tri13R	TTGAAAGCTCCAATGTCGTG			

Tab. 2: Species composition, prevalence of each *Fusarium* species isolated from ears of winter wheat and the occurrence of the examined mycotoxin production genes

No.	Year 2012					Year 2013		
	Species	Number of isolates	*Tri7DON*	*Tri13NIV*	*fum1*	Species	Number of isolates	*Tri7DON*
1.	*F. poae*	1	-	-	-	*F. poae*	32	4
2.	*F. graminearum*	27	16	-	-	*F. graminearum*	19	8
3.	*F. sporotrichioides*	3	-	-	-	*F. sporotrichioides*	8	-
4.	*F. avenaceum*	2	-	-	-	-	-	-
5.	-	-	-	-	-	*F. equiseti*	2	-
6.	*F. oxysporum*	2	-	-	-	*F. oxysporum*	2	-
7.	-	-	-	-	-	*F. tricinctum*	1	-
8.	*F. verticillioides*	3	-	-	3	-	-	-
9.	*F. culmorum*	3	-	1	-	*F. culmorum*	1	-
10.	*F. cerealis*	1	-	-	-	-	-	-
	Total	42	16	1	3	Total	65	12
Total isolates 107								

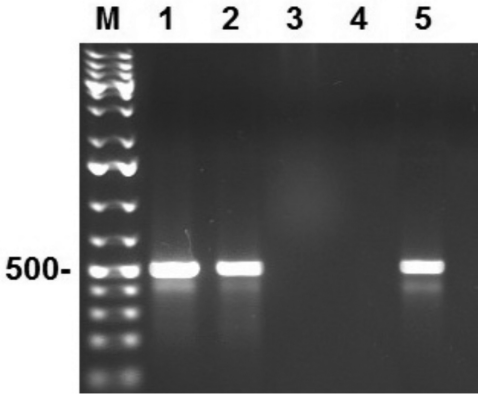

Fig. 1: PCR detection of *Fusarium graminearum* using species-specific primer set: Fgr-F/Fgr-R; M – DNA size marker GeneRuler DNA Ladder Mix (ThermoScientific, Canada); 1, 2, 5 – positively identified *F. graminearum* isolates; 3, 4 – negatively identified *F. graminearum* isolates.

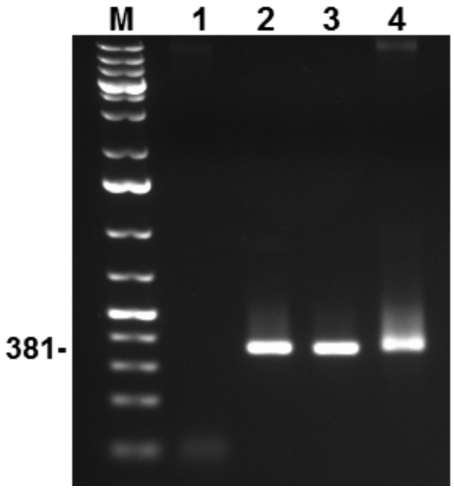

Fig. 2: PCR detection of DON production potential indicated by the presence of Tri7DON marker Tri7F/Tri7DONR. M – DNA size marker GeneRuler DNA Ladder Mix (ThermoScientific, Canada); 1 – negatively identified presence of Tri7DON marker; 2, 3, 4 – positively identified presence of Tri7DON marker.

conducted analysis allowed to determine which of the collected isolates possessed the biosynthesis pathway genes included in the production of deoxynivalenol, nivalenol and fumonisins.

In 2013 the percentage of fungal isolations from wheat ears was greater than in 2012. As shown by other authors, the rate of winter wheat infection is variable and depends on many factors, which were not analyzed in this study, including meteorological conditions, fertilization type, forecrop and the applied plant protection agents (Narkiewicz-Jodko et al., 2005). Jaczewska-Kalicka (2002) in the studies conducted over several years, found significant differences in the incidence of *Fusarium* root rot in wheat. In 1999 this disease was detected in 34% of the examined plants, in 2000 – 27% and in 2001 – 54%. In the studies by Narkiewicz-Jodko et al. (2005) fungi from the genus *Fusarium* were isolated from winter wheat ears with a frequency from 2% to 37%.

In addition to the changes in the frequency of isolation of *Fusarium* spp., the differences in the species composition of the isolated strains were also noted. In 2012 the frequency of isolation of *F. poae* was 2.4%, whereas in 2013 it was already 49.2%, while the frequency of isolation of *F. graminearum* decreased from 64.3% to 29.2% and the remaining species were rarely detected. Ten species were identified in the conducted study: *F. poae, F. graminearum, F. sporotrichioides, F. avenaceum, F. equiseti, F. oxysporum, F. tricinctum, F. verticillioides*, *F. culmorum* and *F. cerealis*. Research carried out in Lithuania confirm the frequent prevalence of these particular species in cereal grains in central-eastern part of Europe (Mačkinaitė et al., 2006). In the study conducted in 2000-2002 by Narkiewicz-Jodko et al. (2005), two species: *F. culmorum* (14-36%) and *F. poae* (3-37%) were identified to be the most prevalent. The share of other species was much smaller: *F. avenaceum* (2-19%) and *F. solani* (1-4%). The studies conducted in northern Europe and Asia indicate the presence of geographical differences in the occurrence of different *Fusarium* species (Yli-Mattila, 2010). It was found that in northern Europe (Scandinavia, Finland and north-western Russia) the predominant species that contaminate cereals include: *F. avenaceum*, *F. arthrosporioides*, *F. tricinctum*, *F. poae*, *F. culmorum*, *F. graminearum*, *F. sporotrichioides* and *F. langsethiae* (Levitin, 2001; Kosiak et al., 2003; Yli-Mattila et al., 2004; Stępień et al., 2008; Nicolaisen et al., 2009). Less frequently occurring species

include: *F. equiseti*, *F. torulosum* and *F. oxysporum* (KOSIAK et al., 2003). In western Siberia *F. avenaceum*, *F. sporotrichioides* and *F. poae* were the most frequently isolated species, while in eastern Siberia *F. acuminatum* and *F. avenaceum* were the predominant ones. Cereals grown in eastern Russia were most frequently infested by two species: *F. graminearum* and *F. poae* (LEVITIN, 2001, 2004). Common prevalence of *F. graminearum* in southern Poland is confirmed by other authors, who indicate that this species is the predominant one in this part of Europe (YLI-MATTILA, 2010). Rapid spread of *F. graminearum* both within the entire European continent and outside Europe has been observed in recent years (NICHOLSON et al., 2003; WAALWIJK et al., 2003; FREDLUND et al., 2008; YLI-MATTILA et al., 2009; YLI-MATTILA and GAGKAEVA, 2010).

The presence or absence of *Tri7DON*, *Tri13NIV* and *fum1* which are the biosynthesis pathway genes of the selected mycotoxins produced by the genus *Fusarium* was verified based on PCR reactions. It was estimated that the *Tri7DON* gene, which participates in the biosynthesis pathway of deoxynivalenol in *Fusarium* spp. was the most frequently detected. Within two years of the study the presence of *Tri7DON* was found in 24 strains of the species *F. graminearum* and in 4 strains of *F. poae*. Three strains of the species *F. verticillioides* possessed the *fum1* gene and 1 strain of *F. culmorum* possessed the *Tri13NIV* gene. *F. graminearum* can produce numerous mycotoxins, including deoxynivalenol, nivalenol and zearalenone (MARASAS et al., 1984), but no moniliformin-producing strains have been found so far (FARBER et al., 1988). In our study, the greatest number of mycotoxin biosynthesis genes were detected within the group of *F. graminearum* strains. *F. poae* is the producer of fusarin X, deoxynivalenol, nivalenol, T-2 toxin, diacetoxyscispenol, monoacetoxyscirpenol, beauvericin and enniatin A, B, B_1 (CHEŁKOWSKI, 1985; EWARDS, 2002), nevertheless it is considered as little toxic or not producing toxins at all. This has been confirmed by the results of our research, as within numerous *F. poae* isolates the presence of DON gene was identified only in 4 cases. The presence of *fum1* gene was found in three (100%) strains of *F. verticillioides*, which can produce significant amounts of fumonisins (GELDERBLOM et al., 1988). It appears paradoxical that the literature data indicate that *F. verticillioides* is not capable of trichothecene production (MARASAS et al., 1984; MIROCHA et al., 1990), although at the same time it possesses genes encoding functional enzymes in the biosynthetic pathway of trichothecenes (KIMURA et al., 2003). *F. culmorum* was the last species, in which the examined genes were found. Within the collected isolates there was one that possessed the *Tri13NIV*, which is one of the nivalenol biosynthesis pathway gene. Apart from nivalenol (CHEŁKOWSKI, 1985), *F. culmorum* can produce moniliformin (SCOTT et al., 1987), deoxynivalenol (MARASAS et al., 1984), fusarin C (FARBER and SANDERS, 1986), zearalenone (MARASAS et al., 1984) and many other toxic compounds (LESLIE and SUMMERELL, 2006). The multitude of mycotoxins produced by *F. culmorum* is an important virulence factor of this species, affecting the spread of *Fusarium* head blight (FHB) (SNIJDERS and PERKOWSJI, 1990; MIEDANER and REINBRECHT, 2001). According to MUTHOMI et al. (2000) the species of *Fusarium* fungi produce different, characteristic sets of multiple mycotoxins. The multitude of factors affecting the rate of mycotoxin synthesis is shown by studies of many other authors (HAIDUKOWSKI et al., 2005; SCHAAFSMA et al., 2005; MARTYNIUK et al., 2010; BATURO-CIEŚNIEWSKA et al., 2011), who indicate the importance of both the plant variety, cultivation site, applied agricultural treatments and the crop rotation.

It still needs to be remembered that the examination of the *Tri7DON*, *Tri13NIV* and *fum1* gene presence reveals only the potential toxicity of *Fusarium* spp., as this is not directly correlated with the actual production of those mycotoxins, because under specific conditions the mycotoxin production genes may not be expressed.

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