Article

Quantification of DNA of *Fusarium culmorum* and Trichothecene Genotypes 3ADON and NIV in the Grain of Winter Wheat

Tomasz Góral [1,*], Jarosław Przetakiewicz [1], Piotr Ochodzki [1], Barbara Wiewióra [1] and Halina Wiśniewska [2]

[1] Plant Breeding and Acclimatization Institute—National Research Institute, Radzików, 05-870 Błonie, Poland; j. przetakiewicz@ihar.edu.pl (J.P.); p.ochodzki@ihar.edu.pl (P.O.); b.wiewiora@ihar.edu.pl (B.W.)
[2] Institute of Plant Genetics, Polish Academy of Sciences, 34 Strzeszyńska str., 60-479 Poznań, Poland; hwis@igr.poznan.pl
* Correspondence: t.goral@ihar.edu.pl; Tel.: +48-22-733-4636

Abstract: Fusarium head blight (FHB) is a wheat disease caused by fungi of the genus *Fusarium*. The aim of the study was to find relationships between the weather conditions in the experimental years and the locations and the amount of *F. culmorum* DNA and trichothecene genotypes, as well as the proportions between them. A three-year field experiment (2017, 2018 and 2019) was established at two locations (Poznań, Radzików). *F. culmorum* DNA was detected in all grain samples in an average amount of 20124 pg per 1 µg of wheat DNA. The average amount of DNA from the 3ADON genotype was 4879 pg/µg and the amount of DNA from the NIV genotype was 3330 pg/µg. In the three experimental years, a large variability was observed in the coefficients of correlation between DNA concentrations and the FHB index, FDK, ergosterol, and the corresponding toxins. There were significant correlations between disease incidence, fungal biomass (quantified as the total amount of fungal DNA or DNA trichothecene genotypes) and toxins (DON, 3AcDON and NIV) concentrations. The 3ADON trichothecene genotype dominates over the NIV genotype (ratio 1.5); however, this varied greatly depending on environmental conditions.

Keywords: chemotype; 3ADON; DNA; *Fusarium*; Fusarium head blight; NIV; real-time PCR; wheat

1. Introduction

Fusarium head blight (FHB) is a wheat disease (*Triticum aestivum* L.) caused by fungi of the *Fusarium* genus. These fungi infect wheat heads, causing necrosis of spikelets, infection and damage of the kernels, and contamination of tissues and grains with *Fusarium* toxins [1]. Several species associated with FHB were identified, but the main species that infect wheat worldwide are *F. graminearum* Schwabe and *F. culmorum* (W.G. Sm.) Sacc. [2]. These species are highly pathogenic and can cause severe epidemics of FHB. The other species are medium- or weakly pathogenic; however, they can also cause contamination of wheat grains with mycotoxins [3–6]. Phylogenetic studies of the collection of strains of *F. graminearum* revealed that *F. graminearum* is a species complex consisting of at least 16 distinct species [7–10]. The most common species worldwide in the *F. graminearum* species complex (FGSC) is *F. graminearum* sensu stricto. In Europe *F. graminearum s.s.* was isolated almost exclusively [7,11]. Both species (*F. culmorum* and *F. graminearum* s.s.) belong to a large *F. sambucinum* species complex (FSAMSC) [12].

Fusarium species produce many toxins of different chemical groups. As small grain cereal contaminants, the most important are type A and type B trichothecenes (mainly deoxynivalenol = DON, nivalenol = NIV, T-2/HT-2 toxins) and the estrogenic compound zearalenone [13,14]. Within the group of type B trichothecene producing species, three trichothecene genotypes (chemotypes) were identified [15]. The NIV chemotype strains can produce NIV and its acetylated derivative. DON chemotype strains can produce DON and its acetylated derivatives (3-acetyl DON and 15-acetyl DON). Two subchemotypes

 doi:10.20944/preprints202211.0279.v1

are present within the DON chemotype: 3ADON strains produce DON and 3AcDON and 15ADON strains produce DON and 15AcDON. All three chemotypes were detected in *F. graminearum* s.s. [10]. In *F. culmorum,* only 3ADON and NIV strains were identified [16–19].

The NIV chemotype is generally considered less aggressive than the DON (3ADON, 15ADON) chemotypes [20–24]. However, the detailed results do not show such clear differences [21]. The pathogenicity of FGSC isolates was found to not depend on the type of toxin produced (DON versus NIV). Their aggressiveness was influenced mainly by the amount of toxins produced. The author stated that this is a key determining factor of the aggressiveness of isolate on wheat. Similar conclusions can be found in the paper by Qu et al. [25] on the pathogenicity of FGSC isolates. In a paper published by Maier et al. [26] the progress of FHB in wheat heads was slower and symptoms were less severe for the NIV-producing isolate compared to the DON-producing isolates. However, both toxins were crucial for head infection, as isolates (DON and NIV) with disrupted *Tri5* gene did not show spread of symptoms beyond inoculated spikelets. In field conditions Mesterhazy et al. [27] compared the aggressiveness of the NIV isolate with wheat using a set of isolates producing DON of *F. culmorum* and found no differences in the severity of symptoms of FHB and total trichothecenes production. Carter et al. [28] found no differences in the pathogenicity to wheat from DON and NIV producing isolates of FGSC (identified later as *F. asiaticum*). Interestingly, the NIV isolates were more pathogenic than the DON isolates to maize.

DON and NIV are both aggressiveness factors and cause similar symptoms in wheat heads [29,30]. Despite this fact, differences were found in the detoxification mechanism of both toxins. [29] suggested that different genes in the *Fhb1* gene cluster may be involved in resistance to these toxins. NIV is less toxic to wheat plants than DON and NIV isolates appear to be less aggressive [31,32]. In contrast, NIV is more toxic to humans and animals [33]. The amounts of NIV detected in agricultural products are lower than the amounts of DON, but both mycotoxins can co-occur and pose a threat to consumers when their total amount is above the maximum limits [34].

Resistance to FHB is a complex trait. Several types (mechanisms) of resistance were identified. In their review article, Foroud et al. [35] described five types of resistance to FHB with two classes for type V. They were as follows. Type I – resistance to initial infection; Type II – resistance to FHB spread in the spike; Type III – resistance to kernel damage (infection); Type IV – tolerance to FHB or trichothecene toxins; Type V – resistance to accumulation of trichothecene toxins subdivided into: Class 1 – by chemical modification (degradation or detoxification); Class 2 – by inhibiting trichothecene synthesis.

The evaluation of resistance type III is to determine the proportion of kernels damaged by *Fusarium* in the grain sample. This is done by dividing the sample into factions: kernels with signs of *Fusarium* damage (shriveled, white-discolored, pink, orange, carmine) and healthy-looking kernels [36]. This type of resistance can also be assessed by determining the content of ergosterol in the grain, which is a component of the cell membranes of fungi [37]. Its quantity indicates the amount of mycelium in the grain, which indirectly determines the degree of infection by *Fusarium* fungi. The amount of mycelium in the kernels can also be specified by measuring the concentration of *Fusarium* DNA in the grain using quantitative PCR (real-time PCR) [6,22,38]. This method is more precise because it can specifically detect DNA from *Fusarium* fungi (or selected *Fusarium* species, or chemotypes) in the grain [3,39,40]. The amount of *Fusarium* DNA can be used as a predictor of the mycotoxins concentration in grains. However, it is strongly dependent on the material analyzed. No relationship was found for naturally infected samples with low toxin content [41]. In samples from inoculated heads or samples containing a large amount of toxins, these relationships were found to be much stronger [42,43].

This research is based on the plant material described by us in the paper of Ochodzki et al.. [44]. We selected 12 lines and cultivars that differ in resistance to FHB and subjected them to real-time PCR analysis for the amount of *F. culmorum* DNA, as well as to quantify the trichothecene genotypes 3ADON and NIV. The purpose of the investigation was to

 doi:10.20944/preprints202211.0279.v1

find relationships between the weather conditions in the experimental years and the locations and the amount of *F. culmorum* DNA and trichothecene genotypes, as well as the proportions between them. DNA concentrations were also related to the resistance parameters to FHB and mycotoxin concentrations shown in our previous article [44].

2. Materials and Methods

2.1 FHB inoculation experiment and mycotoxin analysis

The plant materials included 12 winter wheat lines and cultivars:

- Winter wheat cultivars: Artist, Patras, and RGT Kilimanjaro.
- Breeding lines of wheat susceptible to FHB: KBP 14 16, NAD 10079, and SMH 8816
- Breeding lines/cultivars of wheat resistant to FHB: Fregata, NAD 13014, and NAD 13017
- Lines of wheat resistant to FHB that carry the *Fhb1* resistance gene: UNG 136.6.1.1 and S32.

A three-year field experiment (2017, 2018 and 2019) was established in two locations (Poznań, Radzików). At full anthesis (65 BBCH scale), the wheat lines were inoculated by spraying the heads with a spore suspension. Approximately two weeks after inoculation (depending on the appearance of FHB symptoms) and one week later, the progress of the disease was visually evaluated using the FHB index (FHBi). At harvest, 20 randomly selected heads from each plot at each location were collected and threshed with a laboratory thresher. The percentage of *Fusarium*-damaged kernels (FDK) was visually scored. The weight of the FDK relative to the weight of the entire sample was marked as FDKw and the number of FDK relative to the total sample size was marked as FDK#.

Wheat grain samples from three inoculated plots were mixed and finely ground. The content of type B trichothecenes in the grain (DON, 3-acetyldeoxynivalenol (3AcDON), 15-acetyldeoxynivalenol (15AcDON) and NIV) was analyzed using the gas chromatography technique. Ergosterol (ERG) was chromatographically analyzed via high performance liquid chromatography (HPLC) on a silica column using methanol.

The details of the plant material, the field experiment methodology, and the chemical analysis methodology were described in the previously published article [44].

2.2. DNA quantification

In infected wheat grain samples (72 samples) from experiments in years 2017-2019, the amount of *F. culmorum* DNA and DNA from trichothecene genotypes 3ADON and NIV was analyzed. The real-time PCR technique was used.

Quantitative analysis of plant DNA (a starter specific to the gene encoding the translation elongation factor *EF1α*) and fungal DNA (a starter specific to *F. culmorum*) was performed. (Table 1) [45]. Quantitative DNA analysis of two *F. culmorum* chemotypes (3ADON and NIV) was also performed. Primers specific to the *TRI12* gene belonging to the trichothecene biosynthesis gene cluster were applied [39].

Table 1. Primers used for the real-time PCR amplification of the *F. culmorum,* 3ADON, and NIV chemotypes in the *TRI12* gene and the translation elongation factor gene 1α (*EF1α*).

Target	Primer name	Sequence (5′-3′)
F. culmorum	Fcul F	CACCGTCATTGGTATGTTGTCACT
	Fcul R	CGGGAGCGTCTGATAGTCG
3ADON	3ADONf	AACATGATCGGTGAGGTATCGA
	3ADONr	CCATGGCGCTGGGAGTT
NIV	NIVf	GCCCATATTCGCGACAATGT
	NIVr	GGCGAACTGATGAGTAACAAAACC
Plant EF1α	Hor1F	TCTCTGGGTTTGAGGGTGAC
	Hor2R	GGCCCTTGTACCAGTCAAGGT

 doi:10.20944/preprints202211.0279.v1

Standard curves for *F. culmorum* species and chemotypes were determined using DNA obtained from pure fungal culture and isolates belonging to both chemotypes. Two *Fusarium culmorum* isolates were applied. KF846 was the 3ADON chemotype and KF350 was the NIV chemotype [42]. The isolates were grown on potato dextrose agar (PDA) medium covered with sterile polyethylene circles. The PDA plates were incubated at 22°C with a 12 h photoperiod for one week. Pure mycelium was scraped from the polyethylene surface with a spatula.

2.2.1. DNA extraction

The materials for DNA extraction (mycelium or powdered grains) were ground with two stainless steel beads (5 mm) using a TissueLyser LT mill (Qiagen). Samples (100 mg) were used for DNA extraction, using QIAcube® automatic sample preparation for QIAGEN® spin column kits. DNA was extracted using the Plant Tissue Mini Protocol from the dNeasy Plant Mini Kit (Qiagen) and eluted in 100 uL of AE buffer.

The concentration of DNA from fungal isolates used for the standard curves was determined using a NanoReady Micro UV–Vis Spectrophotometer (Life Real). The same method was used.

2.2.2. Real-time PCR

Real-time PCR was carried out in a total of 10.0 µl consisting of 2.0 µl 5× QUANTUM EvaGreen® HRM Kiter Mix (Syngen Biotech, Wrocław, Poland) and 2.0 µl template DNA. PCR reactions were performed in duplicate on all samples. Genomic DNA from grain samples was diluted 1:10 and pure cultures 1:100 before PCR.

All activities related to the preparation of the PCR reaction, dilution of DNA samples, and the preparation of standard curves were performed using the Myra Liquid Handling System (Bio Molecular System, Upper Coomera, Australia).

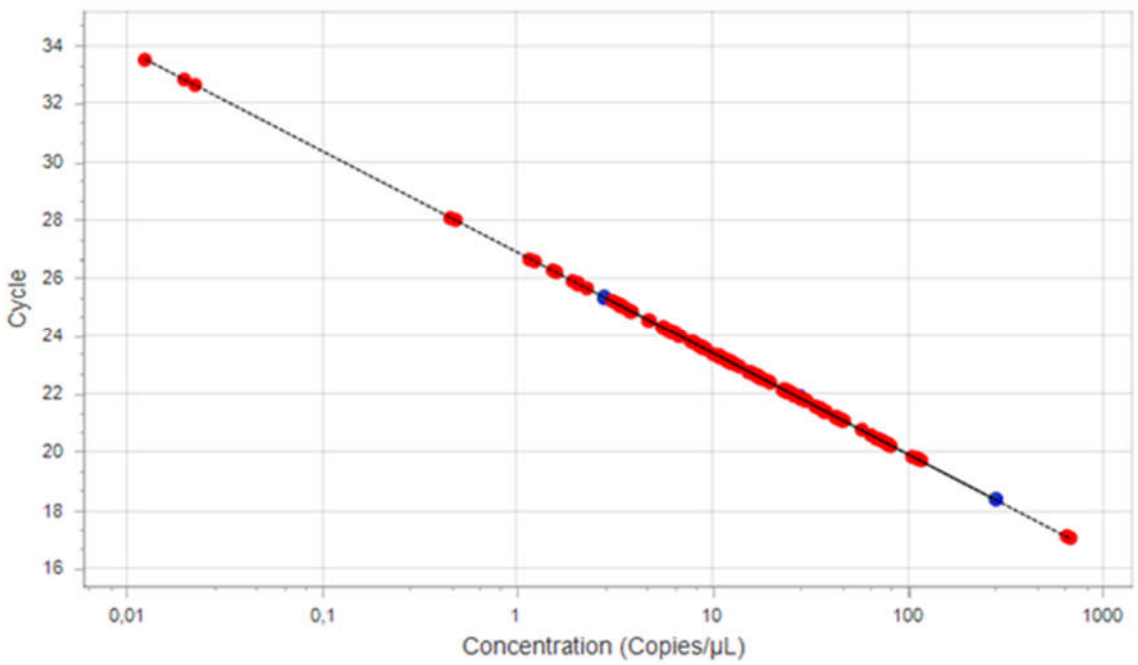
PCR was performed on a Mic qPCR (Bio Molecular System, Upper Coomera, Australia) using the following cycling protocol: 95°C 15 min; 40 cycles of 95°C for 15 s and 62°C for 1 min followed by analysis of dissociation curves at 60 to 95°C. For the plant assay, annealing and extension were carried out at 60°C.

2.2.3. Quantification of Fusarium DNA in plant material

Field samples were analyzed using specific assays together with the plant assay as a positive control. A standard curve was run for each of the assays with pure fungal DNA (Figures 1 and 2). Five-fold dilution series of the isolates were used for standard curves. The same five-fold series of dilutions of plant DNA was used as a standard curve for the plant assay. The amount of fungal DNA was calculated from the values of the cycle threshold (Cq) using the standard curve, and these values were normalized with the estimated amount of plant DNA based on the plant *EF1α* assay.

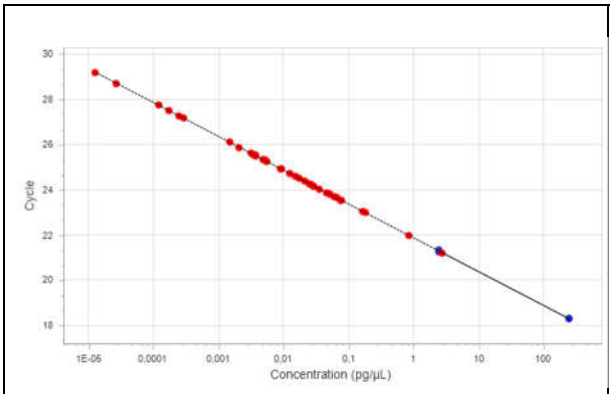
The relative DNA content of *F. culmorum* and chemotypes 3ADON and NIV was calculated in relation to wheat DNA (pg/µg).

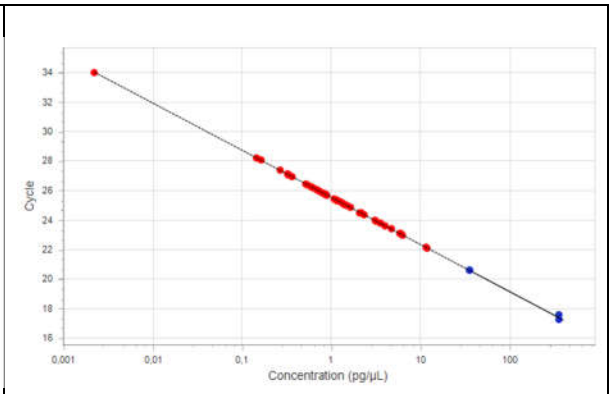
Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 November 2022 doi:10.20944/preprints202211.0279.v1



Target	*F. culmorum* → *Fusarium culmorum* KF 846
R^2	0,9999

Figure 1. Standard curve for DNA from *Fusarium culmorum.*





Target	Trichothecene genotype 3ADON for *F. culmorum* KF 846 → Trichothecene genotypes *TRI12* gene	Target	Trichothecene genotype NIV for *F. culmorum* KF 350 → Trichothecene genotypes *TRI12* gene
R^2	0,9997	R^2	0,9950

Figure 2. Standard curves for DNA of the 3ADON and NIV trichothecene genotypes.

2.3. Statistical analysis

Statistical analysis was performed using XLSTAT Life Science, Version 2021.2.1.1119 (Addinsoft, New York, NY, USA) (Addinsoft 2022).

The concentrations of *F. culmorum* DNA and the 3ADON and NIV chemotypes were analyzed by variance analysis using the XLSTAT procedure: ANOVA. The variables did not follow a normal distribution according to normality tests. Data were transformed using the Box-Cox transformation. Three-way analysis of variance (ANOVA) was performed (year × location × line) was performed. Mean differences were determined according to Fisher's LSD test at $\alpha = 0.05$. Six experimental environments (year/location) were compared using the Kruskal-Wallis nonparametric test and the Steel-Dwass-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 November 2022 doi:10.20944/preprints202211.0279.v1

Critchlow-Fligner multiple pairwise comparison method (XLSTAT procedure: Comparison of k samples (Kruskal-Wallis, Friedman, …)). Relationships between *F. culmorum* DNA and 3ADON and NIV chemotypes, as well as FHBi, FDK, ERG, and mycotoxin concentrations, were investigated using Pearson's correlation tests (XLSTAT procedure: Correlation tests).

3. Results

Weather conditions were highly variable in the experimental years and at the locations. Mostly, in terms of rainfall distribution in the three-month coverage period from wheat heading to full maturity. Summary precipitation was the highest in 2017 at both locations. In Poznań it was similar in the following years; however, the distribution of rainfall was different. Rainfall was very low in June 2019, which was a period of wheat flowering and *Fusarium* inoculations. In Radzików summary precipitation was lowest in 2019, however, rainfall in June was higher than in 2018.

The average temperature was the lowest in 2017 in both locations. It was higher in the next years and similar in locations. In 2019 we observed very high temperatures in June after flowering and inoculation of wheat heads.

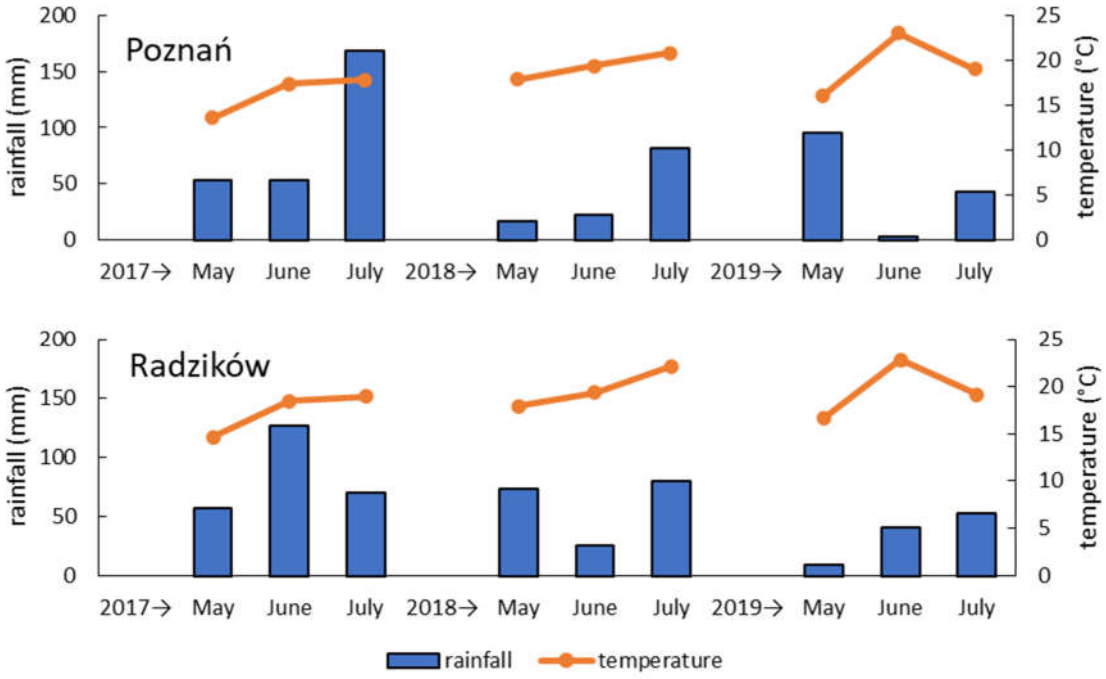


Figure 3. Monthly rainfall and average temperature in May, June and July 2017-2019 in two experimental locations (Poznań, Radzików).

Fusarium culmorum DNA was detected in all grain samples in an average amount of 20124 pg per 1 µg of wheat DNA. The range was from 97015 pg/µg ('SMH 8816', Poznań, 2017) to 110 pg/µg ('RGT Kilimanjaro', Radzików, 2018). The highest amount of *F. culmorum* DNA was detected in 2017 (41484 pg/µg). It was much lower in the following years, 5682 pg/µg in 2018 and 1325 pg/µg in 2019. The means for three years differed statistically significantly. In two experimental locations, the amount of DNA in grain from Poznań was 24862 pg/µg and Radzików 15385 pg/µg. The location means differed significantly.

In the six experimental environments (year / location), the amount of *F. culmorum* DNA was the highest in Pozna in 2017, however it did not differ statistically significantly from the amount in Radzików in this year (Figure 3).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 November 2022 doi:10.20944/preprints202211.0279.v1

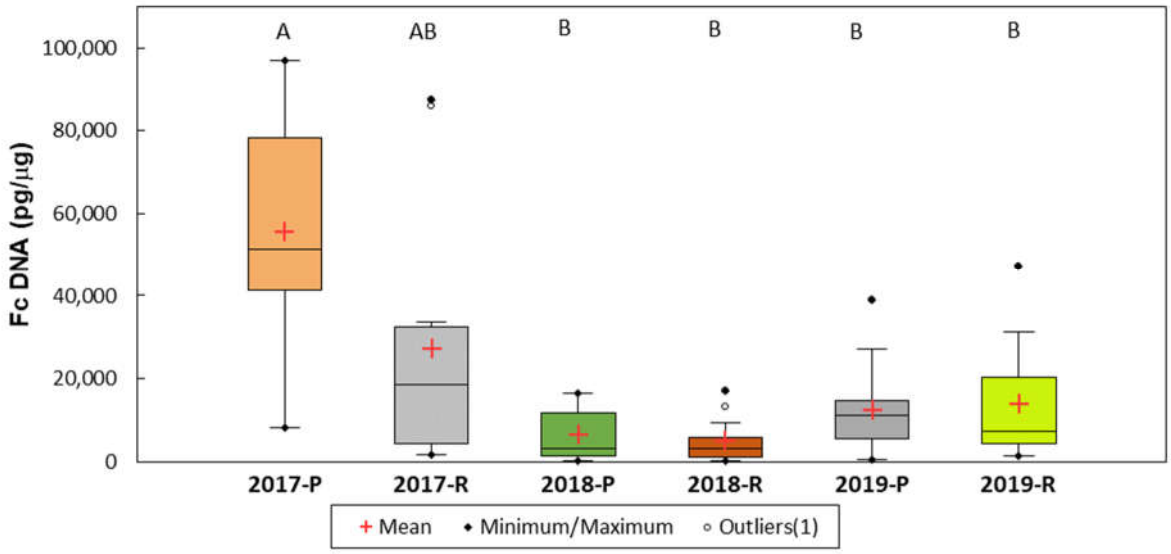


Figure 4. Concentration of *F. culmorum* DNA in wheat grain in six experimental environments (year location). The means marked with the same letter are not significantly different at p = 0.05 according to the Kruskall-Wallis test and the multiple pairwise comparison method of Steel-Dwass-Critchlow-Fligner.

Wheat lines differed significantly in the amount of *F. culmorum* DNA in the grain (Table 2). The lowest concentration was found in the grain of two lines that carry the *Fhb1* resistance gene 'S 32' and 'UNG 136.6.1.1', as well as in the grain of the cultivar 'Fregata' and the breeding line 'NAD 13017'. The highest concentration of *F. culmorum* DNA was detected in the grain of susceptible lines 'DL 358/3/4' and 'KBP 1416'.

Table 2. Amount of DNA (pg fungal DNA/µg plant DNA) of *F. culmorum* and 3ADON and NIV chemotypes in wheat grains and ratios of the amounts of *F.c*, 3ADON, and NIV DNA to concentrations of type B trichothecenes, DON and NIV.

Line	*F. culmorum* DNA (pg/µg)	3ADON chemotype DNA (pg/µg)	NIV chemotype DNA (pg/µg)	*F.c.* DNA/ TCT B[#]	3ADON DNA/ DON[#$]	NIV DNA/ NIV[#]
S 32 (*Fhb1*)	2763 a	1104 a	662 a	1.1	1.5	0.4
Fregata	8118 abc	3094 a	1189 a	2.4	1.1	1.9
UNG 136.6.1.1 (*Fhb1*)	9438 ab	1727 a	2041 a	1.9	1.0	0.6
NAD 13017	9465 abc	2437 a	1641 a	1.6	0.8	0.6
NAD 13014	14625 bcd	2720 a	2961 a	3.1	1.0	1.6
RGT Kilimanjaro	17674 bcd	3068 a	3208 a	3.3	0.9	1.7
Artist	21309 cde	4207 a	3362 a	2.2	0.6	1.3
NAD 10079	21872 cde	7768 a	4574 a	2.7	1.4	1.7
Patras	24355 cde	5010 a	3701 a	2.0	0.6	1.0
SMH 8816	26996 cde	6841 a	4476 a	3.1	1.3	1.3
DL 358 /13/4	41502 de	11236 a	6130 a	2.4	0.9	1.1
KBP 1416	43369 e	9556 a	6019 a	3.5	1.2	1.4
Mean	20124	4897	3330	2.4	1.0	1.2

[#] mycotoxin data from Ochodzki et al.. [44]; [$] sum of DON and 3AcDON; means marked with the same letter are not significantly different at p = 0.05 according to Fisher's LSD test performed on transformed variables

Regarding the trichothecene genotypes, the average amount of DNA from the 3ADON genotype was 4879 pg/µg at a range of 30514 pg/µg ('DL 358/1/34' Radzików,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 November 2022 doi:10.20944/preprints202211.0279.v1

2017) to 0 ('S 32', Radzików, 2019). The highest amount of 3ADON DNA was detected in 2017 (9923 pg/µg). It was much lower in the following years: 2237 pg/µg in 2018 and 2532 pg/µg in 2019. The mean for 2017 differed statistically significantly from the mean for 2018 and 2019. In two experimental locations, the amount of 3ADON DNA in the Pozna grain of Poznań was 4846 pg/µg and in Radzików 4949 pg/µg. The location means did not differ significantly. In the six experimental environments (year / location), the amount of 3ADON DNA was the highest in 2017 in both locations (means difference not significant) (Figure 4). It was lower in 2018 in Radzików and 2019 in Poznań, but not significantly different form value for Radzików in 2017. The amount of 3ADON DNA was very low in 2018 in Poznań and in 2019 in Radzików.

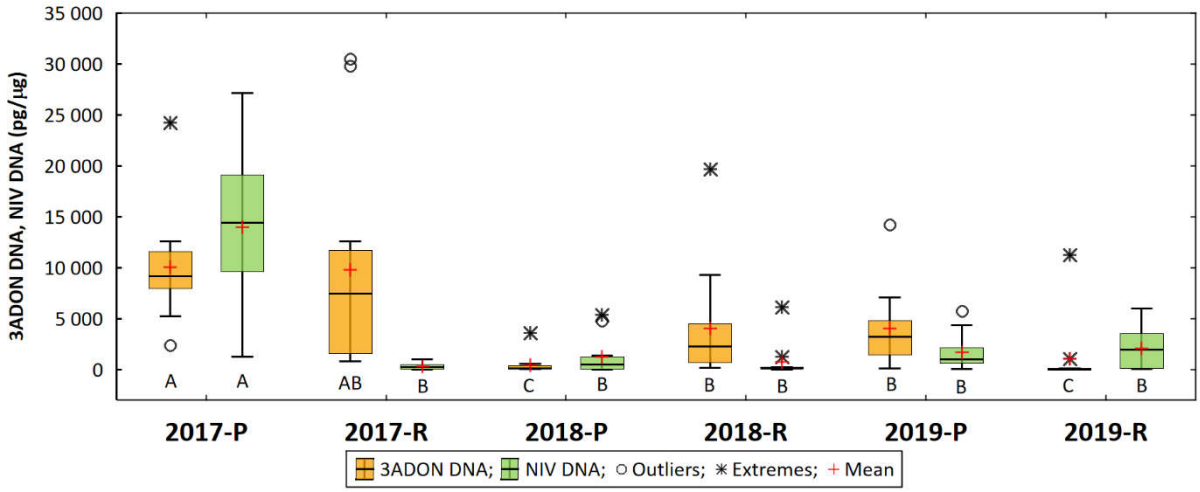


Figure 5. DNA concentration of two trichothecene genotypes of *F. culmorum* (3ADON, NIV) in wheat grain in six experimental environments (year/location). Means marked with the same letter are not significantly different at p = 0.05 according to the Kruskall-Wallis test and the Steel-Dwass-Critchlow-Fligner multiple pairwise comparison method.

The amount of DNA of the NIV genotype was 3330 pg/µg at a range of 27131 pg/µg ('DL 358/1/34' Poznań, 2017) to 8 pg/µg ('SMH 8816', Poznań, 2018). The highest amount of NIV DNA was detected in 2017 (7131 pg/µg). It was much lower in the following years – 1876 pg/µg in 2018 and 985 pg/µg in 2019. The mean for 2017 differed statistically significantly from the mean for 2018 and 2019. In two experimental locations, the amount of NIV DNA in Poznań grain was 5628 pg/µg and in Radzików it was 5 times lower (1033 pg/µg). Location means differed significantly. In the six experimental environments, the amount of NIV DNA was the highest in 2017 in Poznań (Figure 4). It was significantly lower in other environments.

The lowest concentration of 3ADON DNA was detected in grain of two lines with the *Fhb1* gene. It was also low in two lines 'NAD 13017' and 'NAD 13014' and in the cultivars 'Fregata' and 'RGT Kilimanjaro' (Table 2). The highest concentration of 3ADON DNA was detected in the grain of the susceptible lines 'DL 358/3/4' and 'KBP 1416'. The lowest concentration of NIV DNA was detected in the grain of line 'S 32' with the *Fhb1* gene. It was low in the lines 'UNG 136.6.1.1' (with *Fhb1*), 'NAD 13017' and 'Fregata' cultivar. Similarly, for 3ADON DNA, the highest concentration of NIV DNA was detected in the grain of the susceptible lines 'DL 358/3/4' and 'KBP 1416'.

The ratio of 3ADON DNA to NIV DNA was on average 1.5; however, it varied widely depending on year, location, and genotype. The lowest was 0.001 ('RGT Kilimanjaro' and 'S 32', Radzików, 2019) and the highest 93.4 ('NAD 13017', Radzików, 2017). In three years, the ration was as follows: 2017 – 1.4, 2018 – 2.3, 2019 – 1.3. It was five times higher in Radzików (4.8) than in Poznań (0.9). In detail, the ratio in 2017 was 30-times higher in Radzików (31.8) than in Poznań (0.7). In 2018, it was 20 times higher in Radzików (5.6) than in Poznań (0.4). Only in 2019, the ratio was higher in Poznań (2.4) compared to

 doi:10.20944/preprints202211.0279.v1

Radzików (0.5). For wheat lines, the 3ADON/NIV DNA ratio was in the range of 0.8 ('UNG 136.6.1.1') – 2.6 ('Fregata'). We found no relationship between line FHB resistance (head and kernels) and the 3ADON/NIV DNA ratio (Ochodzki et al. 2021). For example, for lines with the *Fhb1* gene, it was 1.7 for 'S 32' and 0.8 for 'UNG 136.6.1.1'.

Next, we calculated the ratio of DNA (*F. culmorum*, chemotypes) to mycotoxin content in grain (data presented by Ochodzki et al. [44]). The ratio of *F. culmorum* DNA to type B trichothecenes was on average 2.4. It ranged from 0.1 ('NAD 13017', 2018, Poznań) to 51.6 ('S 32', 2018, Radzików). In three years, the ratio was as follows: 2017 – 2.6, 2018 – 1.5, 2019 – 3.2. It was similar in two locations – 2.2 in Radzików and 2.8 in Poznań. In detail, the ratio in 2017 was twice higher in Poznań (3.7) than in Radzików (1.7). In 2018, it was similar in two locations (Radzików 1.7, Poznań 1.3). In 2019, the ratio was three times higher in Radzików (6.5) than in Poznań (2.0). There was a relationship of this ratio with phenotypic resistance to FHB [44]. For FHBi, FDKw and FDK# coefficients were 0.527, 0.559, and 0560. They were not statistically significant. The ratio was the lowest for two lines that carried the *Fhb1* resistance gene and the low-infected line 'NAD 13017' (Table 2).

The 3ADON DNA to sum of DON toxins (DON, 3AcDON) ratio was 1.0. It varied from 0.002 ('S 32', 2019, Radzików) to 62.9 ('S 32', 2018, Radzików). In three years, the ratio was as follows: 2017 – 1.0, 2018 – 1.4, 2019 – 0.7. It was higher in Poznań (1.3) than in Radzików (0.8). In detail, the ratio in 2017 was twice higher in Poznań (1.8) than in Radzików (0.7). In 2018, it was similar in two locations (Radzików 1.4, Poznań 1.0). In 2019, the ratio was lower and also similar in locations (Radzików 0.7, Poznań 0.8). There was no relationship of this ratio to phenotypic resistance to FHB [44].

The ratio of NIV DNA to the sum of NIV toxin was 1.2. It varied from 0.001 ('SMH 8816', 2018, Poznań) to 9.1 ('SMH 8816', 2019, Radzików). In three years, the ration was as follows: 2017 – 1.0, 2018 – 1.4, 2019 – 0.7. It was higher in Poznań (1.3) than in Radzików (0.8). In detail, the ratio in 2017 was seven times higher in Poznań (1.5) than in Radzików (0.2). In 2018, the ratio was 40 times higher in Radzików (12.2) than in Poznan (0.3). In 2019, the ratio was also higher in Radzików (3.8) than in Poznań (2.0). There was no relationship of this ratio with phenotypic resistance to FHB; however, it was the lowest for two lines carrying the *Fhb1* resistance gene and the low infected line 'NAD 13017' (Table 2).

The concentrations of *F. culmorum*, 3ADON and NIV DNA were correlated with phenotypic resistance (FHBi, FDK) and the concentration of ergosterol (ERG) and mycotoxins (DON, 3AcDON, NIV) in grain (data from Ochodzki et al. [44]. The amount of DNA from *F. culmorum* was significantly correlated with all variables (Table 3). The highest was the correlation coefficient with FHBi and ERG. Regarding mycotoxins, the coefficients were higher for DON and 3AcDON and lower for NIV.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 November 2022 doi:10.20944/preprints202211.0279.v1

Table 3. Coefficients of correlation between DNA concentrations of *F. culmorum* and trichothecene genotypes (3ADON, NIV) with phenotypic resistance (FHBi, FDK) and ergosterol (ERG) and mycotoxin (type B trichothecenes) concentrations.

Variables (n=72)	Fc DNA	3A DON DNA	NIV DNA	3A DON+ NIV DNA	FHBi$	FDKw$	FDK#$	ERG$	DON$	3Ac DON$	DON+ 3Ac DON$	NIV$
3ADON DNA	0.533											
NIV DNA	0.707	0.370										
3ADON+NIV DNA	0.767	0.801	0.769									
FHBi	0.602	0.268*	0.323**	0.413								
FDKw	0.513	0.384	0.369	0.455	0.655							
FDK#	0.538	0.376	0.404	0.454	0.677	0.985						
ERG	0.619	0.436	0.352	0.477	0.716	0.813	0.829					
DON	0.531	0.552	0.295*	0.552	0.381	0.108ns	0.112 ns	0.344**				
3AcDON	0.609	0.459	0.400	0.549	0.426	0.209 ns	0.216 ns	0.396	0.790			
DON+ 3AcDON	0.534	0.549	0.300**	0.554	0.380	0.108 ns	0.111 ns	0.345**	1.000	0.798		
NIV	0.497	0.277*	0.514	0.460	0.411	0.625	0.665	0.669	0.368	0.494	0.373	
TCT B	0.519	0.488	0.422	0.541	0.417	0.427	0.452	0.608	0.806	0.618	0.808	0.755

The coefficients are significant at $p < 0.001$ except for those marked with * ($p < 0.05$), ** ($p < 0.01$) or ns (non-significant); TCT B – sum of DON, 3AcDON and NIV; $ data from Ochodzki et al. [44]

The correlation coefficients of the trichothecene genotypes (3ADON, NIV) were lower than those of *F. culmorum* DNA. However, the correlation coefficients of 3ADON DNA with DON concentration and NIV DNA with NIV concentration were higher than for *F. culmorum* DNA. The coefficient of correlation of the sum of DNA from the 3ADON and NIV trichothecene genotypes with the sum of trichothecenes was higher than for the DNA of *F. culmorum* or DNA of trichothecene genotypes independently correlated with trichothecenes.

Since the coefficients were low, we analysed the data for particular years separately (Figure 6). The coefficients for 2017 and 2019 were high. The results for 2018 were significantly different from those obtained for the other years. The correlation coefficients of DNA amount with phenotypic resistance and mycotoxins were very low. Similarly, the correlation coefficients of phenotypic resistance with mycotoxins were low.

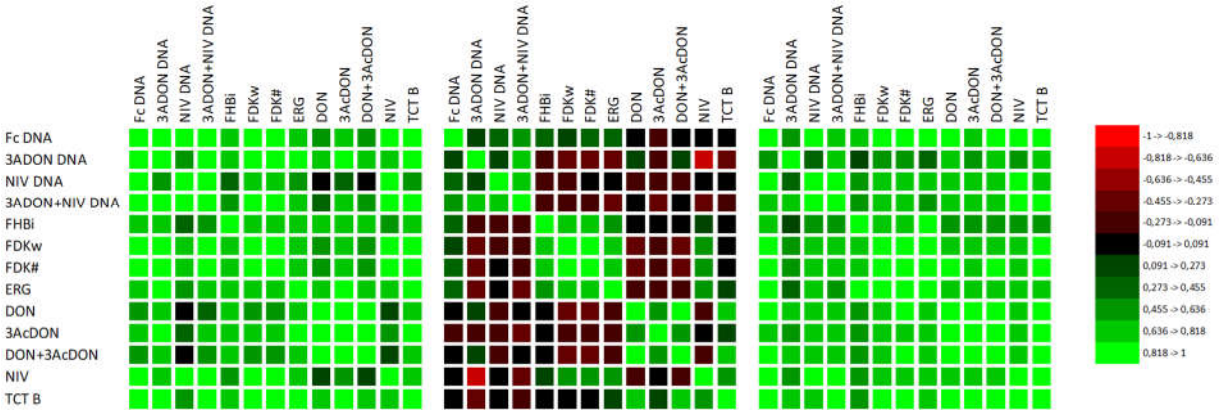


Figure 6. Matrixes of coefficients of correlation of DNA concentrations of *F. culmorum* and trichothecene genotypes (3ADON, NIV) with phenotypic resistance (FHBi, FDK) and ergosterol (ERG) and mycotoxin (type B trichothecenes) concentrations in three experimental years (2017, 2018, and 2019) [44]. The critical value for Pearson's correlation coefficient at $p = 0.05$ is equal to $r = 0.404$.

 doi:10.20944/preprints202211.0279.v1

4. Discussion

The weather conditions strongly affected the development of FHB and the amount of DNA detected in the grain. 2017 was the year most favorable for FHB. This year the amount of *F. culmorum* DNA and 3ADON DNA was the highest in both locations. The story with NIV DNA was different. It was the highest in Poznań in 2017 but in Radzików it was the highest in 2019. It is difficult to explain this, but we can see that the amount of 3ADON genotype DNA was very low in 2019 in Radzików (which was different from previous years). It seems that the weather conditions favored the development of the NIV genotype. We compared daily weather conditions in Radzików in 2018 and 2019. In 2018, no rainfall was recorded after inoculation (beginning of June), but the second half of June was rainy with a lower temperature. On the contrary, in 2019, we recorded some rainfall during and after inoculations, but the end of June was dry and extremely hot. The strong association between environmental conditions and *Fusarium* biomass detected in mature grain was reported in other articles. Hoheneder et al. [46] found that environmental conditions before heading and late after flowering (fourth week) had the highest influence on *F. culmorum* in barley. Before flowering, the most important factors were temperature combined with sufficient rain and relative air humidity. After flowering, the most important factors were the sum of temperature, the sum of precipitation, and the relative humidity of the air. Xu et al. [47] found an increase in the amount of fungal biomass (the total amount of fungal DNA) with an increase in the length of the wetness period and temperature. For F. *culmorum*, it was 36 hours of wetness accompanied by a temperature of 25°C. They used the NIV chemotype of *F. culmorum* and observed that NIV production increased with increasing temperature and duration of wetness. The DON chemotype was the *F. graminearum* species, and in this case the main factor for increased biomass and DON production was leaf wetness.

In three experimental years, we observed large variability in correlation coefficients between *F. culmorum* DNA and phenotypic resistance (FHBi, FDK) and ergosterol (ERG) and mycotoxin (type B trichothecenes) concentrations. In the paper we published, we found a highly significant correlation between the amount of *F. culmorum* DNA and the FHB index, as well as *Fusarium*-damaged kernels [42]. The coefficients for the toxins were more variable, low for DON and high for NIV. In general, they were similar to those obtained in this study (three-year data). However, we did not observe differences between the correlation of DON and NIV with *F. culmorum* DNA.

The amount of trichothecene genotypes was significantly correlated with the corresponding toxins. The correlations 3ADON DNA vs NIV and NIV DNA vs DON were lower. Schner et al. [43] analyzed 300 wheat grain samples differing in DON content. They quantify the amount of DNA of trichothecene-producing *Fusarium* species using primers targeting the trichodiene synthase gene *Tri5*. They found a very high correlation coefficient between DNA and DON (0.956). We compared it with our results for trichothecene genotypes, as we target *Tri12*, the other gene for the trichothecene gene cluster (*Tri5*-cluster) [48]. As Schnerr et al. [43] used raw (nontransformed) data, we compared their results with coefficients calculated on nontransformed results. We found similar high coefficients for the correlations 3ADON DNA vs DON r = 0.803, 3ADON DNA vs 3AcDON r = 0.807 and NIV DNA vs. NIV r = 0.811. Leišová et al. [49] quantified the DNA from *F. culmorum* in wheat and barley and compared its content with the DON content. They found a very high correlation for barley between the Ct values and the DON content. However, these were results for only one year. For wheat, they found a more complicated relationship. The determination coefficient (r^2) varied widely in four experimental years from 0.327 to 0.888. But we must add that the authors omitted results for one cultivar, which results deviated greatly from the linear relationship. Edwards et al. [50] inoculated wheat with different species of *Fusarium.* They did not find a correlation between the severity of FHB and the concentration of DON in the grain, but there was a good correlation (r^2 = 0.76) between the amount of *Tri5* DNA (trichodiene synthase gene) and DON present in the grain. Sarlin et al. [51] analyzed different samples of Finnish barley grains. They found a

 doi:10.20944/preprints202211.0279.v1

significant correlation (r = 0.808) between *Fusarium* DNA and DON level in barley samples artificially inoculated with *F. culmorum*, *F. graminearum*, and *F. poae*. They applied primers that did not target genes involved in trichothecene production.

Under conditions of natural *Fusarium* infection, prediction of mycotoxin concentration from *Fusarium* biomass amount in grain is more problematic. Analyzing wheat samples from four countries, Xu et al. [41] found a weak relationship between the amount of biomass of *F. graminearum* and *F. culmorum* and the concentration of mycotoxins. However, the authors claimed that the linear relationship with DON was nearly significant. In our study, we found a significant relationship between the amount of biomass of *F. graminearum* and the concentration of DON (r = 0.534) and zearalenone (r = 0.672) in wheat grain [52]. Analyzing Canadian wheat samples naturally infected with *F. graminearum* s.s., Demeke et al. [53] found a strong positive correlation between the DNA of *F. graminearum* and DON amount. Furthermore, other researchers observed highly significant correlations between the amount of *F. graminearum* DNA and the concentration of DON in naturally infected wheat grains [54,55]. Mentioned above, Sarlin et al. [51] found a low correlation (r =0.492) between DNA from trichothecene-producing fungi and DON in naturally infected Finnish barley grain. However, they found a high correlation (r = 0.967) for naturally infected barley samples from the USA and Canada. Yli-Matilla et al. [6] also analyzed cereal samples (barley, oats, wheat) from Finland. They found a high correlation between *F. graminearum* DNA and DON concentration in oats, but lower for wheat and barley. The authors explain that in Finland, *F. culmorum* is also an important DON producer in wheat and barley, but not in oats. Sarlin et al. [51] also showed that *F. graminearum* DNA was not correlated with the amount of DON in barley when this species was analyzed separately. Studying Italian wheat grain and whole grain, flour, and bread, Terzi et al. [56] found a significant correlation between *Fusarium* DNA and DON content. The coefficients were high, with r = 0.99 for all samples. The authors applied primers designed on the *Tri5-Tri6* intergenic sequence involved in DON synthesis.

The ratio from the amount of DNA of the 3ADON chemotype to DNA from the NIV chemotype was on average 1.5. It varied widely depending on year, location, and genotype. For head inoculations, we applied a mixture of two 3ADON chemotype isolates and one isolate of NIV chemotype. Thus, without reduction of DNA of particular isolate due to competition ratio of 3ADON/NIV should be 2. The increase in the ratio was mainly due to the conditions in the experimental location in Poznań that were favorable for NIV chemotype in two of three experimental years.

The reduction in the amount of particular isolate in mixed inoculation can be caused by competition between isolates. Xu et al. [47] inoculated wheat heads with *F. avenaceum*, *F. culmorum* , *F. graminearum,* and *F. poae* species. They applied the species separately and in mixtures. Competition between species led to large reductions in fungal biomass compared to single-isolate inoculations. It was up to > 90% reduction for the weaker species. In contrast, mycotoxin production increased noticeably in co-inoculations, by as much as 1000 times. The authors stated that competition resulted in greater production of trichothecene mycotoxins. They compared isolates of different species, but in the article by von der Ohe et al. [57] we can find comparison of two chemotypes of *F. graminearum*, DON and NIV. The authors did not check whether the DON isolates belonged to the 3ADON or 15ADON sub-chemotype. In mixed inoculation with DON and NIV isolates, FHB rating was not significantly different from DON+DON binary mixtures. With regard to mycotoxin production, the application of DON+NIV mixture resulted in the lack of DON in one environment and the production of DON similar as for DON+DON mixtures in the other two environments. The authors did not analyze the NIV content, so we do not know what was the effect on mixed inoculation on amount of this toxin. Explanation can be result of reisolation from inoculated heads. In the first environment (no DON detected), the NIV chemotype dominated, and in the others, the DON chemotype. We observed NIV dominance (measured by DNA amount) in three of six environments (in Poznań in 2017 and 2018 and in Radzików in 2019). This was strongly dependent on weather and experimental conditions (mist irrigation).

Miedaner et al. [58] compared aggressiveness and mycotoxins production of isolates of two pairs of the *F. culmorum* of 3ADON and NIV chemotype. On average, they observed that the aggressiveness of the isolate mixtures was significantly lower than that of the isolates applied individually for inoculation. Similarly, mycotoxin concentrations were significantly lower in the mixtures in most of the comparisons. The authors re-isolated and molecular identified *F. culmorum* isolates from inoculated heads. They found the dominance of the highly aggressive DON isolate over the NIV isolate and the less aggressive DON isolate. However, the more aggressive NIV isolate dominated both the less aggressive NIV and the DON isolates. Unfortunately, the authors did not test mixtures of more aggressive isolates of both chemotypes and presented mycotoxin data only for one environment. Therefore, it is difficult to evaluate the effect of experimental conditions on the frequency of chemotypes and proportions of mycotoxins.

5. Conclusions

The DNA of *F. culmorum* and trichothecene chemotypes (3ADON, NIV) was quantified in winter wheat. Strong effect of weather conditions in three experimental years on DNA amounts was observed. The was significantly of DNA amount correlated with parameters of phenotypic resistance to FHB and with the concentration of trichothecene toxins. The coefficients were very high in the two experimental years. In one year, the correlation coefficients of the amount of DNA with phenotypic resistance and mycotoxins were very low, which showed a strong effect of weather conditions on these relationships. Coefficients were higher for DNA from *F. culmorum.* However, for mycotoxins (DON, NIV, total trichothecenes), coefficients were higher for DNA from trichothecene chemotypes. The proportion from the DNA of 3ADON chemotype to the NIV chemotype was on average 1.5. It varied widely depending on environmental conditions and in some cases the amount of the DNA of NIV chemotype was higher than that of the 3ADON chemotype.

Authors Contributions: Conceptualization, T.G. and H.W.; methodology, T.G., J.P., H.W. and P.O.; investigation, T.G., J.P., P.O. H.W. and B.W..; data curation, T.G., J.P., P.O and B.W.; writing—original draft preparation, T.G.; writing—review and editing, T.G., J.P., P.O., H.W. and B.W.; visualization, T.G.; supervision, T.G.; project administration, T.G. and H.W.; funding acquisition, T.G. and B.W.. All authors have read and agreed to the published version of the manuscript.

Funding: This research was founded by the project of the Ministry of Agriculture and Rural Development of Poland: 'Identification and application of phenotypic, metabolic, and molecular markers in studies of types of resistance to Fusarium head blight in winter wheat accessions differing in resistance'. Project no. 6 (HOR.hn.802.28.2017, HOR.hn.802.19.20l8, HOR.hn.802.3.2019).

Acknowledgments Technical support of Dorota Walentyn-Góral and Elżbieta Dąbrowska is greatly appreciated..

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Snijders, C.H.A. Resistance in wheat to *Fusarium* infection and trichothecene formation. *Toxicol. Lett.* **2004**, *153*, 37–46, doi:10.1016/j.toxlet.2004.04.044.
2. Bottalico, A.; Perrone, G. Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. *Eur. J. Plant Pathol.* **2002**, *108*, 611–624, doi:10.1023/A:1020635214971.
3. Nielsen, L.K.; Jensen, J.D.; Nielsen, G.C.; Jensen, J.E.; Spliid, N.H.; Thomsen, I.K.; Justesen, A.F.; Collinge, D.B.; Jørgensen, L.N. Fusarium head blight of cereals in Denmark: species complex and related mycotoxins. *Phytopathology* **2011**, *101*, 960–969, doi:10.1094/PHYTO-07-10-0188.
4. Uhlig, S.; Jestoi, M.; Parikka, P. *Fusarium avenaceum* – the North European situation. *Int. J. Food Microbiol.* **2007**, *119*, 17–24, doi:10.1016/j.ijfoodmicro.2007.07.021.
5. Yli-Mattila, T.; Paaavanen-Huhtala, P.; Parikka, P.; Hietaniemi, V.; Jestoi, M.; Rizzo, A. Real-time PCR detection and quantification of *Fusarium poae* as compared to mycotoxin production in grains in Finland. In Proceedings of the 2nd International Symposium on Fusarium Head Blight; incorporating the 8th European *Fusarium* seminar; Canty, S.M., Boring, T., Wardwell, J., Ward, R.W., Eds.; Orlando, FL, USA. East Lansing, MI: Michigan State University., 2004; pp. 422–425.

6. Yli-Mattila, T.; Paavanen-Huhtala, S.; Jestoi, M.; Parikka, P.; Hietaniemi, V.; Gagkaeva, T.; Sarlin, T.; Haikara, A.; Laaksonen, S.; Rizzo, A. Real-time PCR detection and quantification of *Fusarium poae*, *F. graminearum*, *F. sporotrichioides* and *F. langsethiae* in cereal grains in Finland and Russia. *Arch. Phytopathol. Plant Prot.* **2008**, *41*, 243–260, doi:10.1080/03235400600680659.
7. Amarasinghe, C.; Sharanowski, B.; Dilantha Fernando, W.G. Molecular phylogenetic relationships, trichothecene chemotype diversity and aggressiveness of strains in a global collection of *Fusarium graminearum* species. *Toxins* **2019**, *11*, 263, doi:10.3390/toxins11050263.
8. O'Donnell, K.; Kistler, H.C.; Tacke, B.K.; Casper, H.H. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proc. Natl. Acad. Sci.* **2000**, *97*, 7905–7910, doi:10.1073/pnas.130193297.
9. O'Donnell, K.; Ward, T.J.; Geiser, D.M.; Kistler, H.C.; Aoki, T. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genet. Biol.* **2004**, *41*, 600–623, doi:10.1016/j.fgb.2004.03.003.
10. Starkey, D.E.; Ward, T.J.; Aoki, T.; Gale, L.R.; Kistler, H.C.; Geiser, D.M.; Suga, H.; Tóth, B.; Varga, J.; O'Donnell, K. Global molecular surveillance reveals novel Fusarium head blight species and trichothecene toxin diversity. *Fungal Genet. Biol.* **2007**, *44*, 1191–204, doi:10.1016/j.fgb.2007.03.001.
11. Talas, F.; Parzies, H.K.; Miedaner, T. Diversity in genetic structure and chemotype composition of *Fusarium graminearum* sensu stricto populations causing wheat head blight in individual fields in Germany. *Eur. J. Plant Pathol.* **2011**, *131*, 39–48, doi:10.1007/s10658-011-9785-3.
12. O'Donnell, K.; Rooney, A.P.; Proctor, R.H.; Brown, D.W.; McCormick, S.P.; Ward, T.J.; Frandsen, R.J.N.; Lysøe, E.; Rehner, S.A.; Aoki, T.; et al. Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. *Fungal Genet. Biol.* **2013**, *52*, 20–31, doi:10.1016/j.fgb.2012.12.004.
13. Perincherry, L.; Lalak-Kańczugowska, J.; Stępień, Ł. *Fusarium*-produced mycotoxins in plant-pathogen interactions. *Toxins* **2019**, *11*, 664, doi:10.3390/toxins11110664.
14. Munkvold, G.P. Mycotoxigenic Fungi. In *Mycotoxigenic Fungi: Methods and Protocols, Methods in Molecular Biology*; Moretti, A., Susca, A., Eds.; Humana Press, New York, NY, 2017; Vol. 1542, pp. 51–105 ISBN 9781493967056.
15. Miller, J.D.; Greenhalgh, R.; Wang, Y.; Lu, M. Trichothecene chemotypes of three *Fusarium* species. *Mycologia* **1991**, *82*, 121–130.
16. Quarta, A.; Mita, G.; Haidukowski, M.; Santino, A.; Mulè, G.; Visconti, A. Assessment of trichothecene chemotypes of *Fusarium culmorum* occurring in Europe. *Food Addit. Contam.* **2005**, *22*, 309–315, doi:10.1080/02652030500058361.
17. Tóth, B.; Mesterházy, Á.; Nicholson, P.; Téren, J.; Varga, J. Mycotoxin production and molecular variability of European and American isolates of *Fusarium culmorum*. *Mol. Divers. PCR-detection Toxigenic Fusarium Species Ochratoxigenic Fungi* **2004**, *125*, 587–599, doi:10.1111/j.1439-0523.2006.01251.x.
18. Jennings, P.; Coates, M.E.; Turner, J.A.; Chandler, E.A.; Nicholson, P. Determination of deoxynivalenol and nivalenol chemotypes of *Fusarium culmorum* isolates from England and Wales by PCR assay. *Plant Pathol.* **2004**, *53*, 182–190, doi:10.1111/j.0032-0862.2004.00985.x.
19. Kammoun, L.G.; Gargouri, S.; Barreau, C.; Richard-Forget, F.; Hajlaoui, M.R. Trichothecene chemotypes of *Fusarium culmorum* infecting wheat in Tunisia. *Int. J. Food Microbiol.* **2010**, *140*, 84–9, doi:10.1016/j.ijfoodmicro.2010.01.040.
20. Liu, Y.Y.; Sun, H.Y.; Li, W.; Xia, Y.L.; Deng, Y.Y.; Zhang, A.X.; Chen, H.G. Fitness of three chemotypes of *Fusarium graminearum* species complex in major winter wheat-producing areas of China. *PLoS One* **2017**, *12*, doi:10.1371/journal.pone.0174040.
21. Goswami, R.S.; Kistler, H.C. Pathogenicity and in planta mycotoxin accumulation among members of the *Fusarium graminearum* species complex on wheat and rice. *Phytopathology* **2005**, *95*, 1397–1404, doi:10.1094/PHYTO-95-1397.
22. Kumar, A.; Karre, S.; Dhokane, D.; Kage, U.; Hukkeri, S.; Kushalappa, A.C. Real-time quantitative PCR based method for the quantification of fungal biomass to discriminate quantitative resistance in barley and wheat genotypes to fusarium head blight. *J. Cereal Sci.* **2015**, *64*, 16–22, doi:10.1016/j.jcs.2015.04.005.
23. Muthomi, J.; Schütze, A.; Dehne, H.-W.; Mutitu, E.W.; Oerke, E.-C. Characterization of *Fusarium culmorum* isolates by mycotoxin production and aggressiveness to winter wheat. *J. Plant Dis. Prot.* **2000**, *107*, 113–123.
24. Shin, S.; Son, J.H.; Park, J.C.; Kim, K.H.; Yoon, Y.M.; Cheong, Y.K.; Kim, K.H.; Hyun, J.N.; Park, C.S.; Dill-Macky, R.; et al. Comparative pathogenicity of *Fusarium graminearum* isolates from wheat kernels in Korea. *Plant Pathol. J.* **2018**, *34*, 347, doi:10.5423/PPJ.OA.01.2018.0013.
25. Qu, B.; Li, H.P.; Zhang, J.B.; Huang, T.; Carter, J.; Liao, Y.C.; Nicholson, P. Comparison of genetic diversity and pathogenicity of fusarium head blight pathogens from China and Europe by SSCP and seedling assays on wheat. *Plant Pathol.* **2008**, *57*, 642–651, doi:10.1111/j.1365-3059.2008.01824.x.
26. Maier, F.J.; Miedaner, T.; Hadeler, B.; Felk, A.; Salomon, S.; Lemmens, M.; Kassner, H.; Schäfer, W. Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (*Tri5*) gene in three field isolates of different chemotype and virulence. *Mol. Plant Pathol.* **2006**, *7*, 449–461, doi:10.1111/j.1364-3703.2006.00351.x.
27. Mesterházy, Á.; Bartók, T.; Mirocha, C.G.; Komoróczy, R. Nature of wheat resistance to Fusarium head blight and the role of deoxynivalenol for breeding. *Plant Breed.* **1999**, *118*, 97–110, doi:10.1046/j.1439-0523.1999.118002097.x.
28. Carter, J.P.; Rezanoor, H.N.; Holden, D.; Desjardins, A.E.; Plattner, R.D.; Nicholson, P. Variation in pathogenicity associated with the genetic diversity of *Fusarium graminearum*. *Eur. J. Plant Pathol.* **2002**, *108*, 573–583, doi:10.1023/A:1019921203161.
29. Lemmens, M.; Koutnik, A.; Steiner, B.; Buerstmayr, H.; Berthiller, F.; Schuhmacher, R.; Maier, F.; Schäfer, W. Investigations on the ability of *Fhb1* to protect wheat against nivalenol and deoxynivalenol. *Cereal Res. Commun.* **2008**, *36*, 429–435, doi:10.1556/CRC.36.2008.Suppl.B.36.

30. Proctor, R.H.; Desjardins, A.E.; McCormick, S.P.; Plattner, R.D.; Alexander, N.J.; Brown, D.W. Genetic analysis of the role of trichothecene and fumonisin mycotoxins in the virulence of *Fusarium*. *Eur. J. Plant Pathol.* **2002**, *108*, 691–698, doi:10.1023/A:1020637832371.
31. Eudes, F.; Comeau, A.; Collin, S.R. Phytotoxicite de huit mycotoxines associees a la fusariose de l'epi chez le ble. *Can. J. Plant Pathol.* **2000**, *22*, 286–292, doi:10.1080/07060660009500477.
32. Foroud, N.A.; McCormick, S.P.; MacMillan, T.; Badea, A.; Kendra, D.F.; Ellis, B.E.; Eudes, F. Greenhouse studies reveal increased aggressiveness of emergent canadian *Fusarium graminearum* chemotypes in wheat. *Plant Dis.* **2012**, *96*, 1271–1279, doi:10.1094/PDIS-10-11-0863-RE.
33. Escrivá, L.; Font, G.; Manyes, L. In vivo toxicity studies of fusarium mycotoxins in the last decade: A review. *Food Chem. Toxicol.* **2015**, doi:10.1016/j.fct.2015.02.005.
34. Alassane-Kpembi, I.; Puel, O.; Pinton, P.; Cossalter, A.M.; Chou, T.C.; Oswald, I.P. Co-exposure to low doses of the food contaminants deoxynivalenol and nivalenol has a synergistic inflammatory effect on intestinal explants. *Arch. Toxicol.* **2017**, *91*, 2677–2687, doi:10.1007/s00204-016-1902-9.
35. Foroud, N.A.; Baines, D.; Gagkaeva, T.Y.; Thakor, N.; Badea, A.; Steiner, B.; Bürstmayr, M.; Bürstmayr, H. Trichothecenes in cereal grains - An update. *Toxins* **2019**, *11*, doi:10.3390/toxins11110634.
36. Argyris, J.; Van Sanford, D.; TeKrony, D. *Fusarium graminearum* infection during wheat seed development and its effect on seed quality. *Crop Sci.* **2003**, *43*, 1782–1788, doi:10.2135/cropsci2003.1782.
37. Perkowski, J.; Buśko, M.; Stuper, K.; Kostecki, M.; Matysiak, A.; Szwajkowska-Michałek, L. Concentration of ergosterol in small-grained naturally contaminated and inoculated cereals. *Biologia* **2008**, *63*, 542–547, doi:10.2478/s11756-008-0083-2.
38. Horevaj, P.; Milus, E.A.; Bluhm, B.H. A real-time qPCR assay to quantify *Fusarium graminearum* biomass in wheat kernels. *J. Appl. Microbiol.* **2011**, *111*, 396–406, doi:10.1111/j.1365-2672.2011.05049.x.
39. Nielsen, L.K.; Jensen, J.D.; Rodríguez, A.; Jørgensen, L.N.; Justesen, A.F. *TRI12* based quantitative real-time PCR assays reveal the distribution of trichothecene genotypes of *F. graminearum* and *F. culmorum* isolates in Danish small grain cereals. *Int. J. Food Microbiol.* **2012**, *157*, 384–392, doi:10.1016/j.ijfoodmicro.2012.06.010.
40. Sohlberg, E.; Virkajärvi, V.; Parikka, P.; Rämö, S.; Laitila, A.; Sarlin, T. Taqman qPCR quantification and *Fusarium* community analysis to evaluate toxigenic fungi in cereals. *Toxins* **2022**, *14*, doi:10.3390/TOXINS14010045.
41. Xu, X.; Parry, D.W.; Nicholson, P.; Thomsett, M.A.; Simpson, D.; Edwards, S.G.; Cooke, B.M.; Doohan, F.M.; Van Maanen, A.; Moretti, A.; et al. Is the amount of mycotoxins in cereal grains related to the quantity of *Fusarium* DNA? *Asp. Appl. Biol.* **2003**, *68*, 101–108.
42. Góral, T.; Wiśniewska, H.; Ochodzki, P.; Nielsen, L.K.; Walentyn-Góral, D.; Stępień, Ł. Relationship between Fusarium head blight, kernel damage, concentration of *Fusarium* biomass, and *Fusarium* toxins in grain of winter wheat inoculated with *Fusarium culmorum*. *Toxins* **2019**, *11*, 2, doi:10.3390/toxins11010002.
43. Schnerr, H.; Vogel, R.F.; Niessen, L. Correlation between DNA of trichothecene-producing *Fusarium* species and deoxynivalenol concentrations in wheat-samples. *Lett. Appl. Microbiol.* **2002**, *35*, 121–5, doi:10.1046/j.1472-765X.2002.01146.x.
44. Ochodzki, P.; Twardawska, A.; Wiśniewska, H.; Góral, T. Resistance to Fusarium head blight, kernel damage, and concentrations of *Fusarium* mycotoxins in the grain of winter wheat lines. *Agronomy* **2021**, *11*, 1690, doi:10.3390/AGRONOMY11091690.
45. Nicolaisen, M.; Suproniene, S.; Nielsen, L.K.; Lazzaro, I.; Spliid, N.H.; Justesen, A.F. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *J. Microbiol. Methods* **2009**, *76*, 234–240, doi:10.1016/j.mimet.2008.10.016.
46. Hoheneder, F.; Biehl, E.M.; Hofer, K.; Petermeier, J.; Groth, J.; Herz, M.; Rychlik, M.; Heß, M.; Hückelhoven, R. Host genotype and weather effects on Fusarium head blight severity and mycotoxin load in spring barley. *Toxins* **2022**, *14*, 125, doi:10.3390/TOXINS14020125/S1.
47. Xu, X.-M.; Monger, W.; Ritieni, A.; Nicholson, P. Effect of temperature and duration of wetness during initial infection periods on disease development, fungal biomass and mycotoxin concentrations on wheat inoculated with single, or combinations of, *Fusarium* species. *Plant Pathol.* **2007**, *56*, 943–956, doi:10.1111/j.1365-3059.2007.01650.x.
48. Kimura, M.; Tokai, T.; O'Donnell, K.; Ward, T.J.; Fujimura, M.; Hamamoto, H.; Shibata, T.; Yamaguchi, I. The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes. *FEBS Lett.* **2003**, *539*, 105–110, doi:10.1016/S0014-5793(03)00208-4.
49. Leišová, L.; Kučera, L.; Chrpová, J.; Sýkorová, S.; Šíp, V.; Ovesná, J. Quantification of *Fusarium culmorum* in wheat and barley tissues using real-time PCR in comparison with DON content. *J. Phytopathol.* **2006**, *154*, 603–611, doi:10.1111/j.1439-0434.2006.01154.x.
50. Edwards, S.G.; Pirgozliev, S.R.; Hare, M.C.; Jenkinson, P. Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat. *Appl. Environ. Microbiol.* **2001**, *67*, 1575–1580, doi:10.1128/AEM.67.4.1575-1580.2001.
51. Sarlin, T.; Yli-Mattila, T.; Jestoi, M.; Rizzo, A.; Paavanen-Huhtala, S.; Haikara, A. Real-time PCR for quantification of toxigenic *Fusarium* species in barley and malt. *Eur. J. Plant Pathol.* **2006**, *114*, 371–380, doi:10.1007/s10658-006-0001-9.
52. Góral, T.; Ochodzki, P.; Nielsen, L.K.; Walentyn-Góral, D. Species of the genus *Fusarium* and *Fusarium* toxins in the grain of winter and spring wheat in Poland. *Biul. Inst. Hod. i Aklim. Roślin* **2021**, *296*, 25–42, doi:10.37317/biul-2021-0011.
53. Demeke, T.; Gräfenhan, T.; Clear, R.M.; Phan, A.; Ratnayaka, I.; Chapados, J.; Patrick, S.K.; Gaba, D.; Lévesque, C.A.; Seifert, K.A. Development of a specific TaqMan® real-time PCR assay for quantification of *Fusarium graminearum* clade 7 and

comparison of fungal biomass determined by PCR with deoxynivalenol content in wheat and barley. *Int. J. Food Microbiol.* **2010**, *141*, 45–50, doi:10.1016/j.ijfoodmicro.2010.04.020.

54. Fredlund, E.; Gidlund, A.; Olsen, M.; Börjesson, T.; Spliid, N.H.H.; Simonsson, M. Method evaluation of *Fusarium* DNA extraction from mycelia and wheat for down-stream real-time PCR quantification and correlation to mycotoxin levels. *J. Microbiol. Methods* **2008**, *73*, 33–40, doi:10.1016/j.mimet.2008.01.007.
55. Waalwijk, C.; Van Der Heide, R.; De Vries, I.; Van Der Lee, T.; Schoen, C.; Costrel-de Corainville, G.; Häuser-Hahn, I.; Kastelein, P.; Köhl, J.; Lonnet, P.; et al. Quantitative detection of *Fusarium* species in wheat using TaqMan. *Eur. J. Plant Pathol.* **2004**, *110*, 481–494, doi:10.1023/B:EJPP.0000032387.52385.13.
56. Terzi, V.; Morcia, C.; Faccioli, P.; Faccini, N.; Rossi, V.; Cigolini, M.; Corbellini, M.; Scudellari, D.; Delogu, G. *Fusarium* DNA traceability along the bread production chain. *Int. J. Food Sci. Technol.* **2007**, *42*, 1390–1396, doi:10.1111/j.1365-2621.2006.01344.x.
57. Von der Ohe, C.; Miedaner, T. Competitive aggressiveness in binary mixtures of *Fusarium graminearum* and *F. culmorum* isolates inoculated on spring wheat with highly effective resistance QTL. *J. Phytopathol.* **2011**, *159*, 401–410, doi:10.1111/j.1439-0434.2010.01778.x.
58. Miedaner, T.; Schilling, A.G.; Geiger, H.H. Competition effects among isolates of *Fusarium culmorum* differing in aggressiveness and mycotoxin production on heads of winter rye. *Eur. J. Plant Pathol.* **2004**, *110*, 63–70, doi:10.1023/B:EJPP.0000010136.38523.a9.