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Natural Contamination of Rice with Ustiloxins and the Connection with Climate Conditions in Southern China

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Abstract: Mycotoxins often contaminate rice, which are the secondary metabolites of fungi. Ustiloxins, a type of mycotoxin that has often been overlooked, pose a significant risk to human health. Therefore, identifying and controlling the pollution of ustiloxins in rice is required. In this study, we examined the natural contamination of rice with ustiloxins and their link to climate conditions. A total of 300 paddy samples were collected from six regions in southern China, and concentrations of ustiloxins A, B, C, D, and F were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Results showed that the occurrence of ustiloxins A, B, C, D, and F in paddies was found to be 55.7%, 41.3%, 29.0%, 93.7%, and 96.7%, respectively. Ustiloxin A had the highest mean (177.8 μg/kg) and maximum (3620.9 μg/kg) concentration, followed, in order, by ustiloxins C, B, D, and F. Furthermore, ustiloxin levels were significantly different depending on their origin, with the highest concentration in rice from Changde and Yueyang. And the regional difference in ustiloxins was related to the diversity of climate. A positive correlation between ustiloxin occurrence and mean humidity and precipitation was found in July and August of different regions, while mean temperature indicated a negative dependence. This is an essential survey of the contamination of rice with ustiloxins throughout southern China. The influence of climatic conditions on ustiloxins contamination was evaluated for the first time in our study. Overall, the rice samples examined in this study exhibited a high distribution of ustiloxins, suggesting that regulatory limits and the establishment of maximum allowable levels of ustiloxins in rice are necessary. This study provides a basis and guidance for the pollution situation and control strategy of ustiloxins in China.

Keywords: ustiloxins; rice; LC-MS/MS; agroclimatic conditions; food safety

Citation: Miao, X.; Miao, Y.; Liu, Y.; Tao, S.; Zheng, H.; Kuang, N.; Wang, J.; Tang, Q. Natural Contamination of Rice with Ustiloxins and the Connection with Climate Conditions in Southern China. *Agronomy* **2024**, *14*, 976. https://doi.org/10.3390/agronomy14050976

Academic Editor: Caterina Morcia

Received: 5 April 2024
Revised: 26 April 2024
Accepted: 3 May 2024
Published: 6 May 2024

1. Introduction

Rice (*Oryza sativa* L.) is a crucial staple grain that provides nutrition for approximately 75% of the global population [1]. As the world population grows, the demand for rice production is increasing. China is the largest rice-producing country in the world, with over 65% of the Chinese population consuming rice [2]. However, the safety of the rice supply is challenged by mycotoxin contamination. Rice is easily infected by molds such as *Fusarium* spp., *Aspergillus* spp., and *Penicillium* spp. These molds produce mycotoxins and can contaminate the rice during both cultivation and storage [3,4]. According to the Food and Agriculture Organization of the United Nations (FAO), over 25% of the world's agricultural commodities are contaminated with mycotoxins [5]. Ferre reported that rice samples across 18 countries contained mycotoxins [6]. Aflatoxin concentrations ranging from 0.2 to 1.8 μg/kg were identified in rice from the United Kingdom and Germany. Over 90% of

the rice samples in Germany contained aflatoxins [7]. In Bahrain, 80% of the rice samples had a zearalenone concentration of 0.7 µg/kg [8]. In China, contamination of mycotoxins in grains was investigated, and the incidence of aflatoxins in rice was 63.5% across all samples [9]. Li et al. reported that 14.5% and 27.6% of cereal samples were contaminated with ochratoxin A and zearalenone in the Yangtze Delta region of China [10]. The losses of global grain in 2018 due to mycotoxin contamination during harvest and storage reached 690 million tons, accounting for 83% of the total newly stored grain [11]. Considering the threats to human health caused by mycotoxins, many countries and organizations have established limits for grain contamination. For instance, the European Union has set a permissible limit of 5 µg/kg for aflatoxin B1 and 10 µg/kg for total aflatoxins in unprocessed rice. Additionally, the maximum level of ochratoxin A in unprocessed cereals was 5 µg/kg [12]. In China, the maximum level of deoxynivalenol in cereals has been set as 1000 µg/kg [13]. However, it is concerning that only a limited number of mycotoxins currently have established legal limits in worldwide.

Rice false smut (RFS), caused by the pathogen *Ustilaginoidea virens*, traditionally was considered to be a minor disease. However, it is now considered one of the most important diseases in rice-growing regions worldwide [14]. The emergence of this disease can be partly attributed to the cultivation of susceptible rice cultivars and the excessive use of nitrogen fertilizers in agricultural production [15]. False smut not only reduces rice grain yield and negatively affects rice quality but also produces mycotoxins known as ustiloxins and ustilaginoidins, both of which pose a threat to human health [16–18]. As shown in Figure 1 [19], ustiloxins are water-soluble heterodetic cyclopeptide, commonly found in false smut balls on the panicles of rice plants. Among ustiloxins, there are five primary compounds: ustiloxins A, B, C, D, and F, where A and B are the most predominant in RFS balls and rice grains [20]. Numerous findings have demonstrated that ustiloxins can inhibit skeletal formation and cell microtubule assembly, resulting in liver, heart, and kidney damage in animals [16,21,22]. Unfortunately, the presence of ustiloxins has often been overlooked, and no legal limits have been established by any country or organization.

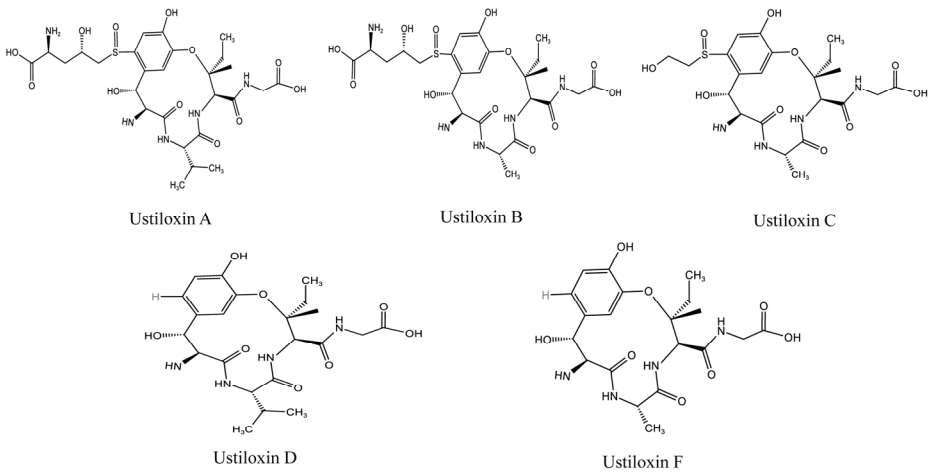

Figure 1. Structures of ustiloxins A, B, C, D, and F [19].

Monitoring ustiloxins is essential for assessing the contamination levels and ensuring the food safety of rice. Various techniques have been applied for the quantitative analysis of mycotoxins in cereals, including immunochemical, omics, chromatography, and spectroscopy methods. For instance, Fu et al. developed an enzyme-linked immuno-sorbent assay for the simultaneous detection of ustiloxins A and B, demonstrating high recovery rates of between 84.1 and 102.5% [23]. In addition, both near and mid-infrared spectroscopy had been utilized for detecting aflatoxins B1 in rice [24]. However, the application of spectroscopic technology is limited due to spectral overlapping and the challenges of interpreting spectral data. The most common techniques for analysis of mycotoxins in food products are solid–liquid extractions followed by chromatographic detection methods such

as high-performance liquid chromatography (HPLC) [25] and HPLC coupled with tandem mass spectrometry (LC-MS/MS). Currently, LC-MS/MS techniques have been successfully used for quantification of aflatoxins, ochratoxins, fumonisins, and ustiloxins in rice [26,27]. In summary, LC-MS/MS technology is the preferred choice for detecting ustiloxins in this study due to its high accuracy, sensitivity, and selectivity.

The severity of mycotoxin contaminations is highly dependent on mold growth. Mold growth can be affected by weather conditions, such as temperature, humidity, and precipitation [28]. For example, hot and dry conditions can promote aflatoxins contamination of crops in the field [29]. For zearalenone, the optimal conditions for synthesis in the field were moderate temperature and high humidity throughout the growing season [30]. Similarly, *Ustilaginoidea virens* infection and ustiloxin production in rice are favored by high humidity and temperatures between 25–30 °C [31]. In some rice-growing locations, particularly in the middle and lower reaches of the Yangtze River in China, such as Changde and Yueyang in Hunan province, extended periods of rainfall are typical during the rice flowering and heading periods, causing favorable environmental conditions for RFS and ustiloxins outbreaks [32]. The higher rainfall in the summer within these regions may be due to factors such as subtropical monsoons and typhoons. Most mycotoxins are stable molecules, which makes them difficult to remove from grain. Consequently, the most effective measure for reducing mycotoxin occurrence is to control fungal diseases and minimize mycotoxin production as much as possible. Unlike other mycotoxins, ustiloxins are mainly produced in the field [33]. Therefore, agronomic control and favorable environmental conditions during rice cultivation are critical to limiting ustiloxins contamination [34]. Exploring the relationship between agroclimatic conditions and the occurrence of ustiloxins can help to achieve this goal.

Ustiloxins have been known for many years [20] but have been the subject of limited investigation. The objective of this study was to develop a highly sensitive and accurate LC-MS/MS method to investigate the occurrence of ustiloxins in paddies from different regions across southern China. Reports on the occurrence of ustiloxins in China are scarce, so this study can provide data support for understanding the ustiloxins distribution in paddies from southern China. Our results are expected to accelerate the establishment of legal limits for ustiloxins in rice. Furthermore, the correlation between agroclimatic conditions and ustiloxin occurrence in different regions was investigated in this study. It is hoped that ustiloxins in rice can be mitigated by appropriate agronomic and field management measures.

2. Materials and Methods

2.1. Chemicals and Reagents

HPLC-grade methanol (purity $\geq$ 99.9%) was obtained from Merck (Darmstadt, Germany), while formic acid (purity $\geq$ 98%) was purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water was purified using a Purelab Chorus Reagent water system (London, UK). The pure reference materials of ustiloxins A, B, C, D, and F were prepared according to the previous work [35]. Thereafter, a 0.1 mg sample of each ustiloxin (purity $\geq$ 88%) was accurately weighed and dissolved in 1 mL of methanol to act as the mother solution with a concentration of 100 μg/mL. Since the response intensity of the five ustiloxins on the LC/MS/MS instrument differed, ustiloxins were separated into two groups (ustiloxins A, B, and C for one group and ustiloxins D and F for the other) when the working standards were prepared. The standard solution (100 μg/mL) of ustiloxin D and F was diluted to 10 μg/mL using methanol. Then, 1 mL of each ustiloxin mother solution was added to a working standard with ustiloxins A, B, and C each at a concentration of 10 mg/L, while ustiloxins D and F were each at 1 mg/L. Ultrapure water was used as the solvent for the working standard. All other reagents used were of analytical grade.

2.2. Samples

A total of 300 late indica rice paddy samples were randomly collected from six different regions in the Hunan Province of China (Figure 2). The collection locations were Taoyuan County, Changde City (29°15′ N, 111°15′ E, S1); Yueyang County, Yueyang City (29°15′ N, 113°30′ E, S2); Sangzhi County, Zhangjiajie City (29°30′ N, 110°15′ E, S3); Hengyang County, Hengyang City (27°15′ N, 112°30′ E, S4); Lukou District, Zhuzhou City (27°30′ N, 113° E, S5); and Shuangfeng County, Loudi City (27°30′ N, 112°15′ E, S6). Rice samples were harvested in 2021, with 50 paddy samples, each weighing approximately 300 g, collected from each region. Before analysis, each paddy sample was milled into a CT410 fine powder (FOSS, Hillerod, Denmark), passed from a sieve with a 1 mm screen, and then stored at 4 °C until needed.

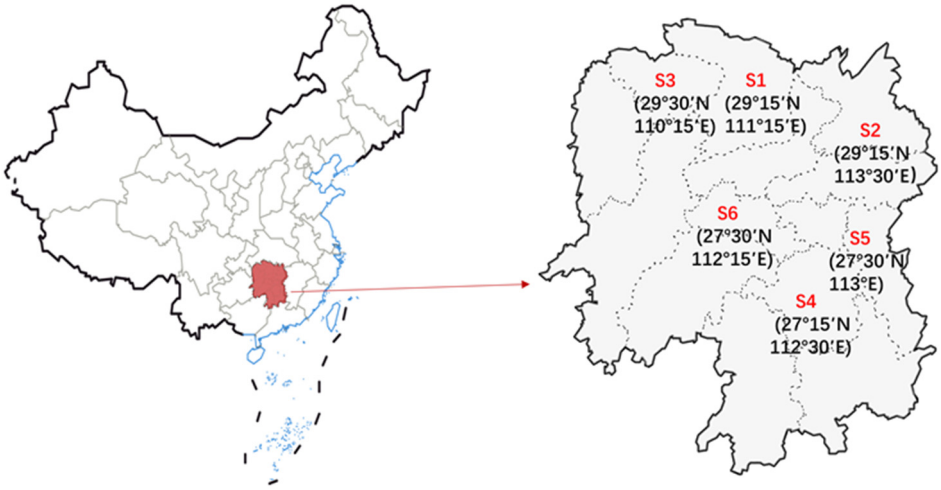

Figure 2. Locations of sampling points in different regions of Hunan Province, China (S1, Changde; S2, Yueyang; S3, Zhangjiajie; S4, Hengyang; S5, Zhuzhou; S6, Loudi).

2.3. Analytical Method

2.3.1. Extraction

Ustiloxins were extracted in paddy samples following the method described by Cao et al. with some modifications [19]. Ustiloxins are soluble in water due to the presence of free carboxyl and hydroxyl groups (Figure 1). Therefore, ultra-pure water was used as the extractant in this study. Briefly, a 5 g sample was weighed and placed in a 50 mL centrifuge tube, along with 25 mL of ultrapure water. After that, the sample was soaked for 10 min at room temperature, followed by vortexing for 1 min and sonicating for 10 min with a KO-400KDE ultrasonic cleaner (Kunshan, China). Then, the tube was centrifuged at 7000 rpm for 15 min with a Thermo Fisher MULTIFUGE X1R centrifuge (New York, NY, USA). Subsequently, 3 mL of the supernatant was transferred into a 15 mL centrifuge tube containing 100 mg C18. After vortexing for 1 min and centrifuging at 7000 rpm for 5 min, the supernatant was filtered through a 0.22 μm membrane for LC-MS/MS analysis.

2.3.2. LC-MS/MS Analysis

The quantification of ustiloxins was performed with a system consisting of an LC-20A chromatograph (Shimadzu, Kyoto, Japan) coupled to an 8040 mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electron spray ionization (ESI) source. The samples were separated using a Shim-pack GIST-HP C18 column (dimensions, 100 mm × 2.1 mm × 3 μm). The mobile phase consisted of two solvents. Solvent A was the mixture of water and formic acid (100:0.1, *v*/*v*), and solvent B was methanol. The gradient elution procedure was as follows: First, the mobile phase gradient was set at 10% solvent B. Then, over the next 5 min, it was adjusted linearly to 80% B. The proportion of solvent B remained at 80% for 3 min, after which solvent B returned to 10% within 0.1 min. The proportions remained at 10% solvent B for the remaining 4.4 min of the chromatogram. The flow rate was set to

0.2 mL/min, and the temperature of the column oven was maintained at 35 °C throughout the analysis. The injection volume was 2 μL, and the total chromatographic time was 12.5 min.

The mass spectrometer was operated in positive ion ionization mode. Nitrogen (N_2) was used as the atomizing and drying gas with a flow rate of 3.0 L/min and 15.0 L/min, respectively. The voltage ion source interface was 4.5 kV, and the desolvation line temperature was 150 °C. The heating module temperature was set to 400 °C. Multiple reaction monitoring modes were used for the quantification of ustiloxins. All instrument parameters were carefully optimized to ensure accurate and reliable quantification of ustiloxins. The retention times and MS/MS parameters (quantification ions, qualitative ions, and collision energy) of the five ustiloxins are listed in Table S1.

2.4. Method Performance

Before analyzing ustiloxins content in rice samples, the performance of the analytical method in this study was validated by determining the parameters, such as linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effects, and recovery. The linearity of five ustiloxins was evaluated for both solvent and matrix-matched calibration curves using at least six concentration levels (10–1000 μg/L for ustiloxins A, B and C; 1–500 μg/L for ustiloxins D and F). LOD and LOQ were calculated based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively. Additionally, matrix effects (MEs) were assessed by analyzing the slope of the matrix-matched calibration curve and solvent calibration curve. The recovery rates were determined at three different levels (50, 100, and 200 μg/kg for ustiloxins A, B, and C; 5, 10, and 20 μg/kg for ustiloxins D and F) by spiking the blank rice samples with the standard ustiloxin solutions. Moreover, precision was evaluated by using intra- and inter-day repeatability of each ustiloxin. The recovery, intraday, and interday precision tests were all performed in six replicates (n = 6). In general, recoveries between 70% and 120% were considered acceptable.

2.5. Data Analysis

In this study, positive samples were defined as those with ustiloxin content above the LOD. Agroclimatic factors such as temperature, precipitation, and relative humidity were obtained from the China Meteorological Administration. Agroclimatic data were only obtained for the period from late rice sowing (June) to harvest (November) of 2021 (Figure S1). The effects of agroclimatic conditions and geographic position on contamination of ustiloxins were analyzed via analysis of variance (ANOVA) with SPSS version 22.0 (IBM, Armonk, NY, USA) and Excel 2010. Least significant difference tests with an alpha level of 0.05 and Pearson correlation analysis were performed to determine significant differences and calculate the Pearson correlation coefficient (r).

3. Results and Discussion

3.1. Validation of the Analytical Method

Table 1 lists the performance data of the analytical method. Obviously, a strong linear response was obtained for each ustiloxin in their respective concentration ranges, with coefficients of determination (R^2) higher than 0.9991. The LOD values of five ustiloxins were found to be 2.68 μg/kg (ustiloxin A), 0.27 μg/kg (ustiloxin B), 4.95 μg/kg (ustiloxin C), 0.06 μg/kg (ustiloxin D), and 0.03 μg/kg (ustiloxin F). The results indicated significant matrix enhancement effects for all ustiloxins, with ME values $\geq$ 30.09%, except for ustiloxin D, which exhibited an ME of 8.96% (Table 1). Our study is consistent with the findings reported by Hu et al., who found that MEs of ustiloxin A and ustiloxin B in rice were 82.1% and 70.5%, respectively [36]. To reduce matrix effects, ustiloxins were quantified using matrix-matched calibrations. The recovery values were satisfactory, ranging between 81.1% and 105.0%, with RSD ranging from 3.8% to 7.8% (Table 1). The intra-day precisions were 1.3–4.3%, and the inter-day precisions were 2.7–10.6%. According to these validation

results, the analytical method established in this study was applicable to the quantification of ustiloxins in rice samples.

Table 1. Analytical performance data for the five ustiloxins in paddy samples.

Mycotoxin	Calibration Curve	Correlation Coefficient (r)	LOQ (μg/kg)	LOD (μg/kg)	Matrix Effect (%)	Recovery ± RSD (%) (*n* = 6)			Intra-Day Precision (%)	Inter-Day Precision (%)
						A1	A2	A3		
Ustiloxin A	y = 429.463x + 269.388	0.9999	8.12	2.68	30.09	96.4 ± 4.8	101.9 ± 6.7	105.0 ± 5.3	4.1	10.6
Ustiloxin B	y = 936.351x + 2949.53	0.9998	0.82	0.27	78.30	85.1 ± 5.4	81.9 ± 7.7	91.2 ± 7.8	4.3	9.9
Ustiloxin C	y = 366.019x + 545.535	0.9997	16.50	4.95	46.76	88.0 ± 7.4	86.2 ± 4.0	84.1 ± 6.4	3.9	8.3
Ustiloxin D	y = 8775.77x − 137.548	0.9999	0.18	0.06	8.96	81.1 ± 7.3	94.5 ± 6.8	93.8 ± 7.9	1.3	2.7
Ustiloxin F	y = 8614.85x + 858.712	0.9998	0.09	0.03	32.64	90.4 ± 5.9	93.0 ± 5.8	91.5 ± 3.8	3.5	4.8

Note: For ustiloxins A, B, and C, the spiked levels (A1, A2, and A3) were 50, 100, and 200 μg/kg, respectively; for ustiloxins D and F, the spiked levels (A1, A2, and A3) was 5, 10, and 20 μg/kg, respectively.

3.2. The Occurrence of Ustiloxins in Paddy Samples

As can be seen in Table 2, the incidence of ustiloxins ranged from 29.0% (ustiloxin C) to 96.7% (ustiloxin F), with 98.7% of all samples being positive for at least one type of ustiloxin. Of 300 samples, 167 (55.7%) were contaminated with ustiloxin A. Among these positive samples, 37 had ustiloxin A levels between 100 and 499 μg/kg, 16 samples had ustiloxin A levels between 500 and 1827.1 μg/kg, and only a single sample had a ustiloxin A level of 3620.9 μg/kg. The detected mean concentration (±SD) of ustiloxin A (177.8 ± 388.7 μg/kg) in rice samples was the highest among the five ustiloxins. In addition, 124 of 300 samples were positive for ustiloxin B with a maximum and mean level (±SD) of 693.7 and 41.8 ± 89.5 μg/kg, respectively. For ustiloxin B, 11 rice samples had levels above 100 μg/kg. Ustiloxin C was observed in 87 of 300 rice samples, ranging from 5.4 to 903.9 μg/kg. For ustiloxin C, 14 rice samples had levels above 100 μg/kg. The incidence of ustiloxin C was 29.0%, with a mean level (±SD) of 72 ± 114.5 ug/kg. Furthermore, ustiloxin D was detected in 281 of 300 (93.7%) of the rice samples, with a mean level (±SD) of 20.6 ± 39.6 μg/kg. The minimum and maximum contents of ustiloxin D were 0.2 μg/kg and 222.5 μg/kg, respectively. The most frequently detected ustiloxin was ustiloxin F (in 96.7% of positive samples), with concentrations ranging from 0.1 to 179.2 μg/kg. However, the mean concentration (±SD) of ustiloxin F was the lowest, with 17.8 ± 34.8 μg/kg.

Table 2. Incidence and occurrence of ustiloxins in paddy samples.

Mycotoxin	No. of Positive/No. of Total Samples	Percentage of Positive Samples (%)	Mean of Positive Samples (μg/kg)	SD (μg/kg)	Min (μg/kg)	Max (μg/kg)
Ustiloxin A	167/300	55.7	177.8	388.7	4.2	3620.9
Ustiloxin B	124/300	41.3	41.8	89.5	1.6	693.7
Ustiloxin C	87/300	29.0	72.0	114.5	5.4	903.9
Ustiloxin D	281/300	93.7	20.6	39.6	0.2	222.5
Ustiloxin F	290/300	96.7	17.8	34.8	0.1	179.2
Total ustiloxins	296/300	98.7	175.8	485.7	0.27	5520.7

Note: Positive samples were those with ustiloxin content above the LOD.

The results of our study indicate that ustiloxin A exhibited the highest mean and maximum concentration among the ustiloxins analyzed, followed, in order, by ustiloxins C, B, D, and F. Although the mean levels of ustiloxin D and F in rice were low, the incidence of these toxins was high. These results could be attributed to the high sensitivity of ustiloxins D and F on the LC-MS/MS instrument. Our findings are consistent with previous studies, which reported that ustiloxin A was the major and common ustiloxin in rice grain and false smut balls [19,37]. According to our results, the contamination of ustiloxins in rice from

southern China is widespread and severe. Further research should be carried out to reduce the human health risks.

3.3. Correlation among the Contents of Five Ustiloxins in Paddy Samples

The correlation heatmap of five ustiloxins in paddy samples can be seen in Figure 3. The results indicate that the concentration of ustiloxin A is highly positively correlated with that of ustiloxin B (r = 0.93) and ustiloxin C (r = 0.97) and moderately positively correlated with the concentrations of ustiloxin D (r = 0.73) and ustiloxin F (r = 0.75). Furthermore, the concentration of ustiloxin B was highly positively correlated with that of ustiloxin C (r = 0.95). The concentration of ustiloxin B was weakly positively correlated with ustiloxin D (r = 0.57) and ustiloxin F (r = 0.62). Moderate positive correlations were observed between concentrations of ustiloxin C and ustiloxin D (r = 0.64) with ustiloxin F (r = 0.69). A strong positive correlation was found between the concentration of ustiloxin D and ustiloxin F (r = 0.96). Studying the correlations among different ustiloxins can provide insights into their chemical synthesis pathways. Notably, ustiloxins A, B, and C contain a thionyl group in the side chain, contributing to their higher toxicity than ustiloxins D and F.

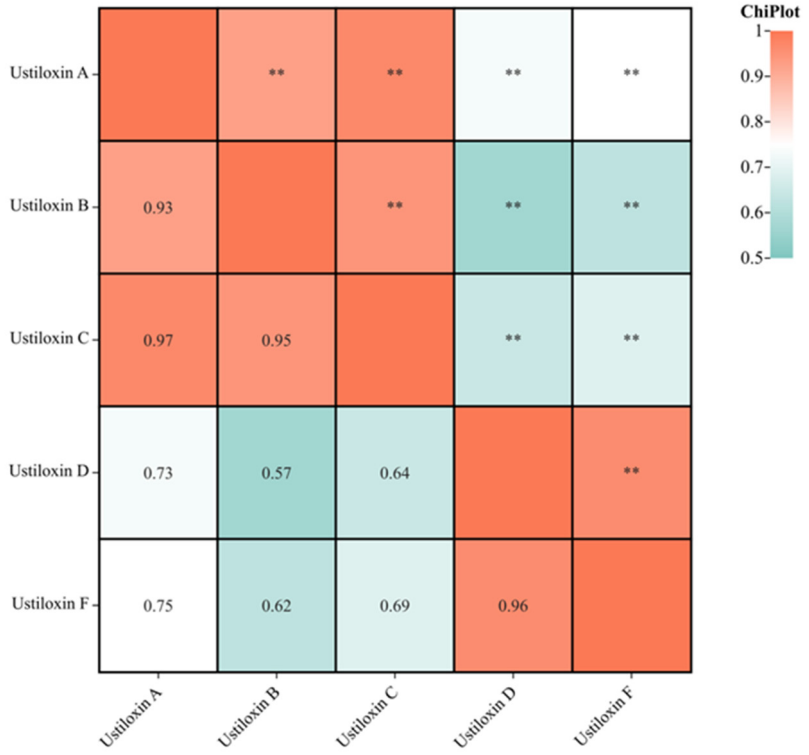

Figure 3. Correlation heatmap of five ustiloxins in paddy samples, ** indicates significance at the 0.01 probability level between the two ustiloxins ($p < 0.01$).

3.4. The Ustiloxins Content in Paddy Samples Varied across Geographic Regions

The prevalence and quantities of mycotoxins in food may vary due to climatic and geographic differences [34,38]. Here, the effect of locality on ustiloxin content was studied. The occurrence of ustiloxins in paddies varied across the six sampled regions in this study (Table S2). The highest incidence of ustiloxin A was found in regions S1 and S2 (100%), followed by region S3 (92.0%), region S5 (26.0%), region S6 (10.0%), and region S4 (6.0%). Similarly, ustiloxin B showed the highest incidence in region S1 (100%), followed by region S3 (50.0%), region S2 (42.0%), region S4 (40.0%), region S5 (12.0%), and region S6 (4.0%). For ustiloxin C, the highest level was observed in region S1 (96.0%), followed by region S2 (44.0%), region S3 (20.0%), region S4 (6.0%), region S5 (6.0%), and region S6 (2.0%). For ustiloxins D and F, the positive sample rates in all six regions were 100%, except in region S4, where the rates were 60.0 and 78.0%, respectively.

Figure 4 shows the distribution of ustiloxins (mean, minimum, and maximum values) in six regions. Overall, the ustiloxins content in paddies was generally higher in regions S1 and S2 and lower in regions S5 and S6. Variance analysis was performed for the ustiloxins content in rice samples from six regions. The results showed that the content of ustiloxins A, B, and C in regions S1 and S2 were significantly greater than in regions S3, S4, S5, and S6 ($p < 0.05$) (Figure 4). Additionally, the contents of ustiloxins D and F in region S1 were significantly higher than in the other five regions ($p < 0.05$) (Figure 4). Paddy samples from region S1 had the greatest levels of ustiloxin, and the mean concentrations of ustiloxins A, B, C, D, and F were 303.0, 53.1, 65.5, 63.9, and 60.5 μg/kg, respectively. Conversely, the lowest ustiloxins concentrations were found in region S6, where the mean concentrations of ustiloxins A, B, C, D, and F were 2.0, 0.4, 0.4, 1.5, and 1.5 μg/kg, respectively. Notably, greater levels of ustiloxin A were reflected in all six regions compared with other ustiloxins. Obviously, the distribution of ustiloxins in rice samples had significant regional differences. The reason for this result is a subject worthy of further discussion.

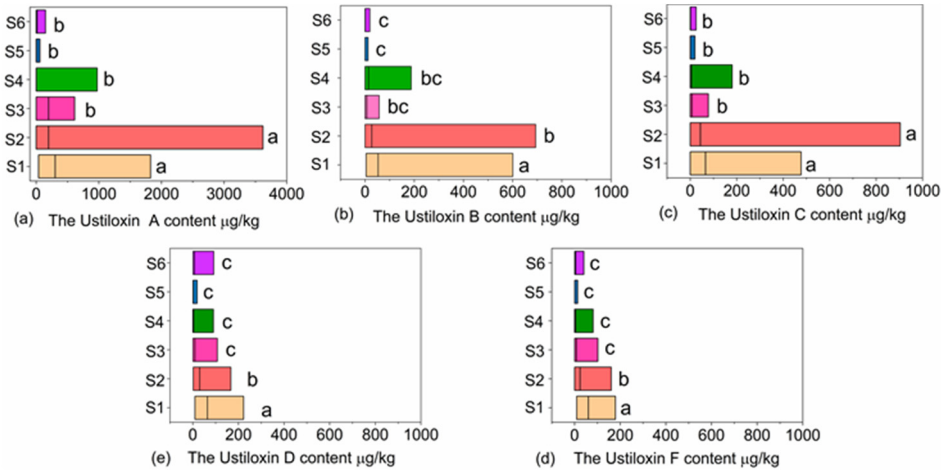

Figure 4. Distribution of ustiloxins (mean, minimum, and maximum values) in six regions. Letters indicate statistically significant differences (LSD test, $p < 0.05$).

3.5. The Effect of Weather Conditions on Ustiloxin Contamination of Paddy Samples

Various studies have indicated that mycotoxin occurrence and concentrations depend on climatic conditions. For instance, weather can impact the extent of aflatoxin contamination, with rainfall during or near harvest leading to greater aflatoxin contamination in grains [39]. Moreover, precipitation influences deoxynivalenol concentration levels at harvest, which is a mycotoxin prevalent in cold and wet climates of Europe [40,41]. Ustiloxins, which primarily occur in the field, may be found in higher concentrations in areas with conducive climates.

To evaluate the effect of weather conditions on ustiloxins contamination of rice, a correlation analysis was conducted between the agroclimatic conditions (temperature, humidity, and precipitation) during rice growth and the occurrence of ustiloxins. The agroclimatic condition data for July, August, and September were acquired from the China Meteorological Administration. Before correlation analysis, the monthly average temperature, precipitation, and relative humidity were obtained. Results showed that the incidence of ustiloxins was highly correlated with local agroclimatic conditions in July, August, and September (Table 3). Both mean concentrations and incidence of ustiloxins were significantly positively correlated with the mean precipitation of July ($p < 0.05$) and August ($p < 0.01$) (Figure 5). This is consistent with Jaime-Garcia et al. [42], which found that aflatoxin contamination in cottonseed was positively correlated with precipitation. No significant correlation was found between the incidence of ustiloxins and the mean precipitation of September, October, and November. Mean concentrations of ustiloxins did not correlate with mean temperatures of any month from July to November. However,

the incidence of ustiloxins was negatively correlated with monthly mean temperatures from July to October ($p < 0.05$), which is consistent with the results of previous studies [43]. Moreover, the incidence of ustiloxins was positively correlated with monthly relative humidity from July to September ($p < 0.05$). Our study further confirms that moderate temperatures and high relative humidity promote fungal growth and increase ustiloxins production. In agricultural production, appropriately reducing rice cultivation density can lower the relative humidity of the field and is expected to decrease the occurrence of ustiloxins.

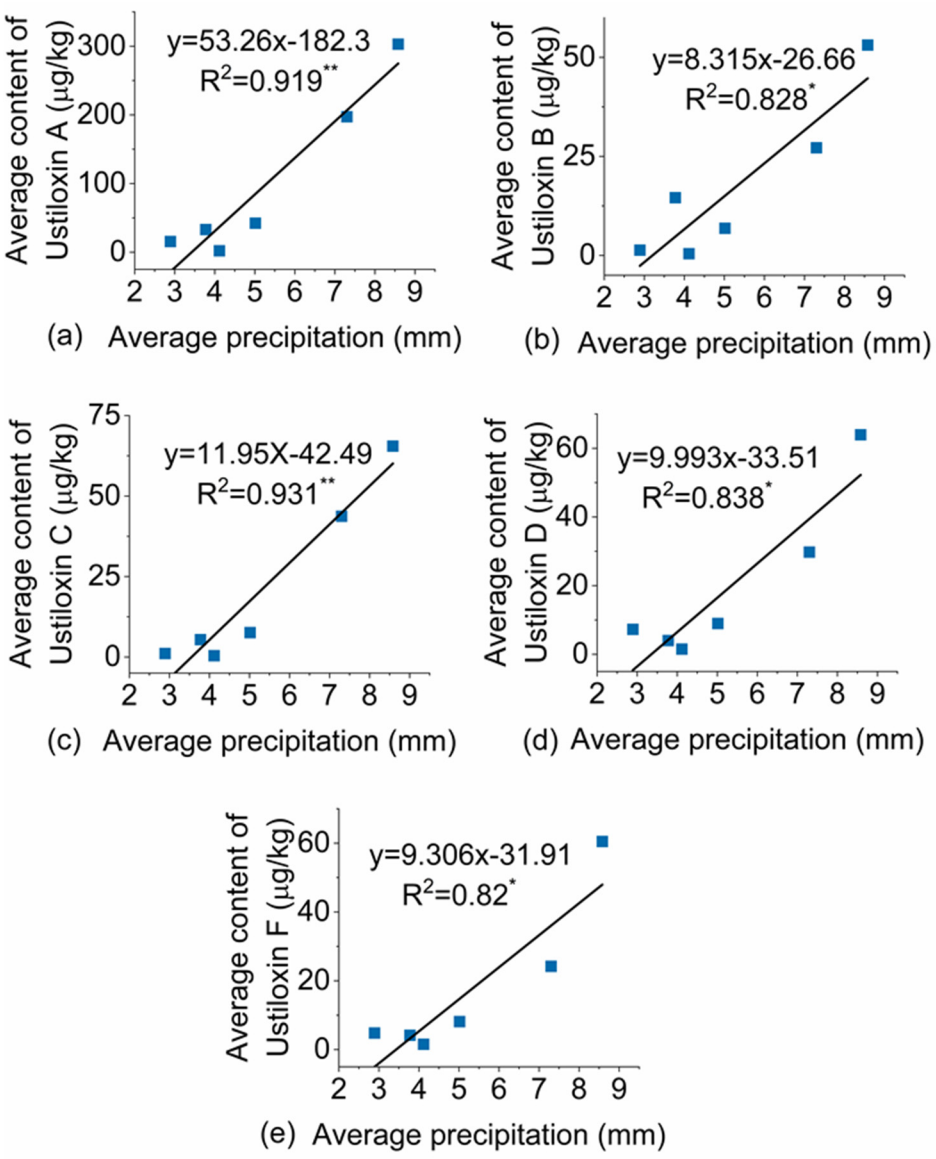

Figure 5. Scatter plot representation of relationships between mean precipitation of August and mean ustiloxins concentrations: (**a**) ustiloxin A, (**b**) ustiloxin B, (**c**) ustiloxin C, (**d**) ustiloxin D, and (**e**) ustiloxin F. Parameters and coefficients of determination (R^2) are shown. * and **, significant at $p < 0.05$ and at $p < 0.01$, respectively.

Table 3. Correlations among agroclimatic factors of July to September and measured ustiloxins.

	July			August			September		
	Mean Precipitation/mm	Air Temperature/°C	Air Humidity/%	Mean Precipitation/mm	Air Temperature/°C	Air Humidity/%	Mean Precipitation/mm	Air Temperature/°C	Air Humidity/%
Mean concentrations of ustiloxins/ μg/kg	0.370 *	−0.185 ^{NS}	0.399 *	0.598 **	−0.292 ^{NS}	0.361 ^{NS}	0.159 ^{NS}	−0.246 ^{NS}	0.394 *
The incidence of ustiloxins/%	0.367 *	−0.363 *	0.492 **	0.507 **	−0.426 *	0.435 *	0.200 ^{NS}	−0.406 *	0.472 **

Note: Spearman's coefficient (p). ** indicates significance at the 0.01 probability level between the two factors ($p < 0.01$), * indicates significance at the 0.05 probability level ($p < 0.05$), and ^{NS} indicates no significant correlation at the 0.05 probability level ($p < 0.1$).

In southern Europe, when rainy or humid weather coincides with the flowering stage of wheat, *Fusarium* head blight infection is likely to occur [44]. Similarly, *Ustilaginoidea virens* mainly infect the filaments of rice panicles and result in RFS disease. The critical infection period of *Ustilaginoidea virens* was at the booting stage of rice. Therefore, it is expected that weather conditions in this period would be closely related to ustiloxin levels. In the Hunan Province of China, late rice typically reaches the booting and heading stage in August. Hence, linear models were developed in this study to explain the relationship between the precipitation of August and ustiloxins concentrations. Regression analysis indicated that an obvious linear relationship existed between the precipitation of August and ustiloxin concentrations, with linear correlation coefficients (R^2) of 0.919, 0.828, 0.931, 0.838, and 0.820 for ustiloxins A, B, C, D, and F, respectively (Figure 5). Studies have reported that the release of pathogen spores was large on rainy days, and the germination rate of *Ustilaginoidea virens* conidia was significantly increased under higher relative humidity conditions [31]. Therefore, rainy and humid climatic conditions during the rice booting stage can increase the severity of RFS disease and further aggravated the contamination of ustiloxins in rice. These findings are useful for the prediction and mitigation of ustiloxins occurrence.

3.6. Comparison of Our Study with Other Studies

Currently, limited reports on ustiloxins contamination in rice have been documented. Cao et al. collected ten rice grain samples from Zhejiang and found six samples containing ustiloxins, with the total content of ustiloxins ranging from 6.6 to 464.1 ng/g [19]. Fu et al. collected 10 grain samples from different regions throughout China to examine ustiloxins A and B as well as the ustiloxin contents in contaminated rice grains, ranging from 3.46 to 190.47 μg/g [23]. However, the number of samples collected in these studies was limited and could not objectively uncover the contamination of ustiloxins in China. Hu et al. reported the natural occurrence of ustiloxins A, B, and G in 206 rice samples from five provinces across China. They found that ustiloxin A was the predominant ustiloxin, with an occurrence and average concentration of 46.1% and 49.71 μg/kg, respectively, followed by ustiloxin B (31.1%, 13.31 μg/kg) [36]. Both the occurrence and average concentration of ustiloxin in their study were lower than in our study. Similar to our research, Hu et al. also uncovered the regional differences of ustiloxins, but they did not conduct an in-depth analysis and discussion of the results. In this study, sufficient representative paddy samples were obtained to analyze the natural contamination of five ustiloxins in southern China. The correlation between regional differences in ustiloxins and climate conditions was also examined, providing novel avenues for the prevention and control of ustiloxins.

4. Conclusions

In this study, a reliable LC-MS/MS method was established for simultaneous determination of ustiloxins A, B, C, D, and F in paddies. After evaluating the performance of method by determining the linearity, sensitivity, recovery, and precision, a total of 300 paddy samples from six different regions were analyzed. Results showed that the incidence of ustiloxins ranged from 29.0% to 96.7%, with 98.7% of all samples being positive for at least one type of ustiloxin. Obviously, the contamination level of ustiloxins in paddies is widespread and should be of grave concern. Among the five ustiloxins, the mean and

maximum concentrations of ustiloxin A were the highest, followed, in order, by ustiloxins C, B, D, and F. Ustiloxins A, B, and C should be the focus of further investigation due to their high concentrations and toxicity.

This study provides essential information on the contamination of paddy with ustiloxins across several geographic regions of southern China. Results showed that the ustiloxins content in paddy samples varied across geographic areas, and agroclimatic conditions were shown to correlate with ustiloxin contamination of rice. Notably, both mean concentrations and incidence of ustiloxins were significantly positively correlated with the mean precipitation of August ($p < 0.01$). This is because August coincides with the booting and flowering stage period of late rice in Hunan Province. As a result, rainy conditions in this period are favorable for the production of RFS and ustiloxins. Adjusting the sowing date of rice in agricultural production at high rainfall areas may help to alleviate this problem.

Given the diverse climatic conditions across regions in China, agricultural producers and local authorities must acknowledge the impact of climate on cereal quality and safety and develop appropriate agronomic measures to reduce the effects of mycotoxins on human health. This is a critical survey about the distribution of ustiloxins in southern China and is helpful for establishing legal limits for ustiloxins in rice. However, our study only investigated the ustiloxin contamination in 2021. Future studies should validate these results by including additional years and regions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14050976/s1, Figure S1: Precipitation, temperature, and relative humidity at rice cultivation (from July to November) in different regions of southern China: (a) Taoyuan County, Changde City; (b) Yueyang County, Yueyang City; (c) Sangzhi County, Zhuangjiajie City; (d) Hengyang County, Hengyang City; (e) Lukou District, Zhuzhou City; and (f) Shuangfeng County, Loudi City. Table S1: Results of retention times and MS/MS parameters for the five ustiloxins. Table S2: Concentrations of ustiloxins in rice harvested in six different regions of Hunan, China.

Author Contributions: Conceptualization, X.M., Y.L. and S.T.; data curation, Y.M.; investigation, N.K. and J.W.; methodology, H.Z.; project administration, Q.T.; supervision, Q.T.; writing—original draft, X.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Hunan Province Science and Technology Innovation Plan Project (2023NK2016); National Key Research and Development Program of China (2021YFD1401100).

Data Availability Statement: Data will be made available on request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Bao, J.; Ying, Y.; Xu, Y.; Zhou, X.; Yan, S.; Pang, Y. Variation in physicochemical properties and nutritional quality in chalky mutants derived from an indica rice. *J. Cereal Sci.* **2020**, *91*, 102899. [CrossRef]
2. Chandravarnan, P.; Agyei, D.; Ali, A. Green and sustainable technologies for the decontamination of fungi and mycotoxins in rice: A review. *Trends Food Sci. Technol.* **2022**, *124*, 278–295. [CrossRef]
3. Reddy, K.R.N.; Reddy, C.S.; Abbas, H.K.; Abel, C.A.; Muralidharan, K. Mycotoxigenic fungi, mycotoxins, and management of rice grains. *Toxin Rev.* **2008**, *27*, 287–317. [CrossRef]
4. Mazaheri, M. Investigating the possibility of the occurrence of mycotoxins in rice and rice flour imported to Iran. *J. Food Compos. Anal.* **2023**, *122*, 105464. [CrossRef]
5. Laganà, A. Introduction to the toxins special issue on LC-MS/MS methods for mycotoxin analysis. *Toxin* **2017**, *9*, 325. [CrossRef] [PubMed]
6. Ferre, F.S. Worldwide occurrence of mycotoxins in rice. *Food Control* **2016**, *62*, 291–298. [CrossRef]
7. Reinhold, L.; Reinhardt, K. Mycotoxins in foods in lower Saxony (Germany): Results of official control analyses performed in 2009. *Mycotoxin Res.* **2011**, *27*, 137–143. [CrossRef] [PubMed]
8. Aydin, A.; Aksu, H.; Gunsen, U. Mycotoxin levels and incidence of mould in Turkish rice. *Environ. Monit. Assess.* **2011**, *178*, 271–280. [CrossRef] [PubMed]
9. Lai, X.W.; Liu, R.C.; Ruan, C.Q.; Zhang, H.; Liu, C.L. Occurrence of aflatoxins and ochratoxin A in rice samples from six provinces in China. *Food Control* **2015**, *50*, 401–404. [CrossRef]

10. Li, R.; Wang, X.; Zhou, T.; Yang, D.G.; Wang, Q.; Zhou, Y. Occurrence of four mycotoxins in cereal and oil products in Yangtze Delta region of China and their food safety risks. *Food Control* **2014**, *35*, 117–122. [CrossRef]
11. Mesterhazy, A.; Olah, J.; Popp, J. Losses in the grain supply chain: Causes and solutions. *Sustainability* **2020**, *12*, 2342. [CrossRef]
12. Commission Regulation (EU). 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. *Off. J. Eur. Union* **2023**, *119*, 103–157.
13. Taghizadeh, S.F.; Rezaee, R.; Badiebostan, H.; Giesy, J.P.; Karimi, G. Occurrence of mycotoxins in rice consumed by Iranians: A probabilistic assessment of risk to health. *Food Addit. Contam. Part A* **2020**, *37*, 342–354. [CrossRef] [PubMed]
14. Khanal, S.; Gaire, S.; Zhou, X.G. Kernel Smut and False Smut: The Old-Emerging Diseases of Rice—A Review. *Phytopathology* **2023**, *113*, 931–944. [CrossRef] [PubMed]
15. Brooks, S.A.; Anders, M.M.; Yeater, K.M. Effect of cultural management practices on the severity of false smut of rice. *Plant Dis.* **2009**, *93*, 1202–1208. [CrossRef]
16. Koiso, Y.; Li, Y.; Iwasaki, S.; Hanaoka, K.; Kobayashi, T.; Fujita, Y.; Yaegashi, H.; Sato, Z. Ustiloxins, antimitotic cyclic peptides from false smut balls on rice panicles caused by Ustilaginoidea virens. *J. Antibiot.* **1994**, *47*, 765–773. [CrossRef] [PubMed]
17. Meng, J.J.; Sun, W.B.; Mao, Z.L.; Xu, D.; Wang, X.H.; Lu, S.Q.; Lai, D.W.; Liu, Y.; Zhou, L.G.; Zhang, G.Z. Main ustilaginoidins and their distribution in rice false smut balls. *Toxins* **2015**, *7*, 4023–4034. [CrossRef] [PubMed]
18. Wang, X.; Wang, J.; Lai, D.; Wang, W.; Dai, J.; Zhou, L.; Liu, Y. Ustiloxin G, a new cyclopeptide mycotoxin from rice false smut balls. *Toxins* **2017**, *9*, 54. [CrossRef] [PubMed]
19. Cao, Z.Y.; Sun, L.H.; Mou, R.X.; Lin, X.Y.; Zhou, R.; Ma, Y.N.; Chen, M.X. Analysis of ustiloxins in rice using polymer cation exchange cleanup followed by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2016**, *1476*, 46–52. [CrossRef]
20. Koiso, Y.; Natori, M.; Iwasaki, S.; Sato, S.; Sonoda, R.; Fujita, Y.; Yaegashi, H.; Sato, Z. Ustiloxin: Aphytotoxin and a mycotoxin from false smut balls on rice panicles. *Tetrahedron Lett.* **1992**, *33*, 4157–4160. [CrossRef]
21. Cheng, S.Y.; Liu, H.; Sun, Q.; Kong, R.; Letcher, R.J.; Liu, C.S. Occurrence of the fungus mycotoxin, ustiloxin A, in surface waters of paddy fields in Enshi, Hubei, China, and toxicity in *Tetrahymena thermophila*. *Environ. Pollut.* **2019**, *251*, 901–909. [CrossRef]
22. Nakamura, K.; Izumiyama, N.; Ohtsubo, K.; Koiso, Y.; Iwasaki, S. Apoptosis induced in the liver, kidney and urinary bladder of mice by the fungal toxin produced by Ustilaginoidea virens. *Mycotoxins* **1993**, *38*, 25–30. [CrossRef]
23. Fu, X.; Wang, W.; Li, Y.; Wang, X.; Tan, G.; Lai, D.; Wang, M.G.; Zhou, L.G.; Wang, B.M. Development of a monoclonal antibody with equal reactivity to ustiloxins A and B for quantification of main cyclopeptide mycotoxins in rice samples. *Food Control* **2018**, *92*, 201–207. [CrossRef]
24. Shen, F.; Wu, Q.; Shao, X.; Zhang, Q. Non-destructive and rapid evaluation of aflatoxins in brown rice by using near-infrared and mid-infrared spectroscopic techniques. *J. Food Sci. Technol.* **2018**, *55*, 1175–1184. [CrossRef] [PubMed]
25. Shan, T.; Sun, W.; Liu, H.; Gao, S.; Lu, S.; Wang, M.; Sun, W.; Chen, Z.; Wang, S.; Zhou, L. Determination and analysis of ustiloxins A and B by LC-ESI-MS and HPLC in false smut balls of rice. *Int. J. Mol. Sci.* **2012**, *13*, 11275–11287. [CrossRef]
26. Liao, C.; Wong, J.; Zhang, K.; Hayward, D.; Lee, N.; Trucksess, M. Multi-mycotoxin analysis of finished grain and nut products using high-performance liquid chromatography-triple-quadrupole mass spectrometry. *J. Agric. Food Chem.* **2013**, *61*, 4771–4782. [CrossRef]
27. Al-Zoreky, N.S.; Saleh, F.A. Limited survey on aflatoxin contamination in rice. *Saudi J. Biol. Sci.* **2019**, *26*, 225–231. [CrossRef] [PubMed]
28. Chhaya, R.S.; O'Brien, J.; Cummins, E. Feed to fork risk assessment of mycotoxins under climate change influences-recent developments. *Trends Food Sci. Technol.* **2022**, *126*, 126–141. [CrossRef]
29. Moretti, A.; Pascale, M.; Logrieco, A.F. Mycotoxin risks under a climate change scenario in Europe. *Trends Food Sci. Technol.* **2019**, *84*, 38–40. [CrossRef]
30. Mahato, D.K.; Devi, S.; Pandhi, S.; Sharma, B.; Maurya, K.K.; Mishra, S.; Dhawan, K.; Selvakumar, R.; Kamle, M.; Mishra, A.K.; et al. Occurrence, impact on agriculture, human health, and management strategies of zearalenone in food and feed: A review. *Toxins* **2021**, *13*, 92. [CrossRef]
31. Song, J.H.; Yan, W.; Chen, L.R.; Zhang, S.J.; Chuan, N.; Zhu, D.; Liang, Y.; Luo, J.F.; Dou, Z.; Gao, H.; et al. Higher relative humidity and more moderate temperatures increase the severity of rice false smut disease in the rice–crayfish coculture system. *Food Energy Secur.* **2022**, *11*, 323.
32. Sun, X.D.; Su, P.; Shan, H. Mycotoxin contamination of rice in China. *J. Food Sci.* **2017**, *82*, 573–584. [CrossRef] [PubMed]
33. Liu, X.Y.; Matsumoto, H.; Lv, T.X.; Zhan, C.F.; Fang, H.D.; Pan, Q.Q.; Xu, H.R.; Fan, X.Y.; Chu, T.Y.; Chen, S.L.; et al. Phyllosphere microbiome induces host metabolic defence against rice false-smut disease. *Nat. Microbiol.* **2023**, *8*, 1419–1433. [CrossRef] [PubMed]
34. Gonçalves, A.; Gkrillas, A.; Dorne, J.L.; Dall'Asta, C.; Palumbo, R.; Lima, N.; Battilani, P.; Venâncio, A.; Giorni, P. Pre- and postharvest strategies to minimize mycotoxin contamination in the rice food chain. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 441–454. [CrossRef] [PubMed]
35. Koiso, Y.; Morisaki, N.; Yamashita, Y.; Mitsui, Y.; Shirai, R.; Hashimoto, Y.; Iwasaki, S. Isolation and structure of an antimitotic cyclic peptide, ustiloxin F: Chemical interrelation with a homologous peptide, ustiloxin B. *J. Antibiot.* **1998**, *51*, 418–422. [CrossRef] [PubMed]

36. Hu, Z.; Qian, S.A.; Fan, K.; Yu, Y.; Liu, X.; Liu, H.; Meng, J.; Zhao, Z.; Han, Z. Natural occurrence of ustiloxins in rice from five provinces in China and the removal efficiencies of different milling steps. *J. Sci. Food Agric.* **2023**, *103*, 6272–6279. [CrossRef] [PubMed]
37. Wang, G.; He, D.; Zhao, F.; Hu, J.; Lee, Y.W.; Shi, J.; Xu, J. Extraction and purification of ustiloxin A from rice false smut balls by a combination of macroporous resin and high-speed countercurrent chromatography. *Food Prod. Process. Nutr.* **2020**, *2*, 29. [CrossRef]
38. Ksenija, N. Mycotoxins–climate impact and steps to prevention based on prediction. *Acta Vet.* **2018**, *68*, 1–15. [CrossRef]
39. Cotty, P.J.; Jaime-Garcia, R. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *Int. J. Food Microbiol.* **2007**, *119*, 109–115. [CrossRef]
40. Franz, E.; Boou, K.; Van Der Fels-Klerx, I. Prediction of deoxynivalenol contentin Dutch winter wheat. *J. Food Prot.* **2009**, *72*, 2170–2177. [CrossRef]
41. Parikka, P.; Hakala, K.; Tiilikkala, K. Expected shifts in Fusarium species' composition on cereal grain in Northern Europe due to climatic change. *Food Addit. Contam. Part A* **2012**, *29*, 1543–1555. [CrossRef] [PubMed]
42. Jaime-Garcia, R.; Cotty, P.J. Aflatoxin contamination in commercial cottonseed in South Texas. *Phytopathology* **2003**, *93*, 1190–1200. [CrossRef] [PubMed]
43. Gagiu, V.; Mateescu, E.; Armeanu, I.; Dobre, A.A.; Smeu, I.; Cucu, M.E.; Oprea, O.; Iorga, E.; Belc, N. Post-harvest contamination with mycotoxins in the context of the geographic and agroclimatic conditions in Romania. *Toxins* **2018**, *10*, 533. [CrossRef]
44. Reyneri, A. The Role of Climatic Condition on Micotoxin Production in Cereal. *Vet. Res. Commun.* **2006**, *30*, 87–92. [CrossRef]