

Article

Trichothecene Genotypes Analysis of *Fusarium* Isolates from di-, tetra- and Hexaploid Wheat

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Abstract: New sources of resistance to fungal diseases, including FHB (*Fusarium* head blight), need to be identified. The results of research investigating ancient wheat species with desirable traits appear promising. The aim of this study was to determine the presence of *Fusarium culmorum* (W. G. Sm.) Sacc., *F. graminearum* Schwabe, *F. poae* (Peck) Wollenw, *F. avenaceum* (Fr.) Sacc. and *F. langsethiae* Torp & Nirenberg in the grain and glumes of diploid *Triticum monococcum* ssp. *monococcum*, tetraploid *T. turgidum* ssp. *dicoccum*, *T. turgidum* ssp. *polonicum* and *T. turgidum* ssp. *durum*, and hexaploid *T. aestivum* ssp. *spelta* and *T. aestivum* ssp. *aestivum* grown in north-eastern and south-eastern Poland and to analyze the trichothecene genotypes of the isolated strains. The results of this study also point to shifts in the genotype of Polish *F. culmorum* and *F. graminearum* populations from 3-ADON to NIV and 15-ADON to 3-ADON genotypes, respectively. Our findings indicate that selected einkorn lines could potentially be used as sources of genetic material for breeding new varieties resistant to FHB. The fungal genotypes should be closely screened in Poland and the neighboring countries to assess the effects of potential genotypes profile change on fungal virulence, toxin loading and host specificity.

Keywords: *Fusarium*; nivalenol; deoxynivalenol; genotype; wheat

1. Introduction

Fusarium culmorum (W. G. Sm.) Sacc. and *F. graminearum* Schwabe are the dominant pathogens of wheat spikes [1]. In wheat, the infections caused by the above species persist for a relatively long time from flowering to grain formation and maturation [2]. In addition to *F. culmorum* and *F. graminearum*, *Fusarium* head blight (FHB) is also caused by *F. avenaceum* (Fr.) Sacc., *F. poae* (Peck) Wollenw and *F. langsethiae* Torp & Nirenberg [3]. The disease compromises the quantity and quality of wheat yields by contaminating grain with mycotoxins, mainly trichothecenes, which pose a serious threat to human and animal health [4]. Trichothecenes are small molecules that can passively cross cell membranes. Ingested trichothecenes are easily absorbed by integumentary and gastrointestinal systems, and they directly affect rapidly proliferating tissues [5]. Trichothecenes are synthesized in a biochemical pathway from the trichodiene precursor [6]. The cluster of *Tri* genes is composed of 12 genes that are responsible for the synthesis of core trichothecene molecules, including *Tri5* (which encodes the enzyme trichodiene synthase that catalyzes the cyclization and isomerization of furanyl phosphate to trichodiene), *Tri4*, *Tri11* and *Tri13* (which encode P450 cytochrome monooxygenase), *Tri3*, *Tri7* and *Tri8* (which encode specific enzymes that add or remove ester groups), *Tri6* and *Tri10* (regulatory genes which encode the regulators of other *Tri* genes) and *Tri12* (which encodes a 14-membrane spanning domain major facilitator superfamily (MFS) transporter associated with trichothecene synthesis and resistance). The functions of genes *Tri9* and *Tri14* have not been elucidated to date [7]. Disruption of *Tri5*, *Tri6* and

Tri10 genes results in the complete elimination of trichothecenes [8]. The trichothecenes produced by fungi of the genus *Fusarium* have been classified into types A and B. All trichothecenes have a tricyclic 12,13-epoxytrichothec-9-ene (EPT) core structure [6] but they differ in substitution at position C-8. Type B trichothecenes have a keto (carbonyl) functional group at C-8 (nivalenol, deoxynivalenol, and trichothecin) [5].

The pathogenesis of FHB differs depending on the type of produced toxin. In wheat infected with isolates of *F. graminearum* and *F. culmorum* with the DON (deoxynivalenol) genotype, the disease spreads throughout the entire spike, whereas infections caused by NIV (nivalenol) genotype isolates are significantly less severe [9]. *Fusarium* species such as *F. poae* and *F. langsethiae* are unable to spread from the point of infection to the adjacent spikelets [10]. However, pathogens with a lower potential for producing mycotoxins, such as *F. poae* and *F. langsethiae*, co-infect plant tissues with the predominant *F. graminearum* and *F. culmorum*, and contribute to the contamination of grain with toxins [3].

Trichothecene chemotype diversity is biogeographically structured and correlated with species diversity [11]. Covarelli et al. [12] proved that climatic changes have a strong impact on the occurrence of the 3-ADON and 15-ADON genotypes. The same study indicates that the NIV genotype occurred regardless of weather conditions. On the other hand, Beyer et al. [13] analyzed the genotype shift of *F. graminearum* from 15-ADON to NIV during drought conditions. The hypothesis stated that drought might induce genotype change. At the same time, the percentage of *F. culmorum* strains with 3-ADON genotype was higher in comparison with previous years suggesting that dry weather was more inhibitive for *F. graminearum*. The drought favored *F. culmorum* of the NIV genotype but at the same time, those conditions suppressed FHB development on wheat heads. This could be an explanation of DON production dominance in European growing regions [13] by *F. graminearum* which is favored over *F. culmorum* in humid conditions [14]. However, there is also reported co-occurrence of NIV and DON producers, for example in Brazil [15], which suggests that genotype shifts might be conditioned by different responses of *Fusarium* strains to drought. In Dong's [16] study, another environmental factor, the temperature, has been investigated. The results indicated that temperature may not be a critical factor in the distribution of *Fusarium* species and genotype composition.

The growing interest in healthy foods has contributed to the revival of ancient kinds of wheat. There is a general scarcity of research into *Fusarium* isolates from the grain of diploid einkorn (*Triticum monococcum* L. ssp. *monococcum*), tetraploid emmer (*T. turgidum* ssp. *dicoccum* (Schrank ex Schübl.) Thell.), tetraploid Polish wheat (*T. turgidum* L. ssp. *polonicum* (L.) Thell.) and hexaploid spelt (*T. aestivum* (L.) ssp. *spelta* Thell.). So far, researchers generally focused on fungal colonization of the most popular kinds of wheat: Tetraploid durum wheat (*T. turgidum* (L.) ssp. *durum* (Desf.) Husn.) and hexaploid bread wheat (*T. aestivum* (L.) ssp. *aestivum*) [17,18].

The aim of this study was to: (1) determine the presence of *F. culmorum*, *F. graminearum*, *F. poae*, *F. avenaceum* and *F. langsethiae* in the grain and glumes of diploid *Triticum monococcum* ssp. *monococcum*, tetraploid *T. turgidum* ssp. *dicoccum*, *T. turgidum* ssp. *polonicum* and *T. turgidum* ssp. *durum*, and hexaploid *T. aestivum* ssp. *spelta* and *T. aestivum* ssp. *aestivum* grown in north-eastern and eastern Poland; (2) analyze the trichothecene genotypes of the isolated strains.

2. Materials and Methods

2.1. Plant Material

Grain and glumes were obtained from the spikes of six spring and winter wheat species with low and moderate susceptibility to *Fusarium* spp. infections. The cultivar/line specific response to FHB disease was evaluated in 2014–2015 (see Table S1). The severity of disease was evaluated under field conditions. The FHB symptoms were determined in two growth stages: Milk maturity (BBCH 75) and hard maturity (BBCH 89). A minimum of 100 plants selected randomly from each line/cultivar were analyzed. The severity of infection was evaluated by calculating the average percentage of infected heads based on the scale developed by the European and Mediterranean Plant Protection

Organization [19]. The disease symptoms of lines classified as susceptible appeared in early stages of development, symptoms of the disease were severe, pathogenic changes were observed on more than 30% of head surface area. The resistant lines were characterized by symptoms appearance in later stages of development, symptoms of the disease were not severe, pathogenic changes were observed on less than 30% of head surface area. In the first year of the experiment, 643 lines of the genus *Triticum* were sown, and in the second year 222 lines with high yield potential, including five lines of *T. monococcum* ssp. *monococcum*, 117 lines of *T. turgidum* ssp. *dicoccum*, 51 lines of *T. turgidum* ssp. *polonicum*, 48 lines of *T. turgidum* ssp. *spelta* and one *T. aestivum* ssp. *aestivum* line. Grain and glume colonization by fungi of the genus *Fusarium* was examined in plants characterized by high productive potential and resistance to infections. Spring wheats were sown in Bałcyny in north-eastern Poland (53°36' N, 19°51' E). The field experiment was carried out in a randomized complete blocks design (RCBD) with two replications. The area of a single plot was 6 m² and the grains/spikelets were sown manually in spacing 10 cm × 20 cm. A plot experiment was established on medium-heavy silty pseudopodsolic soil developed from light loam of quality class IIIa, suitable for the production of wheat (good wheat complex, class 2 according to the Polish soil classification system). The preceding crop was a mixture of cereals and legumes. The seeds of winter durum wheat (*T. turgidum* ssp. *durum*) cvs. *Duroflavus* and *Duromax* were obtained from a commercial farm in south-east Poland (Lublin). The seeds of winter wheat cv. *Komnata* and spring wheat cv. *Torka* and *Zebra* were obtained from experimental plots in Bałcyny [20], and the seeds of winter bread wheat cv. *Bogatka* was obtained from experimental plots in Tomaszkowo [21].

2.2. Isolation and Morphological Identification of *Fusarium* Species

Wheat spikes were manually threshed to separate glumes from grain. The prevalence of *Fusarium* spp. in the grain of winter and spring wheat was determined by plating non-surface-disinfected glumes and kernels and kernels surface-disinfected in 1% NaOCl on potato dextrose agar (PDA, Merck, Poland) in Petri dishes. A total of 52 grain and glume samples were plated. The four technical replications were conducted. The number of *Fusarium* spp. colonies on grain and glumes were counted after seven days of incubation at 24 °C (En 120 Incubator, Warsaw, Poland). Filaments characteristic of *Fusarium* fungi were passaged onto small nutrient agar (SNA) and identified based on the morphological traits of colonies and spores. The isolates were initially identified to species level under a microscope (Nikon Eclipse, Tokyo, Japan) at 400× magnification. The morphological characteristics of *Fusarium* spp. isolates were determined according to Leslie and Sammerell [22]. A total of 305 isolates identified as *F. culmorum*, *F. graminearum* *F. poae*, *F. langsethiae*, *F. avenaceum* were obtained. The vast majority of these isolates belonged to *F. culmorum* and *F. graminearum* species. Molecular analyses were performed on 18 isolates of *F. culmorum*, 13 isolates of *F. graminearum*, 4 isolates of *F. poae*, 3 isolates of *F. langsethiae* and 1 isolate of *F. avenaceum* (Table 1). One technical replicate of PCR was conducted. The percentage structure of molecularly analyzed *Fusarium* isolates reflects the structure of 305 microscopically identified isolates.

Table 1. List of *Fusarium* spp. isolates.

Code of <i>Fusarium</i> Isolates	Species	Collection	Sequence Alignment Analysis	Reference Isolate (NCBI)
Fg35	<i>Fusarium graminearum</i>	UWM	+	KX878931.1 <i>Fusarium graminearum</i>
Fg36	<i>Fusarium graminearum</i>	UWM	N/A	N/A
Fg37	<i>Fusarium graminearum</i>	UWM	+	MF800906.1 <i>Fusarium graminearum</i>
Fg49	<i>Fusarium graminearum</i>	UWM	+	MF800906.1 <i>Fusarium graminearum</i>
Fg71	<i>Fusarium graminearum</i>	UWM	N/A	N/A
Fg74	<i>Fusarium graminearum</i>	UWM	+	KX421420.1 <i>Fusarium graminearum</i>
Fg106	<i>Fusarium graminearum</i>	UWM	N/A	N/A
Fg107	<i>Fusarium graminearum</i>	UWM	+	KU377276.1 <i>Fusarium graminearum</i>
Fg182	<i>Fusarium graminearum</i>	UWM	N/A	N/A

Table 1. Cont.

Code of <i>Fusarium</i> Isolates	Species	Collection	Sequence Alignment Analysis	Reference Isolate (NCBI)
Fg184	<i>Fusarium graminearum</i>	UWM	N/A	N/A
Fg249	<i>Fusarium graminearum</i>	UWM	+	KX421420.1 <i>Fusarium graminearum</i>
Fg19	<i>Fusarium graminearum</i>	UWM	N/A	N/A
Fg39	<i>Fusarium graminearum</i>	UWM	N/A	N/A
Fc321	<i>Fusarium culmorum</i>	UWM	+	KP292806.1 <i>Fusarium culmorum</i>
Fc329	<i>Fusarium culmorum</i>	UWM	+	AY147341.1 <i>Fusarium culmorum</i>
Fc331	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc333	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc335	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc10	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc20	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc21	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc22	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc31	<i>Fusarium culmorum</i>	UWM	+	KX349468.1 <i>Fusarium culmorum</i>
Fc32	<i>Fusarium culmorum</i>	UWM	+	KT318585.1 <i>Fusarium culmorum</i>
Fc56	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc58	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc59	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc60	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fc61	<i>Fusarium culmorum</i>	UWM	+	MF372583.1 <i>Fusarium culmorum</i>
Fc62	<i>Fusarium culmorum</i>	UWM	+	KT992460.1 <i>Fusarium culmorum</i>
Fc64	<i>Fusarium culmorum</i>	UWM	N/A	N/A
Fp21	<i>Fusarium poae</i>	UWM	+	KP271956.1 <i>Fusarium poae</i>
Fp22	<i>Fusarium poae</i>	UWM	+	GU480965.1 <i>Fusarium poae</i>
Fp48	<i>Fusarium poae</i>	UWM	+	AF414967.1 <i>Fusarium poae</i>
Fp206	<i>Fusarium poae</i>	UWM	+	KF889085.1 <i>Fusarium poae</i>
Fl53	<i>Fusarium langsethiae</i>	UWM	+	AB587023.1 <i>Fusarium langsethiae</i>
Fl87	<i>Fusarium langsethiae</i>	UWM	+	NR.121214.1 <i>Fusarium langsethiae</i>
Fl130	<i>Fusarium langsethiae</i>	UWM	+	AF414969.1 <i>Fusarium langsethiae</i>
FaFa9	<i>Fusarium avenaceum</i>	UWM	+	KT362194.1 <i>Fusarium avenaceum</i>
Feq3	<i>Fusarium equiseti</i>	UWM	+	MF166765.1 <i>Fusarium equiseti</i>
Feq25	<i>Fusarium equiseti</i>	UWM	+	KR094440.1 <i>Fusarium equiseti</i>
Feq36	<i>Fusarium equiseti</i>	UWM	+	KU680356.1 <i>Fusarium equiseti</i>
Feq37	<i>Fusarium equiseti</i>	UWM	+	KU680356.1 <i>Fusarium equiseti</i>
Fd13	<i>Fusarium equiseti</i>	UWM	+	KX270351.1 <i>Fusarium dimerum</i>
Fd15	<i>Fusarium equiseti</i>	UWM	+	KX270351.1 <i>Fusarium dimerum</i>

Designations: UWM—University of Warmia and Mazury in Olsztyn, N/A—not applicable.

2.3. Isolation and Amplification of Fungal DNA

Fungal filaments were cultured on PDA (Merck, Warsaw, Poland) for 48 h, then they were transferred to a liquid medium (0.1% beef extract, *w/v*, 0.5% soy peptone, *w/v*, 0.5% sodium chloride, *w/v*, 1% glucose, *w/v*, 0.7% yeast extract, *w/v*, distilled water) in 50 cm³ flasks. DNA was isolated from the filaments of fungi cultured on a liquid medium with the use of the Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Gdansk, Poland) according to the manufacturer's procedure. The quantity and quality of the isolated DNA were determined by measuring absorbance at a wavelength of 260 nm and 280 nm (NanoMaester Gen, Warsaw, Poland). The isolated DNA was amplified by PCR with primers specific for *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. poae*, *F. langsethiae* and *F. equiseti* (Table 2). A total of two biological and two technical replicates were conducted. The amplified products were electrophoresed on 1.2% agarose gel (Prona, Warsaw, Poland) with ethidium bromide (Merck Millipore, Warsaw, Poland) in TBE buffer (Sigma Aldrich, Warsaw, Poland). The products separated on agarose gel were visualized with a transilluminator (UVP, Poznan, Poland). The species identity of 24 isolates of *Fusarium* fungi was determined by PCR with conserved non-coding sequences ITS4 and

ITS5 primers (Table 2). The reaction mixture contained 20 ng of previously isolated DNA. PCR was conducted according to the protocol described by White et al., [23]. The electrophoresis of ITS products was conducted on 1% agarose gel (Prona, Warsaw, Poland) with ethidium bromide (Merck Millipore, Warsaw, Poland) in TBE buffer (Sigma Aldrich, Warsaw, Poland). The separated products were visualized with a transilluminator (UVP, Poznan, Poland). Amplification products were sequenced by Genomed SA in Warsaw. Partial sequences of the small subunit 18S rDNA gene in the 5.8S rDNA region, large subunit 28S rDNA gene and ITS were obtained. Species identification based on sequence similarities was carried out with the BLAST tool in the NCBI database. After sequencing, ITS sequences were added into the test sequence window at the online BLAST program, which provided results in the form of best matches with the available sequences in GeneBank. A homology search was performed within the non-redundant databases of GeneBank using the BLAST algorithm at NCBI.

Table 2. Primer sequences and PCR conditions for species identification based on ITS sequences and the determination of trichothecene genotypes.

Species/Target Gene	Primer	Sequence (5'–3')	Product Size (bp)	Reference	PCR Reaction Condition
<i>F. avenaceum</i>	JIAF (F)	GCTAATTCTTAACCTACTAGGGGCC	220	[24]	94 °C 2 min; [94 °C 30 s, 58 °C 30 s, 72 °C 2 min] × 40; 72 °C 5 min 94 °C 5 min; [94 °C 20 s, 66 °C 1 min, 72 °C 45 s] × 5, [94 °C 20 s, 64 °C 1 min, 72 °C 45 s] × 5, [94 °C 20 s, 62 °C 1 min, 72 °C 45 s] × 25; 72 °C 5 min 94 °C 2 min.; [95 °C 35 s, 66 °C 30 s, 72 °C 30 s] × 35; 72 °C 5 min 94 °C 5 min; [94 °C 20 s, 66 °C 1 min, 72 °C 45 s] × 5, [94 °C 20 s, 64 °C 1 min, 72 °C 45 s] × 5, [94 °C 20 s, 62 °C 1 min, 72 °C 45 s] × 25; 72 °C 5 min 94 °C 2 min; [95 °C 35 s, 61 °C 30 s, 72 °C 30 s] × 35; 72 °C 5 min 94 °C 2 min.; [95 °C 35 s, 61 °C 30 s, 72 °C 30 s] × 35; 72 °C 5 min 98 °C 2 s; [95 °C 10 s, 56 °C 5 s, 72 °C 40 s] × 5, [95 °C 10 s, 54 °C 10 s, 72 °C 40 s] × 5, [95 °C 10 s, 52 °C 20 s, 72 °C 40 s] × 25; 72 °C 5 min 98 °C 2 s; [95 °C 10 s, 56 °C 20 s, 72 °C 40 s] × 30, 72 °C 5 min 94 °C 4 min; [94 °C 40 s, 57 °C 30 s, 72 °C 10 min] × 35, 72 °C 10 min
	JIAR (R)	CTGTAATAGGTTATTACATGGGCG			
<i>F. culmorum</i>	Fc01F (F)	ATGGTGAACCTCGTCGTGGC	570	[25]	
	Fc01R (R)	CCCTTCTTACGCCAATCTCG			
<i>F. equiseti</i>	FeqF (F)	GGCCTGCCCGATGCGTC	990	[26]	
	FeqR (R)	CGATACTGAAACCGACCTC			
<i>F. graminearum</i>	Fg16NF (F)	ACAGATGACAAGATTCAAGGCACA	280	[25]	
	Fg16NR (R)	TTCTTTGACATCTGTCAACCCA			
<i>F. langsethiae</i>	FlangF (F)	CAAAGTTCAGGGCGAAAACT	320	[27]	
	LansporR (R)	TACAAGAAGAGCGTGGCGATAT			
<i>F. poae</i>	FpsF (F)	CGCACGTATAGATGGACAAG	400	[26]	
	FpoR (R)	CAGCGCACCCCTCAGAGC			
TRI5	TRI5 (F)	AGCGACTACAGGCTTCCCTC	544	[28]	
	TRI5 (R)	AAACCATCCAGTTCCTCATCTG			
TRI12 (NIV)	12NF(F)	TCTCCTCGTTGTATCTGG	840		
TRI12 (15-ADON)	12-15F (F)	TACAGCGGTCGCAACTTC	670	[29]	
TRI12 (3-ADON)	12-3F (F)	CTTTGGCAAGCCCGTGCA	410		
TRI12	12CON (R)	CATGAGCATGGTGATGTC			
ITS	ITS5 (F)	GTATCGGACGGAGATCCAGC	550	[30]	

2.4. Determination of Trichothecene Genotypes

The strains identified as *F. culmorum* (18 isolates), *F. graminearum* (13 isolates) and *F. poae* (4 isolates) were screened for their potential ability to produce trichothecenes by detecting the amplification of the *Tri5* gene marker with a specific primer pair (Table 2). *F. culmorum* and *F. graminearum* isolates with the ability to produce trichothecenes were subjected to multiplex-PCR analysis to determine their genotypes with specific primers that target genes involved in the synthesis of either 3ADON, 15ADON or NIV (Table 2). The PCR for *Tri5* gene amplification and genotype identification was conducted according to a previously described procedure [28], and multiplex-PCR assays were conducted with 0.06 μ L of each 100 μ M forward primer and 0.18 μ L of each 100 μ M reverse primer. The described analyses were conducted in one technical replicate. Electrophoretic separation was carried out under the previously described conditions.

2.5. Sequence Homology

The sequences of the 24 analyzed isolates of *Fusarium* spp. were compared with the reference sequences from the NCBI database (Table 1), and their similarity was expressed in percentage terms. The reference material comprised four isolates of *F. equiseti* (Feq 3, Feq 25, Feq 36 and Feq 37) and two isolates of *F. dimerum* (Fd13 and Fd 15) from the seeds of *Camelina sativa* L. Crantz.

2.6. Statistical Analysis

The results were processed statistically in the Statistica program [31]. Data were subjected to ANOVA, and the significance of differences between means was estimated in Tukey's test ($p < 0.05$) after Bliss transformation.

3. Results

3.1. Colonization of Wheat Glumes and Grain by *Fusarium* spp.

Since adhering spores on wheat kernels surface are able to cause infectious diseases, our aim was to compare non-disinfected and disinfected wheat *Fusarium* mycoflora in the same samples. This allowed us to compare *Fusarium* species occurrence between surface contamination and internal invasion. Fungi of the genus *Fusarium* colonized 45.03% of wheat glumes, 27.16% of non-surface-disinfected wheat kernels and 18.45% of surface-disinfected wheat kernels on average (Table 3). The prevalence of *Fusarium* spp. was highest on *T. turgidum* ssp. *polonicum* glumes (70%). The smallest average number of *Fusarium* colonies was observed on the glumes and grain of durum wheat. In emmer, spelt and Polish wheat, grain was significantly less colonized by *Fusarium* fungi (by 32.19%, 32.07% and 45.30%, respectively) than glumes (Table 3). The number of *Fusarium* colonies was significantly lower (by 47.32%) in surface-disinfected than in non-surface-disinfected kernels. *Fusarium* pathogens were detected in surface-disinfected kernels of three einkorn lines (out of six analyzed lines), 20 emmer lines (out of 21), one durum wheat cultivar (out of three) and three bread wheat cultivars/lines (out of three). In einkorn and bread wheat, the number of fungal colonies differed significantly between glumes and surface-disinfected kernels. *Fusarium* pathogens were not detected only in surface-disinfected kernels of three einkorn lines (out of six analyzed lines), one emmer line (out of 21 analyzed lines), two durum wheat cultivars (out of three analyzed cultivars) and three bread wheat cultivars/lines (out of three analyzed cultivars). In contrast to the other analyzed wheat species, einkorn was characterized by the lowest level of *Fusarium* infection. Additionally, the percentage of einkorn glumes and non-disinfected kernels colonized by *Fusarium* fungi is below the mean for other investigated wheats, except for durum wheat.

Table 3. The origin and percentage of glumes and kernels colonized by *Fusarium* spp.

Species of Wheat	Lines/Cultivar	Glumes	Non-Disinfected Kernels	Disinfected Kernels	Mean	Glumes	Non-disinfected Kernels	Disinfected Kernels
		Colonization by <i>Fusarium</i> Fungi (%)				Number of Lines/Cultivars Where <i>Fusarium</i> Colonies were not Isolated		
<i>T. monococcum</i> ssp. <i>monococcum</i>	Tm222, Tm166, Tm 166, Tm219, Tm220, Tm168 Td64, Td1, Td12, Td116, Td14, Td126, Td20, Td15, Td216,	18.09 ^{efg}	6.11 ^{gh}	3.33 ^h	9.65 ^C	0	1	3
<i>T. turgidum</i> ssp. <i>dicoccum</i>	Td71, Td111, Td214, Td60, Td454, Td57, Td235, Td35, Td3, Td115, Td13, Td48	40.95 ^{bc}	27.77 ^{de}	21.31 ^{ef}	30.04 ^B	0	0	1
<i>T. turgidum</i> ssp. <i>polonicum</i>	Tp160, Tp131, Tp138, Tp157, Tp163, Tp161, Tp162, Tp149, Tp154, Tp121, Tp159	70.00 ^a	39.69 ^{cd}	20.91 ^{ef}	43.54 ^A	0	0	0
<i>T. turgidum</i> ssp. <i>durum</i>	cv. Duromax, Duroflavus, Komnata	13.33 ^{c-h}	7.14 ^{fgh}	2.08 ^h	5.71 ^C	0	1	2
<i>T. aestivum</i> ssp. <i>spelta</i>	Ts173, Ts217, Ts212, Ts208, Ts205, Ts202, Ts211, cv. Wirtas	54.58 ^b	37.08 ^{cd}	32.92 ^{cde}	41.53 ^A	0	0	0
<i>T. aestivum</i> ssp. <i>aestivum</i>	cv. Torka, Zebra, Ta 140	30.00 ^{b-e}	11.11 ^{e-h}	0 ^h	13.40 ^C	0	1	3
Mean		45.03 ^X	27.16 ^Y	18.45 ^Z				

Values in columns that did not differ significantly in Tukey's test ($p \leq 0.05$) are marked with identical letters.

3.2. Identification of *Fusarium* Species

Specific PCR and morphological analyses supported the identification of 39 isolates of *Fusarium* fungi to species level. Analyses were performed on 18 isolates of *F. culmorum*, 13 isolates of *F. graminearum*, four isolates of *F. poae*, three isolates of *F. langsethiae* and one isolate of *F. avenaceum*. In the analyzed group of 39 strains, the predominant fungal species was *F. culmorum* (46.2% of all isolates) which was isolated from the grain of Polish wheat (seven isolates), durum wheat (cv. *Komnata* from Bałcyny, six isolates) and spelt (five isolates). From the grain of bread wheat only two isolates of *F. culmorum* have been isolated. *F. graminearum* accounted for 33.3% of all isolates, mainly from the grain and glumes of Polish wheat (seven isolates) and durum wheat (cvs. *Duromax* and *Duroflavus* from Lublin, four isolates), and from einkorn and emmer glumes (two isolates). Wheat grain grown in south-east Poland was colonized exclusively by *F. graminearum*. Isolates of *F. poae*, *F. langsethiae* and *F. avenaceum* accounted for, respectively, 10.3% (einkorn, emmer and Polish wheat), 7.7% (emmer, Polish wheat and bread wheat) and 2.5% (wheat cv. *Bogatka*) of analyzed isolates.

3.3. Genotype Determination in *Fusarium* spp. Isolates

The strains of *F. culmorum* (18 isolates), *F. graminearum* (13 isolates) and *F. poae* (four isolates) were analyzed for the presence of the *Tri5* gene encoding trichothecene synthesis. All of the *F. graminearum* and *F. culmorum* isolates (Table 4, see Figure S1) had the potential ability to produce trichothecenes. None of the tested *F. poae* isolates were capable of producing trichothecenes.

Table 4. Mycotoxin-production ability of *Fusarium* isolates.

Gene	Product	Number (%) of Isolates Capable of Producing Trichothecenes		
		<i>F. culmorum</i>	<i>F. graminearum</i>	<i>F. poae</i>
<i>Tri5</i>	Enzyme responsible for the synthesis of a specific substance that initiates the mycotoxin biosynthesis pathway	18 (100)	13 (100)	0
<i>Tri12</i>	Nivalenol	10 (55.6)	0	0
	15-Acetyl Deoxynivalenol	0 (0)	7 (53.8)	0
	3-Acetyl Deoxynivalenol	8 (44.4)	6 (46.2)	0

The percentage of *F. culmorum* and *F. graminearum* isolates with the products of *Tri5* and *Tri12* genes is indicated in parentheses. None of the analyzed *F. poae* isolates were capable of producing any of the researched molecules of the mycotoxin biosynthesis pathway.

The genotypes of trichothecene-producing isolates were identified based on the presence of the *Tri12* gene encoding the transporter associated with trichothecene synthesis and resistance (Table 4). *Fusarium* genotypes were identified based on *Tri12* gene polymorphism. *Fusarium* isolates differed in the size of amplification products of the *Tri12* gene, which determined their genotype. Only isolates harboring the *Tri5* gene, which encodes the production of different mycotoxins, were analyzed. In the group of 18 *F. culmorum* isolates, 10 isolates belonged to the NIV genotype (products with the size of 840 bp) and the remaining isolates belonged to the 3-ADON genotype (410 bp). The analyzed isolates of *F. graminearum* with the *Tri5* gene belonged to the 3-ADON genotype (six isolates) and 15-ADON genotype (seven isolates) (see Figure S2).

3.4. Sequence Homology

The sequences of conserved ITS1 and ITS2 regions were used to confirm the species identity of *Fusarium* fungi identified by PCR. The nucleotide sequences of 30 own isolates had 90%–99% affinity with the reference sequences (see Table S2), which confirmed their species identity.

4. Discussion

Recent years have witnessed a revived interest in relict wheat species which are characterized by a unique population habit resulting from cultivation in isolated areas and the absence of selection [32]. The rich gene pool of these species can be reflected in increased resistance to pathogen infections,

especially considering that ancient wheat species are referred to as “the covered wheats” since their kernels do not thresh free of the glumes. The objectives of this study were to evaluate the presence of selected *Fusarium* spp. on six wheat species grain and glumes (einkorn, Polish wheat, emmer, spelt and bread wheat) and analyse if *Fusarium* isolates are characterized by potential ability to produce trichothecenes. Most wheats were grown in north-eastern Poland (Region of Warmia and Mazury). The only exception was durum wheat which was grown in south-eastern Poland.

The visual examination of the grain samples constitutes an efficient tool in the selection of wheat grains with higher levels of *Fusarium* infection often caused by *Fusarium* spp. forming trichothecenes. Thus, trichothecene contamination monitoring of such grain is highly recommended before incorporating wheat grains into the food industry. We have investigated the spread of infection of *Fusarium* fungi between glumes and grain of different kinds of wheat including relict species. In this study, we have observed that grain and glumes of all analyzed lines of spelt and Polish wheat were colonized by *Fusarium* fungi that are able to effectively spread from glumes to grain of these wheats. The percentage of Polish wheat and spelt kernels and glumes colonized by toxin-producing *Fusarium* fungi was worryingly high. A similar threat was observed in Góral et al., [33] research in which the spelt genotype was characterized by the highest amount of DON in grain. Also, Wiśniewska and Kowalczyk [34] reported the susceptibility of Polish wheat to *Fusarium* infection. Polish wheat and spelt are characterized by high genetic variation [35–37]. However, genetic variability can lead to the emergence of both resistant and sensitive lines. The results of this study indicate that the analyzed lines of Polish wheat and spelt were susceptible to infections with *Fusarium* fungi; therefore, they should not be considered as potential donors of genetic material for modern wheats.

The pathogens were not determined in the surface-disinfected grain of three einkorn lines, one emmer line, two durum wheat cultivars, and four bread wheat cultivars/lines. This has shown that *Fusarium* spp. isolates present on wheat grains were not able to penetrate the grain tissues. The results of this study may suggest that several einkorn lines and one emmer line could be potentially used as sources of resistance to *Fusarium* fungi. The results of einkorn glumes and kernels colonization are promising due to the low mean values of *Fusarium* fungi colonization. The einkorn grain hulling could be a favorable factor as a morphological barrier that conditions resistance to infection. However, further molecular and biochemical analyses are required to confirm this hypothesis. Einkorn grain is less contaminated with *Fusarium* pathogens, in particular, mycotoxin-producing species, which could increase the popularity of products made of einkorn flour. According to Lombardo et al., [38], einkorn gluten is less immunoreactive; therefore, this wheat species could be a potential candidate in the production of hypoallergenic bakery products. Interestingly, the smallest average number of *Fusarium* colonies was observed on the glumes and grain of durum wheat. There are many findings concerning the paucity of FHB resistance in durum wheats [39–41]. Thus, a low level of *Fusarium* infections on durum wheat grain and glumes may be an indicator of the potential resistance genes presence which might be useful in durum wheat breeding, however that should be investigated in more detail.

Fungi that cause FHB in wheat grain should be closely monitored to guarantee a high degree of food security. In this study, PCR supported the rapid identification of *Fusarium* species and trichothecene-producing isolates, as well as the classification of fungal genotypes. Similar studies are conducted in other countries [42,43].

In north-eastern Poland, *F. culmorum* was isolated predominantly from wheat grain and glumes. The second most-often isolated species was *F. graminearum*. *F. poae*, *F. langsethiae* and *F. avenaceum* constituted the minority of the isolated species (extended data is not shown; the percentage structure of the 39 *Fusarium* spp. isolates analyzed reflects the diversity of 305 isolates previously microscopically identified). Perry et al., [44] and Wachowska et al., [45] observed a predominance of *F. culmorum* in cool and moderately humid regions. The experimental material for this study was obtained from north-eastern Poland which has a similar climate. In a study by Stepień et al., [46], 41 of 57 *Fusarium* species isolated from wheat grain in southern Poland belonged to *F. graminearum* species, and their

identity was confirmed by specific PCR reactions. The climate of southern Poland is characterized by higher average temperatures and higher humidity, which could explain the predominance of *F. graminearum* fungi in that region. In this study, durum wheat grain grown in Lublin (south-eastern Poland) was also colonized exclusively by *F. graminearum* isolates. According to Xu and Nicholson [10], an increased presence of *F. graminearum* isolates decreases the prevalence of *F. poae*. In this research, *F. graminearum* colonies were isolated three times more often than *F. poae*, which seems to confirm the above observation. The prevalence of *F. graminearum* isolates could also be attributed to variations in temperature and humidity during the study. *F. graminearum* thrives in temperate regions with daytime temperatures of 12–24 °C and nighttime temperatures of 5–12 °C, whereas *F. poae* proliferate more readily in warmer and drier climates. In this study, average summer temperatures reached 20–22 °C, and they were conducive to the growth of *F. graminearum*, but not *F. poae*.

The variability of trichothecene genotypes has been thoroughly investigated in many studies of *Fusarium* populations that cause FHB [10,24,26]. *Tri5*, *Tri7* and *Tri12* are the major genes encoding mycotoxin synthesis [12]. Pinson-Gadais et al., [47] and Yörük and Albayrak [48] detected the *Tri5* gene in all analyzed isolates of *F. culmorum*. However, those isolates were obtained from bread wheat grain. In our study, all analyzed *F. culmorum* isolates harbored the *Tri5* gene encoding trichothecene production. The analyzed isolates of *F. culmorum* were obtained mainly from the grain of Polish wheat, durum wheat and spelt. Only two isolates from our collection were obtained from bread wheat grain. *Fusarium culmorum* isolates with the *Tri5* gene synthesized mostly NIV (55.6% of analyzed isolates) and the acetylated forms of deoxynivalenol—3-ADON (44.4% of isolates). More than a decade ago, Stępień et al., [46] reported that in Poland the 3-ADON genotype was most frequent among *F. culmorum* isolates. Similar results were reported by Jennings et al., [25], Stępień et al., [46], Yörük and Albayrak [48] and Covarelli et al., [12] who found that all or most of the analyzed isolates of *F. culmorum* belonged to the 3-ADON genotype. Waalwijk et al., [30], Jennings et al., [25] and Pasquali et al., [49] reported in their studies that both DON and NIV chemotypes were present among the analyzed isolates of *F. culmorum*. Observed in our study high proportion of the NIV chemotype among *F. culmorum* population is consistent with some results present in a European database of *F. graminearum* and *F. culmorum* trichothecene genotypes [50] and other studies [30]. According to research by Pasquali et al., [50], the dominant NIV genotype among *F. culmorum* is characterized for some specific regions in Europe: South England, North France and North-East Germany. Our results could be indicative of a new wave of changes in the genotype of *F. culmorum* isolates. There are many reports on chemotype shifts in certain areas [51,52]. However, more extended studies should be performed to obtain epidemiological information. Several years ago, Pasquali et al., [49] emphasized that wheat grain should be closely monitored for the presence of NIV and DON to improve global food security standards. Grain contamination with NIV is often overlooked because this toxin frequently occurs in low concentrations in wheat [53]. However, NIV is more toxic than DON, it inhibits DNA synthesis and induces cell death. Nivalenol is also more toxic to human blood cells than DON [54].

In our study, all of the *F. graminearum* isolates from our own collection harbored the *Tri5* gene marker and were DON genotype (six isolates belonged to 3-ADON and seven isolates to 15-ADON genotype). 15-ADON was the predominant genotype in *F. graminearum* strains isolated in different regions, including the Benelux countries [27], Poland [46] and Turkey [48]. According to the results of Pasquali et al., [50], the 15-ADON genotype is rarely observed in Northern Europe and is specific to the West, Central, East and South regions of Europe where it is considered the major cause of DON accumulation in wheat. The database from Pasquali et al., [50] showed that in Eastern Poland and South-East Poland *F. graminearum* strains are mostly characterized by 15-ADON genotype. *F. graminearum* strains in this study were collected from Warmia and Mazury region situated in North-East Poland and Lublin positioned in South-East Poland. The majority of these isolates (53.8%) were the 15-ADON genotype, however the authors have also observed a high proportion of 3-ADON isolates (46.2%) which are usually found in Northern regions of Europe. This data also suggests that a new wave of changes in the genotype across European *F. graminearum* strains may be present.

According to the literature, *F. poae* strains capable of producing type B trichothecenes generally belong to the NIV genotype [52], but type A trichothecene-producers capable of synthesizing HT-2 and T-2 toxins have also been identified [55]. In our study, none of the tested *F. poae* isolates were capable of producing trichothecenes. The isolates from our own collection did not produce type B trichothecenes; it appears that they produced mostly type A trichothecenes, which however requires further investigation.

The possible change of trichothecenes genotypes among *F. culmorum* and *F. graminearum* isolates in Poland require further investigation involving greater number of *Fusarium* isolates. However, our data indicate that previously rarely occurring in Poland genotypes of *F. culmorum* (NIV) and *F. graminearum* (3-ADON) are present in *Fusarium* species populations in high proportion thus there is a need to assess the effects of these genotypes on fungal virulence, toxin loading and host specificity that could impact on future disease management and plant breeding. The results of this study suggest that several einkorn lines and one emmer line could be potentially used as sources of resistance to *Fusarium* fungi. The genetic variability of relict wheat species can contribute to new resistant wheat varieties development.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/9/11/698/s1>, Figure S1: Gel image after electrophoresis. EtBr was added to the gel before electrophoresis to visualize products with the expected size of 544 bp. Line 1: 100–1000 bp marker. all lines- exemplary *F.culmorum* and *F. graminearum* isolates with the *Tri5* gene marker; Figure S2, Gel image after electrophoresis. EtBr was added to the gel before electrophoresis to visualize products with the expected size of 410 bp. Lines 1–6: Exemplary *F. graminearum* isolates with the *Tri12* gene producing 3-ADON. Line 7: 100–1000 bp marker; Table S1: Selected lines and cultivars of the genus *Triticum* with varied susceptibility to FHB; Table S2, Sequence alignment in *Fusarium* spp. isolates from own collection and reference isolates from NCBI.

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