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Preharvest Natural Multitoxin Contamination of Winter Wheat Genotypes in Hungary with Special Attention to Aflatoxins and HT-2 Toxin

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Abstract: Toxigenic fungi are among the most significant disease-causing agents in wheat. DON is the most common *Fusarium* mycotoxin, and for a long time, it was the only toxin researched. However, multitoxin data from wheat samples have drawn attention to the fact that much more toxins can be involved in the wheat toxin story than we supposed earlier. For resistance breeding, we need a more detailed approach to identify toxins that occur above the limit and identify the source of the fungal species that produces them. This study analyzed local wheat varieties for fungal infections and natural multitoxin contamination. Eighteen winter wheat genotypes were tested for fungal contaminations across three different locations in 2021 and 2022. Fourteen different mycotoxins—deoxynivalenol, aflatoxins (B1, B2, G1, and G2), fumonisins (B1 and B2), sterigmatocystin, ochratoxin A, zearalenone, T-2, HT-2, and diacetoxyscirpenol—were analyzed using HPLC/triple-quad MS. Toxigenic species such as *Fusarium*, *Aspergillus*, and *Penicillium* had low rates of occurrence, but the toxin contamination was often surprisingly high. Many samples without corresponding fungal infections were also identified as containing mycotoxins. Therefore, the identified fungal infection is less useful for forecasting toxin level. In conclusion, mycotoxin contamination is decisive. Most samples were contaminated by one or more mycotoxins. Although the mycotoxin concentrations typically remained below EU limits, some samples exhibited higher levels, particularly aflatoxins and Ht-2 toxin. Significant variations were observed across year, location, and genotype. For several toxins, significant genotype differences were identified, supporting the hypothesis that resistance may be a useful and suitable control measure. Stability of toxin contamination across years and locations is a very valuable trait; genotypes were identified with low toxin levels and stability (low variance) to all mycotoxins tested. It seems that, in addition to DON, more attention should be given to aflatoxin B1, B2, and G1, which provided similar concentrations. The HT-2 toxin was present in many samples surpassing EU limits. This is the first report on the dangerous occurrence of preharvest-origin aflatoxins and the HT-2 toxin of wheat in Hungary.

Keywords: wheat; mycotoxin; *Fusarium*; *Aspergillus*; multitoxins in wheat; variety responses to toxins; fungal infection

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1. Introduction

Wheat is one of the most important staple foods in the world, but it is jeopardized by many toxigenic fungal species that produce large numbers of mycotoxins with diverse chemical structures. In Hungary, the total wheat production area is approximately one million hectares [1], spanning diverse agronomic and climatic conditions.

Major toxigenic fungi. *Fusarium* species are among the main wheat pathogens in Hungary, posing serious risks to animal and human health [2–6]. The origo or starting point of *Fusarium* science is natural infection and its mass form, epidemics. These give

information about the significance of resistance relations and toxin accumulation and are starting points of all research to reduce damages. The detection of mycotoxins pressed the development of toxin research, and this led to international regulations to control toxin contamination in production, trade, and the food and feed industry. The analysis of toxin relations signalizes which are the ruling toxins, i.e., which *Fusarium* species are responsible for it. The most important control mechanism is surveying the toxin situation with multitoxin methods when it became clear that in most cases, not one but more toxins occur at the same time.

Ear symptoms are easily recognizable, as the diseased spikelets die before ripening, and the grains shrivel. *Fusarium*-damaged kernels (FDKs) often exhibit white, pinkish, or pink discolorations. However, not all dead spikelets indicate infection, as parts of the head can die because of the death of the head axis, preventing nutrient transmission. The spikelets then die, and the grains shrivel but remain healthy, a condition known as bleaching. Hence, on the basis of only a visual assessment, the severity of an epidemic can be easily overestimated. FHB not only leads to a reduction in grain yield but also impairs the quality and usability [7–9]. The main cause of FHB worldwide is *F. graminearum* [10–12], although 15–20 other *Fusarium* spp. can contribute to the production of toxins, and it poses significant risks to food safety [13], sometimes in a dominant manner. *F. graminearum* contamination favors warm, moist conditions during cereal flowering [4,14], while other *Fusarium* species may have different ecological needs. Moreover, changing and warming climatic conditions are promoting the occurrence of *A. flavus* and the production of aflatoxins. *F. graminearum* is found in all of Europe, but in Mediterranean countries [15–18], the first occurrences of aflatoxins of preharvest origin have also been reported. In wheat, this is possible when high temperatures and drought co-occur during June. Aflatoxin in wheat was reported first by Christensen and Kaufmann [19]. Because they classified *A. flavus* as a storage fungus, aflatoxin contamination has traditionally been considered to be of postharvest origin. However, previous results showed that inhibiting factors in wheat's lemma and palea reduced aflatoxin contents by 83% [20], as observed in the wheat variety Sods 12, indicating a potential preharvest resistance factor. The determination of whether freshly harvested grain contains mycotoxins is crucial, as strategies differ for preharvest and postharvest control. If the preharvest character is strong, strategies such as seeking higher resistances and using different toxin-reducing practices, including fungicides, tillage practices, and other tools, should be considered [21]. Data on preharvest contamination of aflatoxins are lacking because of the prevailing storage fungal concept in Hungary. However, this is the case with other Fusarium toxins. Given the recent evidence of a preharvest character in maize [18], hot seasons could potentially support aflatoxin synthesis and other mycotoxins before harvest also in wheat; therefore, this study aims to address this gap.

DON, trichothecenes, and various other mycotoxins [22–24] present significant challenges to food safety. *Aspergillus* species are the most common fungi causing grain spoilage during storage [25], and they can also be found in many crops and soils [26,27]. They primarily infect peanuts, pistachios, maize, and nuts. Reports of aflatoxin in wheat have emerged among others from Egypt [28], Iran [29], Pakistan [30], India [31], Brazil [32], and Nigeria [33,34]. In these countries, the aflatoxin contamination of wheat is a daily problem for human health. I had a PhD student from Pakistan study the economic effect of aflatoxin in wheat. The work was interrupted because of the COVID-19 pandemic, when he had to go home, but it shows the significance of aflatoxin damage. As in the last several years we had 20–25 or more days with top temperatures between 35 and 40 °C, it was reasonable to see whether the problem of aflatoxin is as important in Hungary as in Italy and other Middle Eastern or eastern countries. We had no preharvest data about aflatoxin contamination in wheat. This is true also for T-2 and HT-2 toxins. To start a breeding program, it must be proven that preharvest toxin contamination is a real probability. Therefore, the research was started.

Infection is favored by humid, moderately hot weather, but aflatoxin production sharply increases under dry and hot conditions. Preharvest origin aflatoxin was identi-

fied in maize in Hungary [35], and it was hypothesized that wheat might also have this problem [36]. Dietary exposure to aflatoxins is a major public health concern, and animal husbandry is also severely affected. The main aflatoxins found in food are AFB_1, AFB_2, AFG_1, and AFG_2. Aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2), which are hydroxylated metabolites of AFB_1 and AFB_2, respectively, can be found in milk. AFB_1, the most common and toxic member poses significant health risks [37]. Due to the risks to food and feed safety, several mycotoxins produced by *Fusarium* and *Aspergillus* species are subject to strict EU regulation [38], and many mycotoxins are under consideration for inclusion into the toxin limit list. A more detailed analysis of the mycotoxins is important also to determine which research tasks follow from the data, and a new objective is to see whether variety differences occur in toxin responses of the varieties. So far, we know that such a study was not published until now.

Toxin analyses. With advancements in multitoxin analysis methods [39], increasingly more studies have highlighted complex fungal infections and toxin contamination, along with their food safety risks and long-term effects on human health [40–42]. These methods enable the detection of toxins not only in raw materials but also in human and animal blood and urine [43–45]. Arroyo-Manzanares [46] found over ten mycotoxins in blood and urine samples using an untargeted analysis, suggesting the need for serial targeted tests. Toxins such as T-2, HT-2, OTA (ochratoxin A), dihydrocitrinone, aflatoxins, and NIV (nivalenol) were detected, indicating that DON or ZEN (zearalenone) alone does not fully represent the health risks of a given grain sample. NIV, which is occasionally produced by isolates of *F. graminearum* or *F. culmorum*, is predominantly produced by *F. asiaticum*, causing significant toxin contamination outbreaks in China [10,47]. A previous study [10] also provided valuable data on NIV production by members of the *F. graminearum* clade. For the T-2 and HT-2 toxins, a limit of 10 µg/kg was considered for the EU since 1 07 2024 [48], and values of 15, 25, 50, and 100 ppb were settled upon for different products and uses [49]. Their toxicity (LD_{50} in mice) is 9 µg/kg and has been compared to DON (70 µg/kg for male mice and 49.4 µg/kg for female mice) [50]. Nivalenol is ten times more toxic than DON, making its detection crucial for assessments of food safety risks [50]. HT-2 is at least 5–7 times more toxic. T-2 is considered to be even more dangerous, underscoring its importance in our study; however, the last EC (European Community) [49] set its limits to be the same as those determined for the HT-2 toxin. Unlike aflatoxins, these toxins have not been identified as carcinogens until now. The complexity of these food safety aspects inspired this research to gain an understanding of the problem and, on this basis, to develop better hypotheses [3,51,52] to improve their control.

Alternaria species produce several mycotoxins. As they are not the focus of this study, future research should also investigate this issue [53–56].

High-efficiency liquid chromatography coupled with mass spectrometry, using professional sample preparation, offers the necessary accuracy and precision for today's reliability standards [57–60]. These methods are also employed in testing mycotoxins in blood and urine [46,61,62]. Because these toxins originate from food and feed, investigating the multiplicity of mycotoxin occurrences and identifying their producers are crucial for assessing food safety risks and their extent [49,63].

We have to consider that all epidemics cause highly significant damage and mycotoxin contamination in susceptible varieties, and all EU limits refer to natural contamination [64]. These data provide feedback for growers, such as whether the chosen variety is suitable and whether control methods are as effective as they should be, and for breeders, such as whether a variety has the necessary resistance. Because natural infections or toxin contaminations do not necessarily reflect the level of resistance and do not correspond automatically to toxin levels, without artificial inoculation testing, the problem cannot be managed. We should also consider the behavior of a cultivar as an important aspect.

The main objectives of this study were to identify occurrences of these toxins in different genotypes, locations, and years to obtain solid data on a possible role for resistance in their control, evaluate toxin production for different resistance grades, and identify toxins,

in addition to DON, which we need to control in the future. Determining which toxin was produced in amounts that exceeded the official limit was also important. It was also important to see whether the present fungal identification date is useful to explain toxin contamination or not. This information is essential for developing plant breeding and other protection strategies.

2. Materials and Methods

2.1. Plant Materials and Sample Collection

For the microbiological and analytical tests, 18 winter wheat genotypes from breeding materials of Cereal Research Non-Profit Ltd. (Szeged, Hungary) were used (46.235545, 20.097847). These genotypes were tested in 2021 and 2022 at the following three locations: Szeged in southeast of Hungary, Törökszentmiklós (47.179415, 20.437222) in the middle of the Great Plain, and Iregszemcse (46.684375, 18.181241) in Transdanubia. Figure 1 shows the plant stands in Szeged. Sowing was performed on 5 m^2 plots for four replicated field trials in mid-October of each year. Fungicide was not applied, as the objective was to test the biotic stress resistance. The season's weather profiles are shown in Table S1. The wheat stands in 2021 looked well (Figure 1).

Figure 1. Wheat stands in 7 June 2021 one month after flowering time with no visible FHB infection. In the middle plot, only drying of lower leaves were detected. Because of the dry conditions, only traces of leaf spots were detected in several genotypes; this refers to draught sensitivity.

Head samples were collected a few days before harvest to avoid grain loss in shriveled and small-sized grains by the combine harvester. From each plot, 3 sets of 10 ears were collected separately, threshed, and cleaned by an LD 180 Lab thresher (made by Wintersteiger Seedmech GmbH. Wintersteigerstrasse 1. A-4910 Ried im Innkreis, Austria), taking care to keep every grain independently from its shriveled or infected art. Chaff was removed using an air separator Ets Plaut-Aubry (41290 Conan-Oucques, France) and then prepared for analysis. A total of 324 samples of toxin data were analyzed (18 varieties * 2 years * 3 locations * 3 replicates) for each of the 12 toxins and toxin families as a sum of aflatoxins and fumonisins. This allowed for conducting a regular analysis of variance for each toxin and identify possible variety differences.

2.2. Sample Preparation for Microbiological Analysis

After 10 min surface disinfection with 70% ethanol, the air-dried cereal grains were placed on Dichloran Rose Bengal Agar (DRBC) plates, with 100 grains per genotype and five seeds per Petri dish in a sterile air flow chamber, so 600 grains were tested for each genotype in the two years and three locations. Following one- and two-week incubation on classic PDB agar (Formedium Ltd., The Beeches Barn Fakenham Road, Hillington, King's Lynn, Norfolk PE31 6D, UK, https://formedium.com/, accessed on 12 February 2022), the

internal infection was determined and morphological identification of the colonies was conducted. Fungal colonies designated for further investigation were transferred to fungal-specific DRBC (dichloran Rose Bengal Chloramphenicol) medium. After purification, monosporic isolates were obtained from the fungal wild-type strains. These pure isolates were inoculated in a potato dextrose broth (PDB) solution, and after one week of incubation, the culture was filtered and lyophilized at −110 °C using a Scanvac Coolsafe from Labogene (Lillerod, Denmark). DNA was extracted from lyophilized samples using an "Omega Bio-tek E.Z.N.A. Fungal DNA Mini Kit" (Omega Bio-tek, Inc., Norcross, GA, USA, 400 Pinnacle Way, Suite 450, Norcross, Georgia 30071). Genomic DNA was amplified by PCR using ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′), ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′), TEF1 (5′ ATG GGT AAG GAR GAC AAG AC 3′), TEF2 (5′ GGA RGT ACC AGT SAT CAT GTT 3′), CMD5 (5′ CCG AGT ACA AGG AGG CCT TC 3′), and CMD6 (5′ CCG ATA GAG GTC ATA ACG TGG 3′) primers (Table S2). The amplified DNA was purified using the VWR peqGOLD MicroSpin Cycle-Pure kit. The purified DNA samples were sent to Eurofins GmbH, Germany. The 0.65% severity of *Fusarium* isolates for all species was very low, 62 isolates for 324 grain samples.; this is not suitable for making a correct comparison. By a misunderstanding, species level identification failed in several isolates, but the basic situation did not change.

2.3. Sample Preparation for Analysis

The grain samples were milled to a powder consistency using a Perten Laboratory Mill 3310 (Perten Instruments AB, Stockholm, Sweden). One gram of each sample was weighed into an 8 mL centrifuge tube. The samples were extracted on a vertical shaker (Stuart STR4 rotator, Stuart Equipment, Staffordshire, UK) with 6 mL of acetonitrile:water (AcCN:H_2O, 80:20) solution containing 1% formic acid (HCOOH) for 2 h and 30 min. Following extraction, the samples were centrifuged at 8700 rpm for 10 min using a Heraeus Megafuge 8 (Thermo Fisher Scientific, Waltham, MA, USA). Then, 2 mL of the supernatant was filtered through a Phenex 15 mm, 0.2 µm PTFE syringe filter (Phenomenex, Torrance, CA, USA). The filtered samples were dried using a vacuum evaporation system, and the resultant solid sample was redissolved in 500 µL of the eluent mixture (mobile phases A:B, 50:50). Finally, 5 µL of this solution was injected into the HPLC-MS system.

2.4. Chemicals and Reagents

Ethanol, micro agar, D-glucose monohydrate, potato dextrose agar (PDA), and Dichloran Rose Bengal Agar (DRBC) were purchased from Biolab Ltd. (Budapest, Hungary). Omega Bio-tek's E.Z.N.A. Fungal DNA Mini Kit and the VWR peqGOLD MicroSpin Cycle-Pure kit were purchased from VWR International Ltd. (Debrecen, Hungary). Methanol, acetonitrile, ammonium formate, and formic acid were sourced from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced using an Adrona Connect system (Riga, Latvia). The certified standard solutions were bought from Sigma Aldrich (St. Louis, MO, USA). The working standard solutions of all mycotoxins were prepared by dissolving appropriate volumes of each compound in a mobile phase mixture consisting of 50:50 *v*:*v* H_2O and MeOH, each containing 5 mmol/L ammonium formate and 0.3% formic acid. Stock solutions were subsequently diluted with this mobile phase mixture to obtain the required working solutions for the calibrations. All solutions were stored at −20 °C in covered glass vials in the dark before use.

2.5. HPLC-MS Analysis

The methodology employed was previously described in [63,65,66] and further developed and optimized. An Agilent 1260 Infinity II HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) was used for the chromatographic separation of the selected mycotoxins. A Kinetex Biphenyl column (50 mm × 3 mm i.d. and 2.6 µm particle size), preceded by the use of a SecurityGuard™ ULTRA Holder precolumn, both supplied by Phenomenex (Torrance, CA, USA), facilitated the separation. The mobile phase was a time-programmed

gradient that used H_2O (eluent A) and MeOH (eluent B), both containing 5 mmol L^{-1} ammonium formate and 0.3% (*v*/*v*) formic acid. The gradient elution commenced with 95% eluent A for 1 min; then, eluent B was increased linearly to 100% over 7.5 min and maintained for 2 min. Subsequently, eluent B was reduced linearly to 5% in 1.0 min and equilibrated for 5 min. The flow rate was maintained at 300 µL/min.

The LC system was coupled to an Agilent Ultivo QQQ mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a JET Stream source [67,68]. Triple-quadrupole MS was performed in multiple reaction monitoring (MRM) mode. Agilent MassHunter Workstation software (version 2020) was used to control the HPLC-MS system (Agilent MassHunter Acquisition for LC/TOF and Q-TOF, Quantitative Analysis, and BioConfirm now contain technical controls to support 21 CFR Part 11 and Annex 11 compliance, with our version more).

The capillary voltage and source temperature were set at 3000 V and 120 °C, respectively, with a sheath gas flow rate of 12 L/min and a temperature of 350 °C. Fragmentor and collision energy (CE) values were optimized for each precursor ion and its corresponding product ions. For each compound, at least one precursor and two product ions were used for both identification and quantification. The most abundant product ion was utilized for quantification purposes. Spectrometer parameters for the toxins are given in Table S3.

Validation: To calculate the LOQ values, we used an extract made from a toxin-free blank sample, to which a known amount of pure mycotoxin was added (Table S4). Before starting this experimental series, we received from breeders a larger set of different genotypes between 500 and 1000 g. Based on the results, a genotype was found with no detectable toxin amounts that could be used for spiking and producing the control dilution regression curve to determine the amount of toxins included. This sample was used in the two-year research program. The correlation values were r = 0.9907–0.9976. The LOQ values are given as a signal-to-noise ratio of 1:10. The test was performed with six repetitions. Linearity was tested by evaluating the determination coefficient (R^2). We prepared an eleven-point calibration in a clear solution and by spiked matrix extracts. The requirement was $R^2 > 0.99$, which was achieved for all toxins (0.9918–0.9991). The linearity range covered the detected concentrations in all cases. The calibration curves were examined as a series of 6 independent measurements for all mycotoxins. The selectivity was tested by examining the retention times and the mass spectra of each toxin (Table S2), during 6 independent measurements. The smallest resolution value between two toxins was 1.5. The extraction recovery (R) values were between 85 and 115%, with an average RSD value of 10%. To analyze the matrix effect, we examined the signal suppression/enhancement (SSE) values, which varied between 68 and 113% for each toxin [63,68].

2.6. Statistical Analysis

All analytical data were received with ND remark, where the toxin contamination was lower than the detection limit. This was the case in 30–100% of the cases for the different toxins. For each toxin, 324 data were presented. All tables contain 0.00 instead of ND, as with ND, no statistical analyses can be made. With 0 designation, all data can be considered. The ND allows detectable contamination under the detection limit, with its amount unknown, and does not cause any significant modification but allows the consideration of all data. For us, the ND, e.g., 0.00 values, are extremely important, as for food safety, these are the most important numbers. Their correct use is an obligate task.

For the basic two-way ANOVA, Microsoft Excel's built-in Analysis ToolPak was used. The variety in the differences was clear. From the data, 6 columns (two years and three locations) with three replicates were produced with three independent replicates. Further analysis to separate the location and year was not important, as the LSD 5% value makes a comparison of the significances possible. As this ANOVA does not calculate the replicate effect, we determined the replicate effect for each case, and its SS values and df were extracted from the Within data; therefore, the MQ and F values were recalculated. When the replicate was not significant, the new data were nearly identical with previous

calculations; in cases of significance, larger differences occurred. The replicate effect did not influence the variety, column, and interaction; it only influenced the Within value, which determines the calculations of the MQ and F values. For the replicate effect, we used limit values from the statistical tables in [69], by Svab (1981), and calculated the SS values individually for the replicates and the subtraction number for the ANOVA; for the others, the program provided the significance levels. The correlation analysis was also conducted in Microsoft Excel. The variance was calculated by one-way ANOVA in Microsoft Excel for the six data of the hybrids, and summarized data are presented in the tables of the main text and Supplementary Materials. For principal component analysis, the Statistica 13.0 program was used (Informer Technologies Inc., www.informer.com, Tibco, Santa Clara, CA, USA, accessed on 12 February 2022).

3. Results

3.1. Meteorological Data

The precipitation and temperature data (Table S1) present significant differences on the three locations and two years.

3.2. Microbiological Results, 2021–2022

The severities of the fungal infections were high in Szeged, 53,8 and 45.4% in 2021 and 2022; medium in Törökszentmiklós (39.2 and 40.8%); and the lowest in Iregszemcse (21.6 and 28%). Considering the fact that altogether, 38% of the total grains investigated had infections, the percentual infection percentage was really less infected as 62% of the grains did not develop any colony, so the percentage to the total number of tested grains were 62% less. The grain infection is presented in Figure 2.

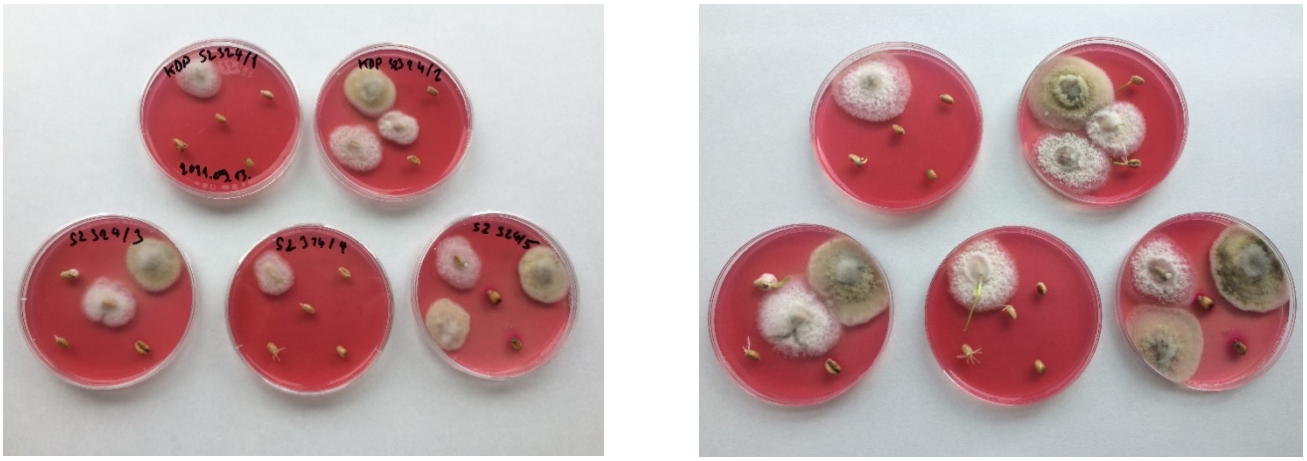

Figure 2. Fungal contamination of a winter wheat sample from Szeged on DRBC medium ((**left**): after one week; (**right**): after two weeks).

The prevalence of toxigenic fungi was low. Among the total isolates, only 1.7% were *Fusarium* species and 0.6% were *Aspergillus* species. Although *Penicillium* was considered as a storage fungus and its toxins were not the focus of this study, the quantity of *Penicillium* isolates was more than double (1.4%) that of *Aspergillus*. *Alternaria* spp. occurs at a high rate (78%); it produces several mycotoxins, but it has never been considered as a severe toxin producer in wheat. Relating this to the total number of tested grains, the proportion of *Fusarium, Aspergillus*, and *Penicillium* spp. is only 0.65, 0.23, and 0.53%, respectively. This low infection severity does not mean a serious infection; 0.65% of 9800 grains is 62 isolates for all species, and at this low number, even with all isolates identified, a correct comparison does not help. This was the main reason why we did not compare the fungal and toxin matrix.

3.3. Analytical Results, 2021–2022

The yearly averages for the 18 genotypes (Table 1) provide an overview of the measured toxin concentrations. The T-2 and HT-2 toxins were detected regularly at low levels [2], but their toxin production could not be controlled 40–50 years ago. Surprisingly, HT-2 had a greater level of significance. In 2021, some samples exhibited exceptionally high concentrations of AFB_1 that were over the limit. Similarly, in 2022, notable concentrations were found for AFB_1, AFB_2, and AFG_1 (Table 2); in many cases, high differences between minimum and maximum values in both years are given. Especially large differences were found for DON; HT-2; ZEN; and aflatoxins, except AFG2.

Table 1. Mean toxin concentrations of 14 mycotoxins in 18 winter wheat genotypes across three locations in Hungary, 2021–2022. Data presented as μg/kg.

Location	Szeged		Törökszentmiklós		Iregszemcse		Mean	Variance
Toxin	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**		
AFG_2	0.00 *	0.00	0.00	0.00	0.00	0.00	0.00	0.0
NIV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0
STC	0.32	0.03	0.29	0.04	0.34	0.04	0.22	0.0
FB_2	1.08	0.07	0.46	0.00	0.58	0.16	0.42	0.4
OTA	0.36	0.00	0.36	0.00	0.29	0.00	0.49	0.3
AFB_2	0.00	0.13	0.00	0.00	0.00	0.50	0.79	1.7
AFB_1	2.00 *	0.21	0.00	0.00	0.00	0.21	0.83	2.5
DAS	0.70	0.20	0.71	0.11	0.64	0.42	0.93	0.0
AFG_1	0.00	0.00	0.00	0.81	0.00	0.38	1.05	1.4
FB_1	8.33	0.23	5.01	0.00	5.52	1.10	2.64	8.3
AFs	2.00 *	0.34	0.00	0.81	0.00	1.10	2.67	1.9
T-2	0.00	0.42	0.00	0.06	0.00	0.00	3.01	21.3
ZEN	8.27	0.11	10.01	0.33	5.70	0.54	13.03	56.6
HT-2	23.63 *	2.46	33.21 *	3.64	31.23 *	7.41	22.52 *	173.9
DON	93.99	0.00	16.39	51.58	11.71	7.55	30.19	1694.8

* Above EU limit: 2 μg/kg for AFB1, for total aflatoxin: 4 μg/kg, for HT-2: 10 μg/kg, * 0.00 = mycotoxin contamination below detection limit.

Table 2. Mycotoxin contaminations in the wheat genotype trials, as determined by multitoxin analysis, 2021–2022, Szeged, Hungary (μg/kg). Number of contaminated samples from 162 (per year) in total, maximum and minimum values, and toxin general means.

Max/Min	No. *	Max	Min	Mean	No.	Max	Min	Mean
Mycotoxin	**2021**				**2022**			
DON	52	987.58	0.00	40.70	17	215.05	0.00	19.71
FB_1	52	15.27	0.00	6.29	7	4.77	0.00	0.44
FB_2	25	2.74	0.00	0.71	4	1.21	0.00	0.07
AFB_1	10	5.68	0.00	0.67	4	2.90	0.00	0.14
AFB_2	0	0.00 **	0.00	0.00	5	3.32	0.00	0.21
AFG_1	0	0.00	0.00	0.00	9	3.93	0.00	0.40
AFG_2	0	0.00	0.00	0.00	0	0.00	0.00	0.00
HT-2	47	72.61	0.00	29.06	25	26.54	0.00	4.59
T-2	0	0.00	0.00	0.00	2	7.55	0.00	0.16
ZEN	23	41.11	0.00	7.99	3	9.68	0.00	0.33
DAS	43	1.75	0.00	0.68	15	4.16	0.00	0.24
OTA	47	1.27	0.00	0.34	0	0.00	0.00	0.00
STC	53	0.49	0.00	0.32	19	0.22	0.00	0.04
NIV	0	0.00	0.00	0.00	0	0.00	0.00	0.00

* For any toxin for a year, we have 3 locations, 3 replicates, and 18 varieties, i.e., 162 data. The No. shows how many from these 162 showed contamination above the detection limit for the toxin in question. In the two years for all toxins, 324 data were evaluated. ** 0.00 = mycotoxin contamination below detection limit.

The principal component analysis determined two factors representing 89.97% of the variation (Figure 3). It seems that the data are determined mostly by Factor 1, where Sz2022 seems to be an independent group; Ir2021 anT2021 and Ir2022 produce another group; and in the third group, Sz2021 and T2022 can be classified. It seems that years and locations have a role only in Iregszemcse; the other data cannot be classified into the same group.

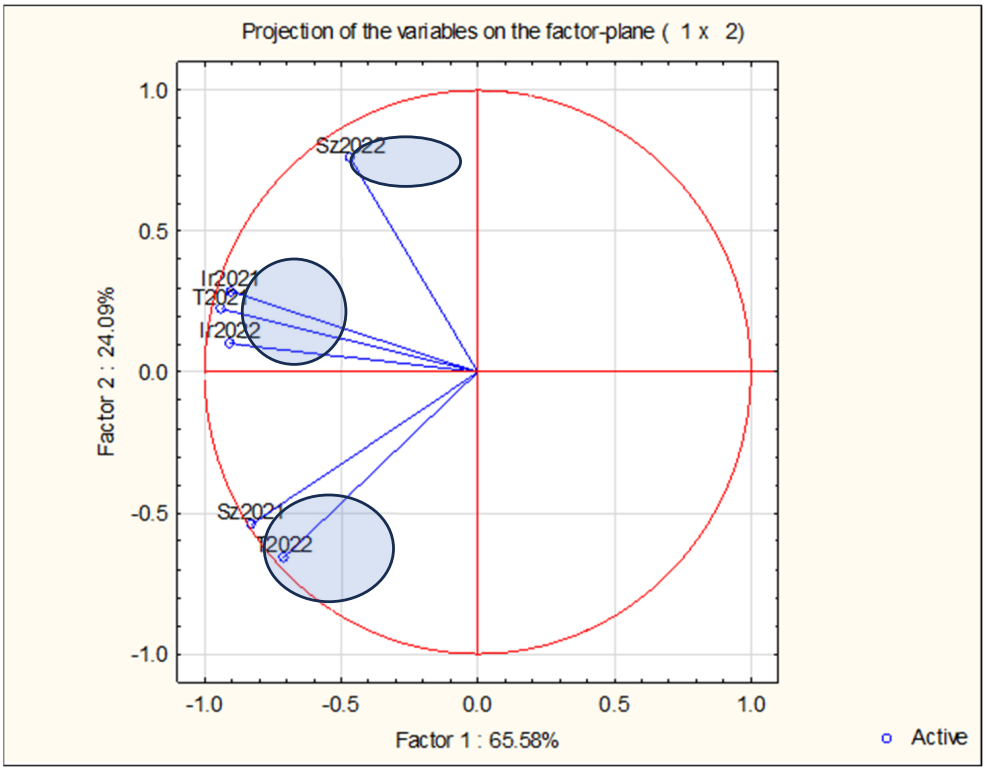

Figure 3. Principal component analysis of toxin contamination for years and locations. Sz = Szeged, Ir = Iregszemcse, T = Törökszentmiklós.

The past two seasons were dry and hot, yet low toxin concentrations were expected. Significant differences among genotypes were noted (Table 3) for DON, FB_2, and their combined totals. The AFB_1 and AFG_1 concentrations did not vary significantly among genotypes, and only AFB_2 had significant differences, though its combined totals did not. From the others, only T-2, DAS, and OTA showed significant differences. The significant differences exhibited by the varieties is one trait to consider when looking at the different toxins.

The correlation analysis (Table 4) of the contamination levels of the different toxins in the genotypes revealed that significant relationships were generally rare. For instance, FB data correlated significantly (except FB_1/FB_2). The combined totals of AFB_1, AFB_2, AFG_1, and AFG_2 were significantly correlated with the concentrations of the three individual aflatoxins (AFB_1, AFB_2, and AFG_1), but no significant correlations among the AF variants were found (Table 4). Additionally, the HT-2 toxin exhibited a significant correlation with ZEN and DAS, and a significant correlation was also observed between AFB_2 and DAS.

Given these results and considering the individual concentrations observed, it is necessary to present the data and ANOVAs for each toxin, mostly provided in the Supplementary Tables but some are in the main text. The genotypes are ranked according to the genotype means in all tables.

Table 3. Mycotoxin contamination in wheat genotype trials as determined by multitoxin analysis, 2021–2022, Szeged, Hungary, µg/kg. Mean data for genotypes across years and locations.

Genotype Code	DON	FB_1	FB_2	$FB_1 + FB_2$	AFB_1	AFB_2	AFG_1	$AFB_1 + AFB_2 + AFG_1$	HT-2	T-2	ZEN	DAS	OTA	STC	Variance
9	8.32	3.82	0.25	4.07	0.62	0.00 *	0.00	0.62	8.32	0.00	0.00	0.17	0.33	0.15	1.90
14	4.73	3.32	0.67	3.99	0.00	0.00	0.00	0.00	18.23	0.00	0.00	0.24	0.14	0.19	2.25
11	6.67	2.77	0.43	3.20	0.55	0.00	0.00	0.55	14.00	0.00	4.13	0.44	0.22	0.19	2.37
4	9.86	2.37	0.34	2.71	0.60	0.00	0.00	0.60	15.00	0.00	2.81	0.44	0.11	0.19	2.50
18	10.21	2.76	0.24	3.00	0.83	0.00	1.16	2.00	14.88	0.00	2.90	0.45	0.22	0.17	2.77
8	7.60	2.92	0.43	3.35	0.61	0.45	0.37	1.42	19.42	0.00	2.36	0.24	0.37	0.19	2.84
7	8.22	2.79	1.00	3.79	0.51	0.00	0.62	1.14	18.37	0.00	6.00	0.17	0.08	0.16	3.06
3	12.63	4.00	0.17	4.17	1.00	0.23	0.00	1.23	20.36	1.26	0.00	0.36	0.11	0.16	3.26
13	25.00	4.39	0.43	4.82	0.00	0.00	0.37	0.37	5.89	0.00	4.94	0.00	0.11	0.15	3.32
2	10.56	3.05	0.00	3.05	0.00	0.00	0.23	0.23	20.86	0.00	8.99	0.53	0.20	0.17	3.42
12	28.70	1.82	0.00	1.82	0.95	0.00	0.22	1.17	13.44	0.00	4.47	0.46	0.14	0.12	3.81
16	17.10	3.28	0.00	3.28	0.76	0.55	0.00	1.32	26.19	0.00	5.58	1.35	0.22	0.18	4.27
10	29.11	3.71	1.03	4.74	0.15	0.14	0.00	0.30	19.59	0.00	3.61	0.46	0.15	0.20	4.51
6	41.69	2.60	0.51	3.11	0.22	0.51	0.39	1.12	20.54	0.00	5.22	0.82	0.19	0.19	5.51
5	52.45	3.81	0.28	4.09	0.00	0.00	0.18	0.18	11.12	0.00	4.59	0.48	0.14	0.20	5.54
15	50.37	3.78	0.49	4.27	0.00	0.00	0.00	0.00	20.06	0.00	2.64	0.37	0.11	0.22	5.88
1	45.98	5.44	0.78	6.22	0.49	0.00	0.00	0.49	31.16	0.19	12.99	0.80	0.07	0.13	7.48
17	174.44	3.94	0.00	3.94	0.00	0.00	0.00	0.00	7.33	0.00	3.66	0.56	0.14	0.21	13.87
Mean	30.00	3.37	0.39	3.76	0.41	0.10	0.20	0.71	16.93	0.08	4.16	0.46	0.17	0.18	4.36
LSD 5% genotype	35.99	ns	0.54	2.11	ns	0.10	ns	ns	ns	0.45	ns	0.39	0.09	ns	1.90

* 0.00 = mycotoxin contamination below detection limit. Aflatoxin G2 is missing as n contamination was found. So, the total aflatoxin contamination is correct.

Table 4. Mycotoxin contamination in naturally infected winter wheat samples. Correlations between the data of Table S3.

	DON	**FB_1**	**FB_2**	**$FB_1 + FB_2$**	**AFB_1**	**AFB_2**	**AFG_1**	**$AFB_1 + AFB_2 + AG_1$**	**HT-2**	**T-2**	**ZEN**	**DAS**	**OTA**
FB_1	0.314												
FB_2	−0.223	0.226											
FB1 + FB2	0.200	0.946 ***	0.527 ***										
AFB_1	−0.411	−0.327	−0.237	−0.364									
AFB_2	−0.121	−0.157	−0.096	−0.169	0.247								
AFG_1	−0.211	−0.336	0.048	−0.278	0.194	−0.007							
$AFB_1 + AFB_2 + AFG_1$	−0.406	−0.433	−0.153	−0.428	0.803 ***	0.483 ***	0.645 **						
HT-2	−0.270	0.153	0.292	0.230	0.197	0.434	−0.124	0.202					
T-2	−0.096	0.281	−0.128	0.202	0.419	0.145	−0.184	0.210	0.215				
ZEN	0.125	0.282	0.136	0.291	−0.150	−0.051	0.060	−0.078	0.483 *	−0.22			
DAS	0.184	−0.001	−0.276	−0.092	0.166	0.550	−0.188	0.188	0.587 **	−0.04	0.43		
OTA	−0.253	−0.268	−0.297	−0.332	0.235	0.414	0.130	0.352	−0.132	−0.22	−0.32	0.00	
STC	0.331	−0.034	0.095	0.002	−0.523	0.146	−0.227	−0.394	−0.085	−0.19	−0.34	0.089	0.055

*** $p = 0.001$, ** $p = 0.01$, * $p = 0.05$.

The DON data (Table S5) were all below 1.25 mg/kg, three samples surpassed the baby food limit (0.2 mg/kg) and one surpassed the adult pig limit (0.9 mg/kg). The piglet limit corresponds to the human baby food limit, which 3 of the 108 samples exceeded. Except Szeged 2022, all other locations and years showed DON contamination in traces or below the detection limit (designed by 0). However, most of the samples contained a high amount of DON; in epidemic years, such as 2019, the maximum levels were between 5000 and 10,000 µg/kg. The differences among the varieties were highly significant, and the variance data clearly show which ones are risky, even under such conditions. The column effect was also highly significant.

F. verticillioides has never been considered a serious wheat pathogen. Out of thousands of wheat isolates, only several items belonging to this fungus have been identified [2]. As this fungus infects maize under warm, dry conditions, the levels of contamination with fumonisins have increased, which led to the question of whether this is also true for wheat. All fumonisin FB1 data (Table S6) showed very low levels; thus, there was no reason to expect serious contamination in wheat. No genotype differences were measured. However, the replicates and columns presented significant deviations. The FB_2 data (Table S7) were much lower, at only 0.47 mg/kg, and FB_1 was at 3.37 (rate of 9:1), much less than that of maize, for which the rate is about 3:1. In spite of this, the genotype differences were significant, but the replicate effect was not. The fumonisin sum (Table S8) did not show significant differences by variety, at $p = 0.05$, but the column differences were significant at $p = 0.1$.

The aflatoxin B_1 data (Table S9), except Szeged, were all lower than the limit of 2.0 µg/kg. In Szeged, eight genotypes were identified with values between 2 and 4 µg/kg, which definitely exceeds the limit for human consumption. Additionally, two genotypes yielded 4.99 and 5.68 µg/kg, which alone surpass the 4 µg/kg aflatoxin limit in humans. The AFB_2 data are surprising (Table S10). For Szeged, two genotypes were found to be below the limit for AFB_2 in 2022; in Iregszemcse, three surpassed the limit of 2 µg/kg; and other locations were free of mycotoxin contamination. The genotype and column data were highly significant, and the replicate effect was below the limit, but the location/genotype data interacted significantly, e.g., the presence of the toxin was genotype- and location-dependent. For AFG_1, six samples surpassed the 2 µg/kg limit (Table S11), but this toxin was not present for Szeged. However, the levels of contamination were within the same range as those found for AFB_1 and AFB_2. AFG_2 was not detected in any of the samples, all with values of zero; therefore, we did not consider these data alone or in sums of the total aflatoxin contamination. The total aflatoxin content (Table 5) shows that aflatoxins were detected in 24 samples, with 15 samples surpassing the 2 mg/kg limit and an additional 5 genotypes summing to higher than 4 µg/kg of the total aflatoxin limit.

Considering the aflatoxin data for the three different toxins, a question arose concerning how the production of different aflatoxins is related among the different genotypes. Moreover, what are the differences in aflatoxin contaminations among the different genotypes. In Table 6, we list data on the three aflatoxin variants with at least one positive data point in the column. There were three varieties that were not contaminated with aflatoxins according to the data, and there were another three with aflatoxin contamination levels below the limit (2 µg/kg); thus, for them, the reaction was stable. It seems that the aflatoxin variants had very similar toxin concentrations, with maximums for Szeged and Iregszemcse of above 4 µg/kg and for Törökszentmiklós between 2 and 3 µg/kg. This differs greatly from maize, for which AFB_1 is normally one hundred times larger than AFB_2. It is remarkable that good producers of aflatoxin B1 may not synthetize the other two toxins, such as genotypes 1, 4, 9, 14, and 16. It happens that an aflatoxin B_1 nonproducer synthetizes the other toxins alone or both like genotypes 2 and 6. Number 7 produces all three toxin variants. Genotype 11 is a good producer of AFB_1 and AFG_1 but negative for AFB_2. The lesson is that, on its own, AFB_1 is not sufficiently significant as a measurement, and all three should be used to avoid poisoning due to a mistaken belief that food is safe.

Table 5. Multitoxin wheat contamination test: Aflatoxin (B_1 + B_2 + G_1) contamination, data presented as µg/kg, 2021/2022.

Variety	Szeged		Iregszemcse		Törökszentmiklós		Mean	Variance
	2021	2022	2021	2022	2021	2022		
5	0.00 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15	0.00	0.00	0.00	0.00	0.00	1.08	0.18	0.20
13	0.00	0.00	0.00	0.00	0.00	1.40	0.23	0.32
10	0.00	1.78	0.00	0.00	0.00	0.00	0.30	0.21
2	0.00	0.00	0.00	0.00	0.00	2.23	0.37	0.83
1	2.96	0.00	0.00	0.00	0.00	0.00	0.49	1.46
14	3.29	0.00	0.00	0.00	0.00	0.00	0.55	1.81
4	3.61	0.00	0.00	0.00	0.00	0.00	0.60	2.18
9	3.75	0.00	0.00	0.00	0.00	0.00	0.62	2.34
6	0.00	0.00	0.00	4.36	0.00	2.36	1.12	2.02
8	3.07	0.00	0.00	3.75	0.00	0.00	1.14	3.14
16	5.68	0.00	0.00	0.00	0.00	1.33	1.17	5.16
3	3.08	4.31	0.00	0.00	0.00	0.00	1.23	2.16
12	2.82	0.00	0.00	5.07	0.00	0.00	1.32	2.54
7	2.82	0.00	0.00	3.51	0.00	2.21	1.42	2.02
11	4.99	0.00	0.00	3.05	0.00	3.93	2.00	5.16
Mean	2.00	0.34	0.00	1.10	0.00	0.81	0.71	1.75
Data above 2 mg/kg limit				Data above 4 mg/kg limit				

ANOVA

Source	SS	df	MS	F	*p*-value	F crit.	LSD 5%
Variety A	103.64	17	6.10	1.17	0.3327624	1.67	ns **
Columns B	160.93	5	32.19	6.20	3.675×10^{-5}	2.26	0.85
Replicates	60.48	2	30.24	5.83	0.01	4.71	0.60
AxB	402.83	85	4.74	0.91	0.7620836	1.33	ns
Within	1111.70	214	5.19				
Total	1839.58	323					

* 0.00 = mycotoxin contamination below detection limit. ** ns: not significant.

The principal component analysis determined based on an eigenvalue above 1 defines six principal components (Figure 4). From these, Factor 1 gives 26.31% of the variance and FB_1, FB sum (FB_1 + FB_2), AB1, AB2, and AF sum are classified in this group. This means that the variety reactions to these toxins have some similarity. HT-2, ZEN, and DAS belong to Factor 2; Factor 4 involves FB2, AG1, and T-2; and DON (Factor 3), STC (Factor 5), and OTA (Factor 6) belong to separate factors. Factor 1 contains fumonisins and aflatoxins, indicating some common basis in variety responses, but FB_2 and AG1 belong to Factor 4. ZEN and DON are in separate factors, even though they are produced by the same fungus. T-2 and HT-2 belong also to different factors, even though they are strongly related compounds and produced by—as currently known—by the same fungi. When we look at the factor loadings in detail, FB2 could belong also to Factor 3, with –0.52072 compared with −0.5509 in Factor 4. OTA could belong also to Factor 1 with its 0.56295. AB2 could also belong, with its value above 0.50, to Factor 2. In its classification, DAS has a value of 0.69723, but in Factor 3, it has a value of 0.61289. These conclusions are similar to those received from the correlation matrix, but possible relations are clearer. However, we should be very careful when drawing final conclusions for genetics, as this is the first step on a long road to better understanding what the results mean.

Table 6. Occurrence of AFB_1, AFB_2, and AFG_1 in field-collected wheat samples for the identification of a preharvest presence across three locations in Hungary, 2021–2022.

Genotype	AFB1 µg/kg		AFB2 µg/kg		AFG1 µg/kg		Mean	Variance
	2021	2022	2022	2022	2022	2022		
	Szeged	Szeged	Szeged	Ireg *	Ireg	Torok **		
5	0.0 *	0.0	0.0	0.0	0.0	0.0	0.00	0.00
17	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
18	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
15	0.0	0.0	0.0	0.0	0.0	1.1	0.18	0.20
13	0.0	0.0	0.0	0.0	0.0	1.4	0.23	0.32
10	0.0	0.9	0.9	0.0	0.0	0.0	0.30	0.21
2	0.0	0.0	0.0	0.0	0.0	2.2	0.37	0.83
1	3.0	0.0	0.0	0.0	0.0	0.0	0.49	1.46
14	3.3	0.0	0.0	0.0	0.0	0.0	0.55	1.81
4	3.6	0.0	0.0	0.0	0.0	0.0	0.60	2.18
9	3.7	0.0	0.0	0.0	0.0	0.0	0.62	2.34
6	0.0	0.0	0.0	3.1	0.0	2.4	0.91	2.02
12	2.8	0.0	0.0	3.3	0.0	0.0	1.02	2.54
8	3.1	0.0	0.0	0.0	3.7	0.0	1.14	3.14
16	5.7	0.0	0.0	0.0	0.0	1.3	1.17	5.16
3	3.1	2.9	1.4	0.0	0.0	0.0	1.23	2.16
7	2.8	0.0	0.0	2.7	0.0	2.2	1.29	2.02
11	5.0	0.0	0.0	0.0	3.0	3.9	2.00	5.16
Mean	2.0	0.2	0.1	0.5	0.4	0.8	0.67	1.75

Highlight: green, zero across all years and sites; light blue, no significant occurrence in all cases; yellow, higher than limit (2 mg/kg) in one or more cases * 0.0 = mycotoxin contamination below detection limit. * Iregszemcse, ** Torokszentmiklos. Bold: highly stable with no or only under EU limit contamination for all aflatoxin versions

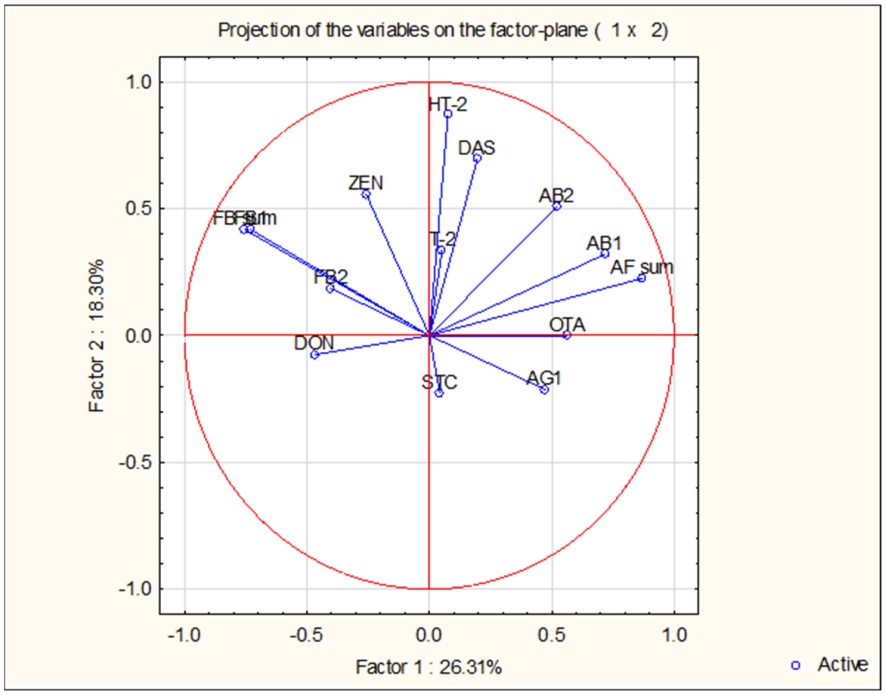

Figure 4. Principal component analysis of the toxins, based on variety data from Table 3. AB1 = AFB_1, AB2 = AFB_2, AG1 = AFG_1, AF sum = AFB_1 + AFB_2 + AFG_1.

The HT-2 and T-2 toxins are newly regulated as of 2024 (EC 2024). The limits are the same for both toxins. As the data show, 2021 was a strong epidemic year for the HT-2 toxin (Table 7), and mean contamination levels varied between 23 and 33 µg/kg. In 2022, ten-fold lower contamination levels were observed. Three genotypes had average concentrations below 10 µg/kg, but two genotypes surpassed the limit of 25 mg/kg. With baby food, concentrations must remain lower than 15 µg/kg. For bread, pastries biscuits, etc., the upper limit is 25 µg/kg. Other milled products have a limit of 50 µg/kg. The differences among the varieties were not significant, but the replicate effect was significant, indicating larger differences among the replicates, whereby the first and other replicate means had larger deviations. On the other hand, the six-fold difference among the varieties leaves hope that, with an improved methodology with artificial inoculation together with natural infection and toxin data, it would be possible to display statistically significant differences. The stabilities varied between 68 and 813; thus, there is the possibility of identifying stable performant genotypes at low toxin levels. The stability (variance) of the HT-2 contamination also varied significantly among genotypes.

Table 7. Multitoxin wheat contamination test: natural infection and total HT-2 toxin, data presented as µg/kg, for 2021/2022.

Variety	Szeged		Iregszemcse		Törökszentmiklós		Mean	Variance
	2021	2022	2021	2022	2021	2022		
2	0.0 *	4.1	0.0	0.0	31.3	0.0	5.9	157.4
17	0.0	0.0	0.0	0.0	33.8	10.2	7.3	185.0
9	0.0	5.1	21.1	0.0	23.7	0.0	8.3	123.3
15	23.3	0.0	22.3	7.6	8.0	5.5	11.1	90.1
16	26.3	12.0	14.6	5.3	18.1	4.3	13.4	68.4
14	36.2	0.0	14.0	4.7	21.8	7.4	14.0	175.3
11	14.8	5.5	12.4	12.0	38.7	5.8	14.9	150.5
4	49.0	0.0	22.6	5.2	13.2	0.0	15.0	351.8
18	17.2	0.0	46.1	0.0	46.0	0.0	18.2	510.1
8	15.6	0.0	51.9	11.6	31.1	0.0	18.4	402.7
7	0.0	0.0	66.1	14.8	35.6	0.0	19.4	719.1
10	31.6	0.0	22.8	9.0	54.2	0.0	19.6	446.0
5	20.5	9.7	45.5	11.7	33.0	0.0	20.1	278.7
3	17.2	0.0	34.1	19.7	51.1	0.0	20.4	394.7
6	0.0	0.0	72.6	20.7	29.9	0.0	20.5	813.0
13	62.5	7.9	31.7	0.0	23.1	0.0	20.9	579.8
12	54.0	0.0	37.3	0.0	59.9	5.9	26.2	763.3
1	57.1	0.0	47.1	11.0	45.2	26.5	31.2	504.4
Mean	23.6	2.5	31.2	7.4	33.2	3.6	16.9	
LSD 5%							n.s.	
LSD 5% col.							10.0	

ANOVA

Source	SS	df	MS	F	*p*-value	F crit.	LSD 5%
Variety A	12,706.76	17	747.46	1.11	0.38	1.67	ns
Columns B	53,503.33	5	10,700.67	15.84	5.53×10^{-13}	2.26	9.93
Replicates	5030.58	2	2515.29	3.72	0.01	3.04	7.02
AxB	47,200	85	555.29	0.82	0.88	1.33	ns
Within	144,592.7	214	675.67				
Total	263,033.8	323					

* 0.00 = mycotoxin contamination below detection limit. Highlight: dark green, lower than 15 µg/kg; light green, between 15 and 25 µg/kg; orange, worse than the highest limit value µg/kg; white, value between 25 and 50 µg/kg, can be used for some animals and special products [49].

The T-2 toxin data show extremely low levels (Table S12) compared to the HT-2 toxin data. For 16 genotypes, the results were zero, and in genotypes 1 and 3, a one–one sample indicated traces of contamination. In spite of this, a single genotype differed significantly from the other 17 genotypes. It is remarkable that these two closely related toxins exhibit such great differences in their contamination levels. On the basis of earlier pilot tests, no zearalenone (Table S13) was found in the freshly harvested wheat samples, even those originating from artificial inoculation and often with high levels of FDK. This data set suggests that more attention must be paid to this toxin because in six samples, the ZEN concentrations surpassed the baby food limit, a concentration which can cause thelarche. Three genotypes were found to have no contaminations, and a further seven had a single contaminated sample, out of six, of between 10 and 20 μg/kg. Of the ANOVA sources, only the column effect was significant, for which 2021 showed higher values, whereas 2022 presented only zeros and traces. DAS was similar to T-2, with most data points being lower than one μg/kg, with the three replicates not being significantly different despite these low values (Table S14), and its level of significance was very moderate. The situation was similar for OTA (Table S15), for which all values, except one, was lower than 1 μg/kg. The varieties and columns differed significantly from each other. It seems that OTA is not the most important preharvest toxin. The STC (sterigmatocystin) seems to be a special case (Table S16). All genotypes in 2021 had similar values between 0.11 and 0.42 μg/kg, and in 2022, between 0 and 0.2 μg/kg, with means between 0.12 and 0.22 μg/kg without significant differences. Only the two years differed significantly from each other.

3.4. Comparison of the Fungal and Toxin Data

As the number of *Fusarium* isolates was very low (n = 62), the fungal isolation and mycotoxin data in many cases did not agree, mostly because of the low number of identified fungal species; the data will not be analyzed, but the consequences will be commented upon in the Section 4.

4. Discussion

4.1. Fungal Contamination

The pattern of fungal contamination in our study was typical, with high rates of *Alternaria* and other genera, but toxigenic fungi such as *Fusarium* spp. and *Aspergillus* spp. represented only 1.7% and 0.6% of the isolates, respectively; 38% of the total tested grains showed fungal infection. Therefore, only 0.65% and 0.23% had severe infections compared to the total number of grains tested. In Szeged, we evaluated the natural FHB infection in the field; in essence, no heads had visible infection (incidence, percentage of heads showing infected spikelets). As nearly all FHB studies deal with *F. graminearum* and DON, but as the recent mycotoxin analyses from human blood samples detected in many cases a high number of mycotoxins [43–45], we felt the need to test multitoxin presence in wheat grains. In dry years, *Fusarium* infection is normally low; therefore, this was not a surprise.

The focus was on toxins produced by the *Aspergillus* and *Fusarium* species. It was observed often that toxins can be present in samples even in the absence of fungal strains being isolated. This mirrors findings in maize for which aflatoxin levels varied significantly irrespective of visible fungal growth [7]. Multiple *Fusarium* species can synthesize the same toxin [68], complicating the attribution of specific toxins to individual species. The same complexity applies to *Aspergillus* species, of which not only *A. flavus* but also *A. parasiticus* and other members of the section *Flavi* can be involved, making it difficult to predict aflatoxin levels solely on the basis of the occurrence of *A. flavus*. The HT-2 and T-2 toxins are mostly produced by *F. sporotrichioides*, *F. sambucinum*, *F. poae* [68], and *F. langsethiae* [70], but to start a breeding program, it is important to know which is the main producer of these toxins. However, T-2 and HT-2 cannot be explained by several *F. sporotrichioides* isolates and this does not explain the wide occurrence of the HT-2 toxin and low contamination by T-2 toxin. Zearalenone contamination seems to be an easier problem to solve, as its producer is the known DON producer *F. graminearum* and *F. culmorum*; therefore, we suppose that

a resistance to DON may mean a resistance to ZEN also, but no direct scientific results support this hypothesis. However, these two toxins landed in different principal component factors. Additionally, most toxins have masked forms, and as their numbers are reasonably high, we did not discuss them and they were not tested here, but to develop a full food safety risk profile, their presence should also be investigated.

In Hungary, the primary producers of HT-2 and T-2 toxins are *F. sporotrichioides* and *F. acuminatum* [2,71]. *F. acuminatum* is another possible producer, but no toxin data from Hungary were presented until now. In Northern Europe, *F. langsethiae* is also known for producing these toxins [72], and *F. poae*, more commonly associated with nivalenol production, can synthesize T-2 and HT-2, typically at lower levels [73]. The concentrations of T-2 and HT-2 in Hungary was similar to those in other countries [71]. According to the EFSA, they have the same level of toxicity [48,49]. However, our results indicate differing behaviors between these two toxins in wheat; T-2 appears infrequently and at low concentrations, whereas HT-2 is consistently found at higher levels, with no genotype being entirely free from contamination. This suggests varying ecological preferences, genetic factors, or pathogens influencing their production. Previous tests on *F. sporotrichioides* resistance showed a medium pathogenicity; even highly susceptible genotypes were identified with significant *Fusarium*-damaged kernels (FDKs), but no toxin tests were made. The conclusion is that the fungal composition does not say too much about the possible toxin levels; therefore, the direct toxin measurements cannot be spared.

We have another problem, too. For *Fusarium* spp. identification, we can start from identifying all *Fusarium* colonies developed; in this case, 3–5% infection can be regularly achieved also in non-epidemic years. This was the case for [12], and they identified a significantly twice higher infection rate for *F. poae* than for *F. graminearum* in Hungary: *F. graminearum* 0.33% and *F. poae* 0.73%. We published [2] *Fusarium* spp. from visible infected spikelets or grains as we wanted to know the real disease-causing species (n = 3619) for 1970–1983, and 2310 *F. graminearum*, 75 *F. sporotrichioides*, and 53 *F. poae* isolates were identified. This means that a general *Fusarium* species evaluation may be misleading regarding the real significance of the given *Fusarium* spp. Therefore, according to [12], the dominating species becomes *F. poae* in Hungary and not *F. graminearum*. The only problem is that the two databases that were compared originated from a population isolated from diseased species or considered all isolates from all grains, including those without visible infection). At that time, nobody spoke about toxins, so the food safety significance could not be presented. However, we know from national epidemics in 1997–1999, 2008, 2010, 2015, and 2019 that the dominant pathogen was *F. graminearum* and no other *Fusarium* spp. were comparable to it. As the toxins were in the blood samples, we have to take them seriously rather than looking only at the rates of occurrence.

The conclusion for this paper is that it is important to identify the agents causing toxin contamination, but it would be important to select isolates from diseased plant material and check their toxin-producing properties to make conclusions about their suitability to evaluate the food safety risks of the treated genotypes under artificial inoculation conditions. Similarly important is to check the genotypes in commercial production as the possibility is high that at selection, only head symptoms were considered and no toxin contamination was measured.

4.2. Toxin Contamination

The observed genotype differences suggest a potential genetic basis for resistance against toxin-producing fungi. However, data on natural infections are often insufficient to establish genetically significant differences due to the varying conditions during the flowering period of a genotype. Multiple artificial inoculations under diverse weather conditions are necessary for reliable data. Rain and temperature fluctuations greatly influence the severity of an infection, which advocates for the use of classic artificial inoculation methods to evaluate resistance components when necessary. This approach

also explains why we refrain from naming specific varieties, as the genetic reliability of data from natural infection scenarios is uncertain.

Most data on wheat mycotoxins are derived from randomly collected samples, which are mainly single samples that can cover many varieties and regions. This approach provides a good picture of the number of existing mycotoxins under different conditions and is therefore more reliable. For many reasons, they are less suitable for resistance screening, but even so, significant differences in the resistance to mycotoxin contaminations can be demonstrated for several toxins when the databases are large enough. This can possibly also be conducted for several cases in which the toxin differences were 5–10 fold, but because of the variations within the three replicates, the significance could not be proven. In this respect, further improvements in head sampling may be necessary, as the genotype differences were significant in several cases but not for all toxins; this might be explained by the toxin x environment and plant genetic interactions. Looking the data of the three replicates in the supporting tables for toxins separately, sampling could also be further improved. We know that five aflatoxin-contaminated grains of 1 kg of maize (out of a total of about 3500 grains) can cause aflatoxin contamination that exceeds the limit, and we may face a similar problem also with wheat. The data clearly prove that aflatoxins and HT-2 occur in Hungarian wheats; therefore, we should research preharvest control methods.

Nonetheless, these data are crucial for detecting preharvest mycotoxin contaminations, which is highly relevant for the breeding, commercial production, control methods, and selection of more resistant genotypes, if possible. Following such studies, there is the possibility of developing a local multitoxin method that is suitable for general screening in the form of targeted screening. However, nontargeted analyses are needed to detect possible changes in the mycotoxin spectrum, following Spanic et al. [74], in which 36 fungal metabolites were identified in wheat samples. In epidemic years, it is necessary to also screen for the mycotoxin matrix, the amounts of which will differ significantly from those in this study. Determinations of preharvest toxin contaminations are crucial in deciding which toxins need to be tested for in semi-finished and finished products. Exceeding a toxin limit not only has implications for food safety but also affects farmers from an economic aspect, who might face reduced selling prices or full income loss. Therefore, conducting toxin tests at harvest is essential to obtain an accurate assessment of the grain's quality, to separate the healthy and contaminated lots before filling the silos and other storage facilities, providing valuable feedback for breeders, farmers, and others in the food production chain. In this way, the homogeneity of the grain can be significantly improving storability, increasing prices and income.

Recent studies highlight the prevalence of the multitoxin contamination of grain [75], as well as in urine and blood samples [76], often identifying multiple mycotoxins in a single sample, sometimes up to ten or more. This suggests that in wheat, DON is not the only toxin of great importance; rather, there could be a greater number of toxins that impact food safety, making it a more complex issue than previously thought. DON was found in the highest concentrations, yet they were significantly lower than the EU limit of 1250 μg/kg. The concentrations of fumonisins were also very low, which is consistent with *F. verticillioides* not being considered a serious wheat pathogen [2]. *F. sporotrichioides* and *F. acuminatum* were isolated in Hungary [2], but their toxins were not controlled until now. *Fusarium graminearum*, besides its well-known DON producer and its ZEN production, is known in maize. Surprisingly, they were classified by PCA to different factors. In artificial inoculation, we did not find ZEN (it needs low temperatures to be produced) for wheat and so it was not a subject for research. It seems that we have to take this toxin more seriously than we did until now. The most surprising finding pertained to aflatoxins. Although the EU limit for AFB_1 is 2 μg/kg and the total aflatoxin contamination should not exceed 4 μg/kg for human consumption, the averages surpassed the EU limits in only one instance, but in individual genotypes responded often with higher aflatoxin and HT-2 toxin contamination than the EU limits (first identified in Hungary from preharvest origin). On

the other hand, it is probable that fumonisins, T-2 toxin, DAS, OTA, and STC will not cause too much trouble due to preharvest origins. *Penicillium* toxins need further tests because of the occurrence of *Penicillium* spp. in the fungal population is higher than the *Aspergillus* infection level, and this would justify to check *Penicillium* toxins from freshly harvested grains This can be ensured in FHB epidemic years and confirmed using multitoxin methods.

Another question is whether the individual toxin values are above the toxin limits. A third problem is that the occurrence of the toxins strongly differed between locations and years, the interaction of which seems to be a significant point for further analysis. Toxins were not identified above the detection limit in every location and year, and given that fumonisins occurred at very low levels, these toxins have not been considered as serious threats to wheat production. It is important that the cultivars have large stability differences: some have low values and stable performance, while some others show significantly higher values, with much higher five-tenfold or higher differences.

Our unpublished findings in maize indicate that although a substantial proportion of *Aspergillus* isolates can produce aflatoxins in vitro in rice, only a fraction of them do so under field conditions. This suggests that the presence of an *A. flavus* isolate does not guarantee aflatoxin production. This uncertainty underscores the need for an extended analysis that includes toxins from *Alternaria*, *Penicillium*, and other detected fungi in subsequent research stages.

We observed that the three aflatoxin variants (AFB_1, AFB_2, and AFG_1) occurred at similar concentrations in wheat. Unlike in maize, in which AFB_1 typically dominates [77], our findings align with studies that primarily report more AF toxins being significant in wheat [78]. AFB_1 is known for its high toxicity, with an LD_{50} of 1.2 mg/kg in adult male rats. AFG_1 shares similar toxicity levels, underscoring its significance in wheat [79]. In India, wheat samples exhibited AFB_1 levels ranging up to 606 µg/kg, with 16% exceeding the official limit of 30 µg/kg and 40% surpassing 5 µg/kg [31]. This variability in levels of aflatoxin contamination is further evident in the different local state limits, ranging from 1.7 to 55.8 µg/kg. A study in Pakistan involving 185 samples found AFB_1 in 26% of samples (0.05–4.78 µg/kg) and AFB_2 in 7% (0.02–0.48 µg/kg), with only five samples exceeding the EU limit of 4 µg/kg for AFB_1 and AFB_2 [30]. These results suggest that although aflatoxin is not currently a significant threat to consumer health in Hungary, ongoing monitoring and development of control procedures are essential. Our data indicate that AFB_2 occurs less frequently than AFB_1 in wheat genotypes, but when present, its concentrations are similar. Some genotypes were significantly contaminated with AFB_2 and AFG_1 but not AFB_1 as outlined in Table 5. Similar results were published by Giray et al. [34]. These findings imply that all four aflatoxin variants should be considered a potential food safety issue in wheat. The higher AFG_1 concentrations compared to AFB_1 emphasize the need for comprehensive testing of all aflatoxin variants for accurate assessments of food safety. Further research is necessary to fully understand and address this complex issue. For these reasons, we cannot rely on AFB_1 only when discussing the risks posed by aflatoxins in wheat compared to maize. Here, at present knowledge, the measurement of AFB_1 contamination seems to be correct.

The significances of aflatoxins and the T-2 and HT-2 toxins in Hungarian wheat samples, particularly their preharvest presences, were previously unknown. Their very different occurrences are also new. The HT-2 toxin deserves more attention as many of the detested concentrations significantly exceeded the 15, 20, or 50 µg/kg limit suggested by EC (2024) [49] for different uses.

During epidemic years, the toxin spectrum may differ considerably from the data for the two years studied, with potentially higher DON contaminations, but the presence of other toxins remains uncertain. The interactions among these toxins are largely unknown, yet their co-presence in blood and urine samples suggests probable interactions, highlighting the need for significant research in this area [3]. It is crucial to examine how wheat genotypes resistant to *F. graminearum* respond to other important *Fusarium* species and their toxins. Additionally, the effects of fungicides on the *Fusarium* spp. complex require further

exploration, along with the potential of microbial products against various toxigenic fungi, including *A. flavus*.

The principal component analysis showed that the toxin patterns do not follow the year and location pattern; only Iregszemcse data from 2021 and 2022 belonged to the same group. Szeged and Törökszentmiklós differed. Only two PC components were found, with 89% of the variance explained. So, in the future, we should not be surprised when different locations give diverging results. Analyzing the responses of the varieties to different toxins, six factors were identified, explaining 86% of the variation. The system is rather complicated; there are similarities within factors, but related toxins were classified to separate factors, and several responses could be classified with small differences to different factors at the same time. These findings suggest that related mycotoxins only occasionally correlate with each other, indicating that it is not feasible to predict the presence or contamination level for other mycotoxins, for example, based on DON data alone. This is the first paper with indications on variety responses to different toxins, but this is only the first step. One thing is sure, the resistance to a multitoxin background should be understood much better to find an acceptable diagnosis about the causes and the possible solutions.

Attention must also be given to the vulnerability of infants, young animals, and pregnant or nursing mothers to toxin exposure. The allowable level of toxin contamination in baby food is typically much lower than for adults, often set at 20% of adult limits. In piglets and other young animals, this has much greater importance than currently thought and seems to be overlooked.

In the context of increasingly stringent regulations, breeding wheat varieties with enhanced resistance to fungal diseases is crucial. Such varieties can produce safer crops even during epidemic periods, potentially reducing the need for more potent fungicides and contributing to safer food production, as we found high levels of aflatoxin contamination in seemingly symptomless maize ears [30].

The presence of preharvest multitoxin contaminations in Hungarian wheat samples is proven. In both years of the study period, June experienced hot and dry conditions, favoring aflatoxin contamination. This the first preharvest aflatoxin report from Hungary. It is also significant that the HT-2 toxin frequently exceeds the EU food safety thresholds. These findings highlight the need to consider the control of these toxins through breeding strategies and in combination with other technologies.

The research on preharvest natural toxin contamination is a highly important task. It provides feedback for breeders and farmers about whether their varieties were well chosen. The preharvest toxin screening informs us which toxins have importance in control. Long-term studies will inform us about the changes in toxin matrix, will help in epidemic forecasts, and can be used to check the influence of different agronomic and other practices on mycotoxin situation. We think that the DON alone is not enough to consider, and other toxins should also be followed to describe the real food safety risk of a given variety; therefore, multitoxin tests will be wide spread in grain production and also in breeding. As in 50% of the mycotoxins, significant genotype differences were shown, breeding for more resistant varieties seems to be possible. For several genotypes, we found high stability of toxin response, which is good news for breeding. More and more toxins are on the list of binding limits all over the world. All refer to quantifying the toxin contamination of cereals and determining the market value of the grain produced. Thus, wheat production will need more R + D investment and will be more complicated than it was before. We think that these need much more attention, and breeders and farmers should be informed about the multitoxin contamination sensitivity or resistance to toxigenic fungi and the toxins in their varieties to provide safer food for consumers and safer feed for animals.

5. Conclusions

Multitoxin analyses of grains, in addition to blood and urine tests, have revealed that mycotoxin syndrome is more complex than previously supposed. In addition to DON, other toxins are also important, and effective control method(s) must be found as soon as

possible. In a survey, the preharvest presence of aflatoxins and the HT-2 toxin surpassed EU food security limits; therefore, effective control measures are necessary. The warmer climate is partly responsible for this issue, and aflatoxins are signs, or biomarkers, of such climate change. In our tests, six genotypes showed no aflatoxin contamination, and the remaining 12 exhibited total aflatoxin contaminations exceeding the 2 or 4 mg/kg limits in at least one year and location. Therefore, in spite of the nonsignificant genotype effect, resistance behavior requires further in-depth research, balancing the negative influence of the higher temperature seasons as happens in maize [3,7]. Resistance alone is unlikely to solve the problem, but supporting practices in agronomy and plant protection can significantly improve the efficacy of control measures. Stability seems to be present is several genotypes to different mycotoxins. Its research will have high importance when this is connected with low toxin values. We need varieties with low toxin production for the most important mycotoxins and stable performance under different epidemic conditions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture14112024/s1, Tables S1–S16, Multitoxin wheat contamination tests, natural infection, including Table S1. Meteorological characteristics of the three locations in 2021 and 2022; Table S2: Fungal PCR primers; Table S3: LC/MSMS parameters; Table S4: LOQ values for toxins; Table S5: DON; Table S6: Fumonisin B1; Table S7: Fumonisin B2; Table S8: Fumonisin B1 + B2; Table S9: Aflatoxin B1; Table S10: Aflatoxin B2; Table S11: Aflatoxin G1; Table S12: T-2 toxin; Table S13: Zearalenone (ZEN); Table S14: Diacetoxyscirpenol (DAS); Table S15: Ochratoxin A (OTA); Table S16: Sterigmatocystin (STC).

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Abbreviations

AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2

AF sum	Total aflatoxin contamination
DAS	Diacetoxyscirpenol
DON	Deoxynivalenol
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB sum	(FB1 + FB2)
HT-2	HT-2-toxin
NIV	Nivalenol
OTA	Ochratoxin
STC	Sterigmatocystin
T-2.	T-2 toxin
ZEN	Zearalenone

References

1. Central Statistics Office. *The Sowing Area of the Most Important Crops*; Central Statistics Office: Budapest, Hungary, 2022.
2. Mesterházy, Á. *Fusarium* species of wheat in South Hungary, 1970–1983. *Cereal Res. Commun.* **1984**, *12*, 167–170.
3. Mesterházy, Á. What Is Fusarium Head Blight (FHB) Resistance and What Are Its Food Safety Risks in Wheat? Problems and Solutions—A Review. *Toxins* **2024**, *16*, 31. [CrossRef] [PubMed]
4. Bockus, W.W.; Bowden, R.L.; Hunger, R.M.; Morrill, W.L.; Murray, T.D.; Smiley, R.W. *Compendium of Wheat Diseases and Pests*; The American Phytopathological Society: St. Paul, MN, USA, 2010; p. 171.
5. 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. [CrossRef]
6. Moschini, R.C.; Pioli, R.; Carmona, M.; Sacchi, O. Empirical predictions of wheat head blight in the northern Argentinean Pampas region. *Crop Sci.* **2001**, *41*, 1541–1545. [CrossRef]
7. Sayler, T. Study: $2.6 billion, 501 million bushels lost to scab 1991–1996. *Prairie Grains* **1998**, *11*, 12.
8. Goswami, R.S.; Kistler, H.C. Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* **2004**, *5*, 515–525. [CrossRef]
9. Ács, K.; Lehoczki-Krsjak, S.; Varga, M.; Kótai, C.; Ács, E.; Salgó, A.; Mesterházy, Á. Reduction of deoxynivalenol (DON) contamination by improved fungicide use in wheat. Part 3. Reduction of Fusarium head blight and influence on quality traits in cultivars with different resistance levels. *Eur. J. Plant Pathol.* **2018**, *151*, 21–38. [CrossRef]
10. Tóth, B.; Kászonyi, G.; Bartók, T.; Varga, J.; Mesterházy, Á. Common resistance of wheat to members of the *Fusarium graminearum* species complex and *F. culmorum*. *Plant Breed.* **2008**, *127*, 1–8. [CrossRef]
11. Doohan, F.M.; Parry, D.W.; Jenkinson, P.; Nicholson, P. The use of species-specific PCR based assays to analyze Fusarium ear blight of wheat. *Plant Pathol.* **1998**, *47*, 197–205. [CrossRef]
12. Xu, X.-M.; Parry, D.; Nicholson, P.; Simpson, D.; Edwards, S.; Cooke, B.; Doohan, F.; Brennan, J.; Monaghan, S.; Moretti, A.; et al. Predominance and association of pathogenic species causing Fusarium ear blight in wheat. *Eur. J. Plant Pathol.* **2005**, *112*, 143–154. [CrossRef]
13. Desjardins, A.E. *Fusarium Mycotoxins, Chemistry, Genetics and Biology*; APS Press: St. Paul, MN, USA, 2006.
14. Mesterházy, Á. Types and components of resistance to *Fusarium* head blight of wheat. *Plant Breed.* **1995**, *114*, 377–386. [CrossRef]
15. Paterson, R.R.M.; Lima, N. How will climate change affect mycotoxins in food? *Food Res. Int.* **2010**, *43*, 1902–1914. [CrossRef]
16. Miraglia, M.; Marvin, H.; Kleter, G.; Battilani, P.; Brera, C.; Coni, E.; Cubadda, F.; Croci, L.; De Santis, B.; Dekkers, S. Climate change and food safety: An emerging issue with special focus on Europe. *Food Chem. Toxicol.* **2009**, *47*, 1009–1021. [CrossRef] [PubMed]
17. Battilani, P.; Toscano, P.; Van der Fels-Klerx, H.J.; Moretti, A.; Camardo Leggieri, M.; Brera, C.; Rortais, A.; Goumperis, T.; Robinson, T. Aflatoxin B_1 contamination in maize in Europe increases due to climate change. *Sci. Rep.* **2016**, *6*, 24328. [CrossRef]
18. Mesterházy, Á.; Szieberth, D.; Toldiné Tóth, E.; Nagy, Z.; Szabó, B.; Herczig, B.; Bors, I.; Tóth, B. Updating the Methodology of Identifying Maize Hybrids Resistant to Ear Rot Pathogens and Their Toxins—Artificial Inoculation Tests for Kernel Resistance to *Fusarium graminearum, F. verticillioides,* and *Aspergillus flavus*. *J. Fungi* **2022**, *8*, 293. [CrossRef]
19. Christensen, C.M.; Kaufmann, H.H. *Grain Storage: The Role of Fungi in Quality Loss*; University of Minnesota Press: Minneapolis, MN, USA, 1969.
20. Ghanem, K.M.; Lotfy, W.A.; El-Shaer, M.M.; Elassar, S.A. The Inhibitory Effect of Wheat Husks Addition on Aflatoxins Production by *Aspergillus flavus* in liquid culture with various wheat compositions as carbon sources. *Front. Microbiol.* **2020**, *11*, 1448. [CrossRef]
21. Nada, S.; Nikola, T.; Bozidar, U.; Ilija, D.; Andreja, R. Prevention and practical strategies to control mycotoxins in the wheat and maize chain. *Food Control* **2022**, *136*, 108855. [CrossRef]
22. Desjardins, A.; Proctor, R. Molecular biology of Fusarium mycotoxins. *Int. J. Food Microbiol.* **2007**, *119*, 47–50. [CrossRef]
23. Terzi, V.; Tumino, G.; Stanca, A.M.; Morcia, C. Reducing the incidence of cereal head infection and mycotoxins in small grain cereal species. *J. Cereal Sci.* **2014**, *59*, 284–293. [CrossRef]

24. Pestka, J. Toxicological mechanisms and potential health effects of deoxynivalenol and nivalenol. *World Mycotoxin J.* **2010**, *3*, 323–347. [CrossRef]
25. Tournas, V.H.; Niazi, N.S. Potentially toxigenic fungi from selected grains and grain products. *J. Food Saf.* **2018**, *38*, e12422. [CrossRef]
26. Zhao, J.; Cheng, T.; Xu, W.; Han, X.; Zhang, J.; Zhang, H.; Wang, C.; Fanning, S.; Li, F. Natural co-occurrence of multi-mycotoxins in unprocessed wheat grains from China. *Food Control* **2021**, *130*, 108321. [CrossRef]
27. Smith, M.C.; Madec, S.; Coton, E.; Hymery, N. Natural co-occurence of mycotoxins in foods and feeds and their in vitro combined technological effects. *Toxins* **2016**, *8*, 94. [CrossRef] [PubMed]
28. Hathout, A.S.; Abel-Fattah, S.M.; Abou-Sree, Y.H.; Fouzy, A.S.M. Incidence and exposure assessment of aflatoxins and ochratoxin A in Egyptian wheat. *Toxicol. Rep.* **2020**, *7*, 867–873. [CrossRef]
29. Ghasemi-Kebria, F.; Joshaghani, H.; Taheri, N.S.; Semnani, S.; Aarabi, M.; Salamat, F.; Roshandel, G. Aflatoxin contamination of wheat flour and the risk of esophageal cancer in a high-*risk* area in Iran. *Cancer Epidemiol.* **2013**, *37*, 290–293. [CrossRef]
30. Asghar, M.A.; Ahmed, A.; Iqbal, J.; Zahir, E.; Nauman, H. Fungal flora and aflatoxin contamination in Pakistani wheat kernels (*Triticum aestivum* L.) and their attribution in seed germination. *J. Food Drug Anal.* **2016**, *24*, 635–643. [CrossRef]
31. Toteja, G.S.; Mukherjee, A.; Diwakar, S.; Padam, P.; Singh, B.N.; Saxena, B.N.; Sinha, K.K.; Sinha, A.K.; Kumar, N.; Nagaraja, K.V.; et al. Aflatoxin B1 contamination in wheat grain samples collected from different geographical regions of India: A Multicenter Study. *J. Food Prot.* **2006**, *69*, 1463–1467. [CrossRef]
32. Trombete, F.M.; de Ávila Moraes, D.; Porto, Y.D.; Santos, T.B.; Direito, G.M.; Fraga, M.E.; Saldanha, T. Determination of aflatoxins in wheat and wheat by-products intended for human consumption, marketed in Rio de Janeiro, Brazil. *J. Food Nutr. Res.* **2014**, *2*, 671–674. [CrossRef]
33. Okafor, S.E.; Eni, A.O. Microbial quality and the occurrence of aflatoxins in plantain/yam and wheat flours in Ado-Odo Ota. In Proceedings of the 8th International Biotechnology Conference, Exhibition and Workshop, Ota, Nigeria, 11–13 March 2018.
34. Giray, B.; Girgin, G.; Engin, A.B.; Aydın, S.; Sahin, G. Aflatoxin levels in wheat samples consumed in some regions of Turkey. *Food Control* **2007**, *18*, 23–29. [CrossRef]
35. Mesterházy, Á.; Szabó, B.; Szél, S.; Nagy, Z.; Berényi, A.; Tóth, B. Novel insights into the inheritance of Gibberella Ear Rot (GER), deoxynivalenol (DON) accumulation, and DON production. *Toxins* **2022**, *14*, 583. [CrossRef]
36. Tóth, B.; Török, O.; Kótai, E.; Varga, M.; Toldiné, T.É.; Pálfi, X.; Háfra, E.; Varga, J.; Téren, J.; Mesterházy, Á. Role of *Aspergilli* and *Penicillia* in mycotoxin contamination of maize in Hungary. *Acta Agron. Hung.* **2012**, *60*, 143–149. [CrossRef]
37. International Agency for Research on Cancer IARC monographs on the evaluation of carcinogenic risks to humans; vol 56, Chemical agents and related occupations. *IARC Lyon.* **2012**, *100*, 1–599.
38. Selamat, J.; Iqbal, S.Z. *Food Safety. Basic Concepts, Recent Issues, and Future Challenges*; Springer: Cham, Switzerland, 2016; p. 160, ISBN 978-3-319-39251-6. [CrossRef]
39. Berthiller, F.; Crews, C.; Dall'Asta, C.; Saeger, S.D.; Haesaert, G.; Karlovsky, P.; Oswald, I.P.; Seefelder, W.; Speijers, G.; Stroka, J. Masked mycotoxins: A review. *Mol. Nutr. Food Res.* **2013**, *57*, 165–186. [CrossRef] [PubMed]
40. De Coli, L.; De Ruyck, K.; Abadallah, M.F.; Finnan, J.; Mullins, E.; Kildea, S.; Spink, J.; Elliott, C.; Danaher, M. Natural co-occurrence of multiple mycotoxins in unprocessed oats grown in Ireland with various production systems. *Toxins* **2021**, *13*, 188. [CrossRef] [PubMed]
41. Chiotta, M.L.; Fumero, M.V.; Cendoya, E.; Palazzini, J.M.; Alaniz-Zanon, M.S.; Ramirez, M.L.; Chulze, S.N. Toxigenic fungal species and natural occurrence of mycotoxins in crops harvested in Argentina. *Rev. Argent. Microbiol.* **2020**, *396*, 9. [CrossRef]
42. Baddi, M.; Nassik, S.; Alali, S.; El Hraiki, A. Co-occurrence of mycotoxins in poultry feed and feed-ingredients marketed in Morocco. *Rev. Marocaine Des Sci. Argon. Omiques Vétérinaires* **2021**, *9*, 348–352.
43. Lemming, E.W.; Montano Montes, A.; Schmidt, J.; Cramer, B.; Humpf, H.-U.; Moraeus, L.; Olsen, M. Mycotoxins in blood and urine of Swedish adolescents' possible associations to food intake and other background characteristics. *Mycotoxin Res.* **2020**, *36*, 193–206. [CrossRef]
44. Beccari, G.; Caproni, L.; Tini, F.; Uhlig, S.; Covarelli, L. Presence of *Fusarium* species and other toxigenic fungi in malting barley and multi-mycotoxin analysis by liquid chromatography−high-resolution mass spectrometry. *J. Agric. Food Chem.* **2016**, *64*, 21. [CrossRef]
45. Schmidt, J.; Cramer, B.; Turner, P.C.; Stoltzfus, R.J.; Humphrey, J.H.; Smith, L.E.; Humpf, H.-U. Determination of urinary mycotoxin biomarkers using a sensitive online solid phase extraction-UHPLC-MS/MS method. *Toxins* **2021**, *13*, 418. [CrossRef]
46. Arroyo-Manzanares, N.; Campillo, N.; Lopez-Garcia, I.; Hernandez-Cordoba, M.; Vinas, P. High-resolution mass spectrometry for the determination of mycotoxins in biological samples. A review. *Microchem. J.* **2021**, *166*, 106197. [CrossRef]
47. Dong, F.; Xing, Y.W.; Lee, Y.W.; Mokoena, M.P.; Olaniran, A.O.; Xu, J.H.; Shi, J.R. Occurrence of Fusarium mycotoxins and toxigenic *Fusarium* species in freshly harvested rice in Jiangsu, China. *World Mycotoxin J.* **2020**, *13*, 1–12. [CrossRef]
48. Arcella, D.; Gergelova, P.; Innocenti, M.L.; Steinkellner, H. Human and animal dietary exposure to T-2 and HT-2 toxin. *EFSA J.* **2017**, *15*, 4972.
49. European Commission, Commission Regulation (EU) 2024/1038 of 9 April 2024 amending Regulation (EU) 2023/915 as regards maximum levels of T-2 and HT-2 toxins in food. *Off. J. Eur. Union* **2024**, 1–5.
50. Vesonder, R.F.; Golinski, P. Metabolites of Fusarium. Fusarium mycotoxins, taxonomy and pathogenicity. In *Fusarium Macotoxins, Taxonomy and Pathogenicity*; Chelkowski, J., Ed.; Elsevier: Amsterdam, The Netherlands, 1989; pp. 1–39.

51. Leslie, J.F.; Moretti, A.; Mesterházy, Á.; Ameye, M.; Audenaert, K.; Singh, P.K.; Richard-Forget, F.; Chulze, S.N.; Del Ponte, E.M.; Chala, A.; et al. Key Global Actions for Mycotoxin Management in Wheat and Other Small Grains. *Toxins* **2021**, *13*, 725. [CrossRef]
52. Alisaac, E.; Mahlein, A.-K. Fusarium Head Blight on Wheat: Biology, Modern Detection and Diagnosis and Integrated Disease Management. *Toxins* **2023**, *15*, 192. [CrossRef]
53. Escrivá, L.; Font, G.; Manyes, L.; Berrada, H. Studies on the presence of mycotoxins in biological samples: An overview. *Toxins* **2017**, *9*, 251. [CrossRef]
54. Gashgari, R.; Ameen, F.; Al-Homaidi, E.; Gherbawy, Y.; Al Nadhari, S.; Vijayan, V. Mycotoxigenic fungi contaminating wheat; toxicity of different *Alternaria compacta* strains. *Saudi J. Biol. Sci.* **2019**, *26*, 210–215. [CrossRef]
55. Bottalico, A.; Logrieco, A. Toxigenic *Alternaria* species of economic importance. In *Mycotoxins in Agriculture and Food Safety*; Marcel Dekker: New York, NY, USA; Basel, Switzerland; Hong Kong, China, 1998; pp. 65–108.
56. Liu, G.T.; Qian, Y.Z.; Zhan, P.; Dong, W.H.; Qi, Y.M.; Guo, H.T. Etiologic role of *Alternaria alternata* in human esophageal cancer. *Chin. Med. J.* **1992**, *105*, 394–400.
57. Verma, J.; Johri, T.S.; Swain, B.K.; Ameena, S. Effect of graded levels of aflatoxin, ochratoxin and their combinations on the performance and immune response of broilers. *Br. Poult. Sci.* **2004**, *45*, 512–518. [CrossRef]
58. Serrano, A.B.; Capriotti, A.L.; Cavaliere, C.; Piovesana, S.; Samperi, R.; Ventura, S.; Laganà, A. Development of a Rapid LC-MS/MS method for the determination of emerging Fusarium mycotoxins enniatins and beauvericin in human biological fluids. *Toxins* **2015**, *7*, 3554–3571. [CrossRef]
59. Capriotti, A.L.; Caruso, G.; Cavaliere, C.; Foglia, P.; Samperi, R.; Laganà, A. Multiclass mycotoxin analysis in food, environmental and biological matrices with chromatography/mass spectrometry. *Mass Spectrom. Rev.* **2012**, *31*, 466–503. [CrossRef] [PubMed]
60. Sulyok, M.; Berthiller, F.; Krska, R.; Schuhmacher, R. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2649–2659. [CrossRef] [PubMed]
61. Huang, Q.; Guo, W.; Zhao, X.; Cao, H.; Fan, K.; Meng, J.; Nie, D.; Wu, Y.; Han, Z. Universal screening of 200 mycotoxins and their variations in stored cereals in Shanghai, China by UHPLC-QTOF MS. *Food Chem.* **2022**, *387*, 132869. [CrossRef] [PubMed]
62. De Santis, B.; Debegnach, F.; Toscano, P.; Crisci, A.; Battilani, P.; Brera, C. Overall exposure of European adult population to mycotoxins by statistically modelled biomonitoring data. *Toxins* **2021**, *13*, 695. [CrossRef]
63. De Santis, B.; Debegnach, F.; Gregori, E.; Russo, S.; Marchegiani, F.; Moracci, G.; Brera, C. Development of a LC-MS/MS method for the multi-mycotoxin determination in composite cereal-based samples. *Toxins* **2017**, *9*, 169. [CrossRef]
64. European Commission. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union* **2006**, *364/5*.
65. Lauwers, M.; De Baere, S.; Letor, B.; Rychlik, M.; Croubel, S.; Devreese, M. Multi LC-MS/MS and LC-HRMS Methods for Determination of 24 mycotoxins including major phase i and ii biomarker metabolites in biological matrices from pigs and broiler chickens. *Toxins* **2019**, *11*, 171. [CrossRef]
66. Elaridi, J.; Yamani, O.; Al Matari, A.; Dakroub, S.; Attieh, Z. Determination of ohratoxin A (OTA), ochratoxin B (OTB), T-2, and HT-2 toxins in wheat grains, wheat flour, and bread in Lebanon by LC-MS/MS. *Toxins* **2019**, *11*, 471. [CrossRef]
67. Malachova, A.; Krska, R.; Berthiller, F.; Sulyok, M.; Beltran, E. Multi-toxin determination in food—The power of "dilute and shot" approaches in LC-MS-MS. *LCGC Eur.* **2015**, *28*, 542–555.
68. Logrieco, A.; Mulè, G.; Moretti, A.; Bottalico, A. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. *Eur. J. Plant Pathol.* **2002**, *108*, 597–609. [CrossRef]
69. Sváb, J. *Biometriai Módszerek a Kutatásban (Methods for Biometrics in Research)*, 3rd ed.; Mezogazdasági Kiadó (Agr. Publ. House): Budapest, Hungary, 1981; p. 557. ISBN 963 231 0136.
70. Imathiu, S.M.; Edwards, S.G.; Ray, R.V.; Back, M.A. *Fusarium* langsethiae—A HT-2 and T-2 toxins producer that needs more attention. *J. Phytopathol.* **2012**, *161*, 1–10. [CrossRef]
71. Varga, J.; Toth, B.; Mesterhazy, A.; Teren, J.; Fazekas, B. Mycotoxigenic fungi and mycotoxins in foods and feeds in Hungary. In *An Overview on Toxigenic Fungi and Mycotoxins in Europe*; Logrieco, A., Visconti, A., Eds.; Kluwer Academic Publishers: Dordecht, The Netherlands, 2004; pp. 123–139.
72. Uhlig, S.; Jestoi, M.; Parikka, P. *Fusarium* avenaceum—The North European situation. *Int. J. Food Microbiol.* **2007**, *19*, 17–24. [CrossRef] [PubMed]
73. Tini, F.; Covarelli, L.; Cowger, C.; Sulyok, M.; Benincasa, P.; Beccari, G. Infection timing affects *Fusarium poae* colonization of bread wheat spikes and mycotoxin accumulation in the grain. *J. Sci. Food Agric.* **2022**, *102*, 6358–6372. [CrossRef] [PubMed]
74. Spanic, V.; Katanic, Z.; Sulyok, M.; Krska, R.; Puskas, K.; Vida, G.; Drezner, G.; Šarkanj, B. Multiple Fungal Metabolites Including Mycotoxins in Naturally Infected and *Fusarium*-Inoculated Wheat Samples. *Microorganisms* **2020**, *8*, 578. [CrossRef]
75. Tolosa, J.; Graziani, G.; Gaspari, A.; Chianese, D.; Ferrer, E.; Mañes, J.; Ritieni, A. Multi-mycotoxin analysis in durum wheat pasta by liquid chromatography coupled to quadrupole orbitrap mass spectrometry. *Toxins* **2017**, *9*, 59. [CrossRef]
76. Viegas, S.; Osteresch, B.; Almeida, A.; Cramer, B.; Humpf, H.U.; Viegas, C. Enniatin B and ochratoxin A in the blood serum of workers from the waste management setting. *Mycotoxin Res.* **2018**, *34*, 85–90. [CrossRef]
77. Bennett, J.W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, *16*, 497–516. [CrossRef]

78. Mesterhazy, Á.; Lemmens, M.; Reid, L.M. Breeding for resistance to ear rots caused by *Fusarium* ssp. in maize—A review. *Plant Breeding* **2012**, *131*, 1–19. [CrossRef]
79. EFSA panel on contaminants in the food chain (CONTAM); Schrenk, D.; Bignami, M.; Bodin, L.; Chipman, J.K.; Del Mazo, J.; Grasl-Kraupp, B.; Hogstrand, C.; Hoogenboom, L.R.; Leblanc, J.C.; et al. Risk assessment of aflatoxins in food. *EFSA J.* **2020**, *18*, 6040. [CrossRef]

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