

Article

Baseline Sensitivity and Resistance Risk Assessment of the *Fusarium graminearum* Species Complex to Pyrifenox in China

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Abstract

Pyrifenox is a pyridine fungicide belonging to the pyridine subgroup of demethylation inhibitor (DMI) fungicides (FRAC group 3), and the resistance risk of the *Fusarium graminearum* species complex (FGSC) to this fungicide remains poorly understood. In this study, 122 FGSC isolates were collected from five provinces across China during 2024–2025. The baseline sensitivity was characterized using a mycelial growth assay, yielding a mean EC₅₀ value of 3.300 ± 0.227 mg/L. Four phenotypically resistant variants were generated via fungicide adaptation combined with UV mutagenesis, with resistance factors ranging from 5.53 to 450.26, covering low, moderate, and high resistance phenotypes. After ten serial transfers on fungicide-free medium, the resistance of these variants decreased significantly. These variants also exhibited reduced sporulation and pathogenicity compared with their parental isolates. However, because these assays were performed on tenth-generation variants that had already lost most of their resistance, these changes cannot be directly attributed to resistance. Cross-resistance assays revealed positive cross-resistance only with tebuconazole, whereas no cross-resistance was found with prothioconazole, pyraclostrobin, and three other fungicides. Target gene sequencing identified no mutations in *CYP51A*, *CYP51B*, or *CYP51C*, implying non-target-site resistance. Overall, the FGSC presents a low resistance risk to pyrifenox. However, species identity within the FGSC was not resolved in this study, and this conclusion should be interpreted with caution. This study established, to our knowledge, the first sensitivity baseline for pyrifenox against the FGSC in China, providing fundamental data for rational field application to control Fusarium head blight.

Keywords: Fusarium head blight; fungicides; sensitivity baseline; pyrifenox; fungicide resistance



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1. Introduction

Fusarium head blight (FHB) is a devastating fungal disease that poses a serious threat to global wheat production [1]. Infection by *Fusarium* pathogens not only causes direct yield losses, including spikelet blight, reduced seed set, and decreased thousand-kernel weight, but also leads to the accumulation of various mycotoxins in the grain [2,3]. In China, FHB of wheat is predominantly caused by the FGSC [4]. Other *Fusarium* species, including *F. oxysporum* and *F. poae*, as well as *Microdochium nivale* and *F. verticillioides*, are also capable of causing the disease [5]. When harvested grains from infected plants are processed into wheat-based products, they exhibit reduced flour yield and dull flour color, and the resulting food products suffer from significantly deteriorated taste, thereby

severely compromising food processing quality [6,7]. In addition, the disease reduces seed germination rates and retards seedling growth, resulting in inferior plant performance throughout the entire growth period compared with healthy plants [8]. Under favorable environmental conditions, infected seeds can also serve as primary inoculum sources, further spreading the disease to surrounding healthy wheat plants [9,10]. Among the mycotoxins accumulated in infected grains, deoxynivalenol (DON) is the most abundant and toxic, representing the most prominent toxin species of concern [11]. Chronic or excessive consumption of contaminated wheat can induce toxic symptoms such as vomiting, diarrhea, and immunosuppression, and may even adversely affect growth, development, and reproductive health [12].

Chemical control applied during the wheat anthesis stage is the primary strategy for managing FHB [13]. However, the prolonged and exclusive reliance on chemical fungicides has led to the emergence of resistant pathogen populations [14]. In China, field isolates with fungicide resistance were detected many years ago, and their frequency has been increasing annually. The frequencies of MBC-R *F. graminearum* isolates collected from conventional fields in Jiangsu Province reached 61.32%, 58.03%, and 56.80% in 2016–2018, respectively [15]. In 2020, among 2910 the FGSC isolates collected from fields in Jiangsu, Henan, and Anhui provinces, 67 (2.3%) were identified as fludioxonil-resistant isolates [16].

Pyrifenoxy is a pyridine DMI fungicide (FRAC group 3) with both protective and curative properties, developed by R. Maag Ltd. (Dielsdorf, Switzerland) (Figure 1). It is effective against a range of plant pathogens belonging to the Ascomycetes, Basidiomycetes, and Deuteromycetes. Previous studies have demonstrated the efficacy of pyrifenoxy in controlling plant fungal diseases. Research using *Ustilago maydis* as a model organism revealed that this compound inhibits C-14 demethylation, thereby disrupting ergosterol biosynthesis [17]. Biochemical studies have shown that pyrifenoxy potently inhibits 14 α -demethylation during ergosterol biosynthesis. This reaction is catalyzed by CYP51 (sterol 14 α -demethylase), a cytochrome P450 enzyme that serves as the primary target of DMI fungicides. Pyrifenoxy binds to the CYP51 enzyme and blocks the demethylation step, thereby disrupting ergosterol biosynthesis and impairing membrane integrity. The interaction between pyrifenoxy and CYP51 provides the mechanistic basis for investigating whether resistance in the FGSC is associated with target-site alterations in the CYP51 genes [18].

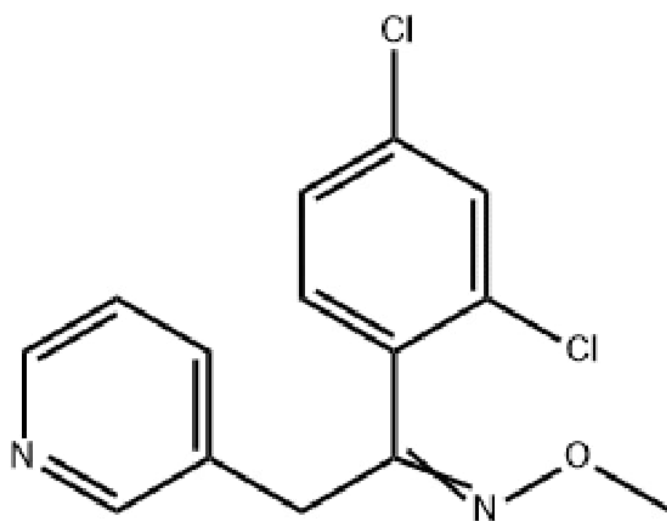


Figure 1. Structural formula of pyrifenoxy (PubChem CID: 55790; <https://pubchem.ncbi.nlm.nih.gov/>, accessed on 12 September 2026). Drawn using InDraw (v 7.1.1).

This study aimed to determine the sensitivity of the FGSC isolates collected from different regions of China to pyrifenoxy and to establish the baseline sensitivity, thereby

providing a reference for future field resistance monitoring. Resistant variants of the FGSC to pyrifenoxy were obtained through induction. The *CYP51* gene, which encodes the target enzyme of DMI fungicides, was sequenced in all obtained variants to determine whether resistance was associated with target-site mutations. The fitness of the resistant variants was evaluated in terms of cross-resistance, stability, pathogenicity, fecundity, and mycelial growth rate, allowing for a preliminary assessment of the resistance risk of the FGSC to pyrifenoxy. The findings of this study provide a scientific basis for the development of resistance management strategies.

2. Materials and Methods

2.1. Fungicides and Culture Media

Pyrifenoxy (a.i. 96%) was provided by Shaanxi Nuozheng Biotechnology (China) Co., Ltd. (Weinan, China). Prothioconazole (a.i. 95%) was provided by Shandong Hailir Chemical Co., Ltd. (Weifang, China). Tebuconazole (a.i. 96%) and hexaconazole (a.i. 96%) were provided by Ningbo Sunjoy Agrosience (China) Co., Ltd. (Ningbo, China). Pyraclostrobin (a.i. 98%) was provided by Shandong Haililai Chemical Technology Co., Ltd. (Linyi, China). Thiophanate-methyl (a.i. 98%) and carbendazim (a.i. 98%) were provided by Shaanxi Nuozheng Biotechnology (China) Co., Ltd. (Weinan, China). All fungicides were dissolved in acetone and prepared as 1×10^4 mg/L mother liquor. The final acetone concentration in the medium did not exceed that corresponding to the highest fungicide concentration tested (10 mg/L), and a solvent control was included in all assays.

2.2. Collection and Isolation of Fungal Isolates

From 2024 to 2025, wheat spikes infected with FHB were collected from Shandong, Hebei, Henan, Anhui, and Jiangsu provinces in China. Diseased spikelets exhibiting reddish mold layers were subjected to tissue isolation. The samples were first rinsed with sterile water, surface-disinfected with 75% ethanol for 2 min, and then rinsed three times with sterile water. The disinfected tissues were placed on potato dextrose agar (PDA) plates and incubated at 25 °C in the dark. After mycelial growth was observed, hyphal tips were excised from the margin of actively growing colonies and transferred to fresh PDA plates. This purification step was repeated three times to obtain pure cultures, yielding a total of 122 FGSC isolates (Supplementary Table S1) [19–21]. All isolates were identified as FGSC based on morphological characteristics. Morphological identification was performed by examining colony morphology and conidial characteristics on PDA plates. In addition, 21 representative isolates were further confirmed by molecular identification. Genomic DNA was extracted, and the fungal universal primers ITS1/ITS4 were used to amplify the internal transcribed spacer (ITS) region [22]. The amplified products were sequenced and compared with reference sequences in GenBank. All 21 isolates showed $\geq 99\%$ sequence identity to the reference FGSC sequences, confirming their identity as the FGSC.

2.3. Toxicity Assay of Pyrifenoxy Against the FGSC and Establishment of Baseline Sensitivity

The effective concentration for 50% inhibition (EC_{50}) of the fungicide was determined using the mycelial growth rate method. The EC_{50} value represents the fungicide concentration that inhibits mycelial growth by 50% relative to the fungicide-free control, and is widely used as a quantitative indicator of fungal sensitivity to a given fungicide [5,23,24]. Cryopreserved isolates were first inoculated onto PDA plates and activated at 25 °C for 4 days. Mycelial plugs (7 mm in diameter) were cut from the colony margin using a cork borer and transferred onto media containing different concentrations of the fungicide, as well as onto fungicide-free control media. The fungicide was added to the medium at concentrations of 0, 0.625, 1.25, 2.5, 5, and 10 mg/L. Each treatment was performed in

duplicate. All fungicides were dissolved in acetone. The final acetone concentration in the medium corresponded to that of the highest fungicide concentration tested (10 mg/L), and a solvent control containing the same volume of acetone but no fungicide was included. The solvent control showed the same mycelial growth as the fungicide-free control, indicating that the acetone concentration used had no detectable effect on mycelial growth. The plates were sealed and incubated at 25 °C in the dark. When the colony diameter in the control group reached approximately two-thirds of the plate, the colony diameters in all treatments were measured using the cross-intersection method. The inhibition rate was calculated using the following formula:

$$\frac{\text{colony diameter of control} - \text{colony diameter of treatment}}{\text{colony diameter of control} - \text{mycelial plug diameter}} \times 100\%$$

Mycelial growth inhibition rates were calculated from the colony diameters of each treatment relative to the control. The inhibition rate was plotted against the logarithm of the fungicide concentration, and the toxicity regression equation was fitted using DPS 9.01. The EC₅₀ value was calculated from the fitted regression equation as the concentration that inhibited mycelial growth by 50%. A frequency distribution histogram of the sensitivity of the tested isolates was constructed, and the Shapiro–Wilk normality test was performed. The mean EC₅₀ value was used as the baseline sensitivity of the FGSC to pyrifenoxy [25]. It should be noted that the concentration series used for the baseline sensitivity assay (0.625–10 mg/L) did not cover the EC₅₀ values of two isolates with reduced sensitivity, HA-4 (22.704 mg/L) and YC-5. For these two isolates, the EC₅₀ values were extrapolated from the fitted regression equation and should therefore be regarded as approximate.

2.4. Induction of Resistance to Pyrifenoxy in the FGSC

Three isolates of the FGSC, WF-2 (EC₅₀ = 0.352 mg/L), HA-4 (EC₅₀ = 22.704 mg/L), and XT-2 (EC₅₀ = 2.012 mg/L), representing the most sensitive, the least sensitive, and an intermediate level of sensitivity, were selected. Mycelial plugs (7 mm in diameter) were prepared and inoculated onto media containing pyrifenoxy at concentrations of 50, 100, 200, and 400 mg/L, with three replicates per treatment, to determine the minimum inhibitory concentration (MIC) [26]. A blank control (medium without fungicide, receiving an equal volume of sterile water) was included. Resistant variants were generated using a combined approach of fungicide adaptation and UV mutagenesis. The parental isolates were inoculated onto PDA plates containing pyrifenoxy at the MIC (400 mg/L) and exposed to UV irradiation using a 15 W germicidal lamp at a distance of 20 cm for 20 min, followed by incubation at 25 °C in the dark for 7 days [27]. Mycelial growth was then observed; any isolate capable of normal growth or exhibiting sector mutations at the MIC was considered a putative resistant mutant. Resistant strains were further confirmed by re-screening on MIC plates. The EC₅₀ values of the variants were determined using the mycelial growth rate method, with fungicide concentrations of 0, 0.625, 1.25, 2.5, 5, and 10 mg/L, the same concentration series used for the baseline sensitivity assay. Each treatment was performed in duplicate, and the EC₅₀ values were calculated from the fitted regression equations. The resistance factor (RF) was calculated as the ratio of the EC₅₀ of the resistant mutant to the EC₅₀ of its corresponding parental isolate. Resistance levels were classified as follows: RF ≤ 2, sensitive; 2 < RF ≤ 10, low resistance; 10 < RF ≤ 100, moderate resistance; RF > 100, high resistance [28]. It should be noted that the MIC assay and the EC₅₀ assay were performed under different conditions and used different evaluation criteria. The MIC was defined as the lowest concentration that completely inhibited mycelial growth, whereas the EC₅₀ values were calculated from fitted regression equations over a lower concentration range. Therefore, similar EC₅₀ values do not necessarily imply identical

growth at 400 mg/L. In addition, HA-4 was a naturally less sensitive field isolate, whereas XT-2X and WF-2XX were obtained by UV mutagenesis; these two types of variants may differ in their resistance mechanisms, so their response to a high concentration such as 400 mg/L may differ even when the EC_{50} values are similar [22].

2.5. Determination of Genetic Stability

The resistant variants were successively subcultured on fungicide-free PDA medium for ten generations. The EC_{50} values of both the first-generation and tenth-generation variants were determined using the mycelial growth rate method. The resistance factors of the first- and tenth-generation variants were calculated and compared to evaluate the genetic stability of resistance [29]. Resistance was considered stable if the RF of the tenth generation did not differ significantly from that of the first generation ($p > 0.05$, LSD test) and remained within the same resistance category. As shown in Table 1, the resistance of all four variants declined markedly after ten successive transfers on fungicide-free medium, with EC_{50} values of the tenth-generation variants ranging from 1.759 to 2.772 mg/L; WF-2X, XT-2X, and HA-4X reverted to the sensitive or low-resistance category, and XT-2X and HA-4X even became more sensitive than their parental isolates. These results indicate that the resistance acquired by the variants was not stably inherited.

Table 1. Resistance level of the pyrifenoxy-resistant variants.

Sample Number	Sensitivity to Pyrifenoxy	EC_{50} (mg/L)		Resistance Factor 1st	Resistance Factor 10th
		1st	10th		
WF-2	Sensitive	0.352	—	—	—
WF-2X	Resistant	158.490	1.981	450.26	5.06
WF-2XX	Resistant	17.028	2.772	48.38	7.88
XT-2	Sensitive	2.012	—	—	—
XT-2X	Resistant	22.701	1.759	11.28	0.87
HA-4	Sensitive	22.704	—	—	—
HA-4X	Resistant	125.558	1.944	5.53	0.09

Note: RF_{1st} and RF_{10th} refer to the resistance factors of the first-generation and tenth-generation variants, respectively, calculated as the EC_{50} of the variant divided by the EC_{50} of its parental isolate.

2.6. Comparison of Biological Fitness Between Resistant Variants and Their Parental Isolates

Uniform mycelial plugs were prepared from actively growing tenth-generation variants and parental isolates using a cork borer (7 mm in diameter). It should be noted that the tenth-generation variants had already lost most of their resistance (Table 1); therefore, the fitness parameters measured here reflect the phenotypic characteristics of the variant lines rather than a direct fitness cost of resistance. The plugs were inoculated upside down onto fungicide-free PDA plates, with two replicates per isolate, and incubated at 25 °C in the dark for 4 days. Colony diameters were measured using the cross-intersection method, and the mean values were calculated. The mycelial growth rate was determined based on the colony diameter and the incubation time to evaluate the effect of resistance on mycelial growth [30].

Ten uniform mycelial plugs of each isolate were inoculated into mung bean liquid medium and incubated on a rotary shaker at 150 r/min and 25 °C for 5 days. Mung bean medium was prepared by boiling 30 g of mung beans in 1000 mL of distilled water for 30 min. The mixture was filtered through double-layered gauze, and the filtrate was made up to 1000 mL with distilled water and autoclaved at 121 °C for 20 min [31]. The culture filtrate was filtered through double-layered gauze, serially diluted, and loaded onto a hemocytometer for microscopic counting. Each isolate was counted ten times, and the spore yield per milliliter was calculated to compare sporulation capacity among isolates.

Wheat grains were surface-disinfected, soaked in sterile water, and incubated at 20 °C under high humidity for 3 days until the coleoptiles reached 2–3 cm in length. The coleoptile tips were excised, and the wounds were wrapped with filter paper strips saturated with spore suspension at a concentration of 1×10^6 conidia/mL, with sterile water used as the control. The inoculated coleoptiles were incubated at 25 °C under a 12 h light/12 h dark photoperiod. Ten coleoptiles were inoculated per isolate, and lesion lengths on each coleoptile were measured at 5 days after inoculation. Pathogenicity was classified as follows: lesion length 0–0.5 cm, low pathogenicity; 0.5–1.0 cm, moderate pathogenicity; >1.0 cm, high pathogenicity [32].

DON production was induced by culturing the isolates in trichothecene biosynthesis induction (TBI) medium. The TBI medium contained the following components per liter: 30 g sucrose, 1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KCl, 10 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 800 mg putrescine, and 200 μL trace element solution, with the pH adjusted to 4.5. The trace element solution consisted of 5 g citric acid, 5 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 50 mg H_3BO_3 , and 50 mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ per 100 mL. Cultures were incubated at 28 °C in the dark with shaking at 150–180 rpm for 7 days. The DON toxin content of the isolates was determined using the RIDASCREEN® DON ELISA kit (R-Biopharm, Catalog No. R5906) (Darmstadt, Germany). Each isolate was tested with two replicates. According to the manufacturer's specifications, the cross-reactivity of this kit is 100% for deoxynivalenol, 100% for 3-acetyldeoxynivalenol (3-ADON), and approximately 19% for 15-acetyldeoxynivalenol (15-ADON). Therefore, the measured values may represent the sum of DON and its acetylated derivatives rather than DON alone. A standard curve was constructed by plotting absorbance against the logarithmic values of DON concentration. The DON concentration of each sample was calculated based on its absorbance value according to the standard curve and expressed as ng/mL of culture. It should be noted that DON values were expressed per milliliter of culture rather than per unit of mycelial dry weight. This approach is consistent with previous studies on DON production in liquid culture, in which DON yields were also expressed as concentrations in the culture medium [33,34]. Because the variants differed in growth rate, this may have influenced the comparison of DON production among isolates, and this is acknowledged as a limitation [35].

2.7. Determination of Cross-Resistance

The sensitivity of the resistant FGSC isolates to prothioconazole, tebuconazole, hexaconazole, pyraclostrobin, thiophanate-methyl, and carbendazim was analyzed using Pearson correlation analysis. The concentrations used were as follows: hexaconazole at 0, 0.375, 0.75, 1.5, 3, and 6 mg/L; prothioconazole at 0, 0.3125, 0.625, 1.25, 2.5, and 5 mg/L; tebuconazole at 0, 0.3125, 0.625, 1.25, 2.5, and 5 mg/L; pyraclostrobin at 0, 0.5, 1, 2, 4, and 8 mg/L; thiophanate-methyl at 0, 1.25, 2.5, 5, 10, and 20 mg/L; and carbendazim at 0, 0.075, 0.15, 0.3, 0.6, and 1.2 mg/L. For pyraclostrobin, salicylhydroxamic acid (SHAM) was not added to inhibit the alternative oxidase pathway, which is a limitation of this study. The criteria for interpreting cross-resistance were as follows: $p > 0.05$, or $p < 0.05$ with $R^2 < 0.3$, indicated no cross-resistance between the two fungicides; $p < 0.05$ with $R^2 > 0.8$ indicated strong positive or negative cross-resistance; $p < 0.05$ with R^2 between 0.5 and 0.8 indicated the presence of cross-resistance; and $p < 0.05$ with R^2 between 0.3 and 0.5 indicated a low probability of cross-resistance between the two fungicides [36]. All EC_{50} values in Table 2 were obtained from tenth-generation variants, which had already lost most of their resistance; the correlation analysis therefore reflects the sensitivity patterns of these variant lines rather than cross-resistance associated with the resistant phenotype. It should be noted that the correlation analysis was based on only seven isolates (four

variants and three parental isolates), and the statistical power is therefore limited. The cross-resistance results should be interpreted with caution.

Table 2. Sensitivity of pyrifeno-resistant variants and their parental isolates to six fungicides.

Strain Number	EC ₅₀ (mg/L)						
	Pyrifeno	Hexaconazole	Prothioconazole	Tebuconazole	Pyraclostrobin	Thiophanate-Methyl	Carbendazim
HA-4	22.704	0.936	0.856	0.355	0.715	5.422	0.574
HA-4X	125.558	0.656	0.765	0.583	0.749	11.287	0.636
XT-2	2.012	0.494	0.128	0.137	0.286	6.729	0.307
XT-2X	22.701	0.652	0.363	0.219	0.228	6.871	0.515
WF-2	0.352	0.315	0.113	0.159	0.284	6.066	0.592
WF-2X	158.490	0.510	0.196	0.302	0.466	3.074	0.347
WF-2XX	17.028	0.161	0.133	0.296	0.196	4.865	0.355

2.8. Investigation of the Resistance Mechanism of the FGSC to Pyrifeno

PCR primers were designed based on the sequences of three 14- α -demethylase *CYP51* genes associated with triazole fungicide resistance (*CYP51A*, FGSG_04092; *CYP51B*, FGSG_01000; *CYP51C*, FGSG_11024) [37]. All primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) (Table 3).

Table 3. PCR primers used for amplification of the three *CYP51* genes in the FGSC (Note: The primers amplify the regions of the *CYP51* coding sequences that contain the previously reported DMI resistance-associated codons; they do not cover the complete coding sequences).

Gene Name	Primer	Sequence	Amplicon Size (bp)
<i>CYP51A</i>	FGSG-04092-F	ATGTTCCATCTACTCATCTATC	1521 bp
<i>CYP51A</i>	FGSG-04092-R	CTATATCTTCTTCTACGCTCC	1521 bp
<i>CYP51B</i>	FGSG-01000-F	TCCTCCTTACAACACCCAACC	1578 bp
<i>CYP51B</i>	FGSG-01000-R	ACAGCCCAGCCATTACAAAA	1578 bp
<i>CYP51C</i>	FGSG-11024-F	TGCCCTGGAGTTAGTAGCGG	1551 bp
<i>CYP51C</i>	FGSG-11024-R	CCTGGTGGATTCACGGATTG	1551 bp

The primers did not cover the complete coding sequences of *CYP51A*, *CYP51B*, and *CYP51C*. The amplified regions included the codons encoding the previously reported DMI resistance-associated amino acid positions, but the remaining regions of the coding sequences were not examined. This is acknowledged as a limitation.

Genomic DNA was extracted using the Ezup Column Fungal Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. Briefly, 50–100 mg of fresh mycelium was ground to a powder in liquid nitrogen, lysed in Buffer Digestion containing β -mercaptoethanol and Proteinase K at 56 °C for 1 h, and then purified through the spin column following the manufacturer's protocol. DNA was eluted with 50 μ L of TE Buffer and stored at −20 °C.

PCR amplification was performed in a total volume of 25 μ L containing 2 μ L of genomic DNA, 12.5 μ L of 2 $\times$ Taq Master Mix, 1 μ L of each primer (10 μ M), and 8.5 μ L of nuclease-free water. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55–58 °C for 30 s, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min, and holding at 4 °C.

PCR products were analyzed by 2% (*w/v*) agarose gel electrophoresis in 1 $\times$ TAE buffer. A suitable DNA molecular weight marker was run in parallel. After electrophoresis, gels were stained with ethidium bromide and visualized under UV light (312 nm) using a gel

documentation system. Successful amplification was confirmed by the presence of a single band of the expected size.

Subsequent gene sequencing was performed by Sangon Biotech (Shanghai) Co., Ltd. The resulting sequences were aligned with each other and with the corresponding sequences of the reference strain, FGSC PH-1 (GenBank assembly accession GCF_000240135.3), using MEGA 7.0.14. The alignments were inspected manually for base substitutions, insertions, or deletions between the variants and their parental isolates. The *CYP51A*, *CYP51B*, and *CYP51C* sequences obtained in this study have been added to Supplementary Text S1.

2.9. Data Analysis

All data obtained in this study were analyzed using Data Processing System (DPS 9.01). Data were first subjected to one-way analysis of variance (ANOVA). When a significant difference was detected among treatments ($p < 0.05$), Fisher's least significant difference (LSD) test was performed for multiple comparisons. The experimental unit was a Petri dish, and each treatment was performed with two replicates. The standard errors and significant differences were obtained using Fisher's LSD test ($p = 0.05$).

2.10. Use of Artificial Intelligence Tools

Artificial intelligence tools were used only for language refinement and for the preparation of graphical abstract illustrations; they were not used to generate, analyze, or interpret any scientific data. Specifically, DeepSeek (V3.2) was used to refine the language of Sections 2.4, 2.6 and 2.7 of Section 2, including grammar, spelling, and formatting. The authors reviewed and edited all AI-generated suggestions and accept full responsibility for the final content. In addition, Nano Banana 2 was used to create illustrative icons for the graphical abstract, including icons representing a UV lamp, a beaker, a gene, and a magnifying glass. No AI tool was used for data collection, statistical analysis, figure preparation, or the interpretation of results. The correlation analysis and scatter plots were generated using Origin 2021.

3. Result

3.1. Baseline Sensitivity of the FGSC to Pyrifenox

The EC_{50} values of the 120 FGSC isolates ranged from 0.352 to 9.367 mg/L, with a mean EC_{50} of 3.300 ± 0.227 mg/L. The Shapiro–Wilk test indicated that the untransformed EC_{50} values deviated significantly from a normal distribution ($W = 0.9365$, $p < 0.0001$), whereas the $\log_{10}$ -transformed EC_{50} values did not deviate significantly from normality ($W = 0.9924$, $p = 0.7623$) (Supplementary Table S2). The frequency distribution of the 120 isolates displayed a continuous unimodal curve (Figure 2).

3.2. Resistance Levels and Genetic Stability of Resistant Variants

The minimum inhibitory concentration (MIC) of pyrifenox for the wild-type isolates WF-2, XT-2, and HA-4 was 400 mg/L. Through simultaneous fungicide adaptation and UV mutagenesis, four pyrifenox-resistant variants were obtained, designated WF-2X, WF-2XX, XT-2X, and HA-4X. As shown in Table 1, the EC_{50} of WF-2X was 158.490 mg/L, exhibiting a 450.26-fold difference compared with its parental isolate WF-2, with a resistance factor exceeding 100; thus, it was classified as a highly resistant isolate. The EC_{50} values of WF-2XX and XT-2X were 17.028 and 22.701 mg/L, representing 48.38-fold and 11.28-fold differences relative to their respective parental isolates WF-2 and XT-2, with resistance factors between 10 and 100, thus classified as moderately resistant isolates. The EC_{50} of HA-4X was 125.558 mg/L, with a 5.53-fold difference compared with its parental isolate HA-4, and a resistance factor between 2 and 10, thus classified as a low-level resistant

isolate. These resistance levels refer to the first-generation variants. After ten successive transfers on fungicide-free medium, the resistance of all variants declined substantially (Table 1), and the tenth-generation variants were no longer resistant.

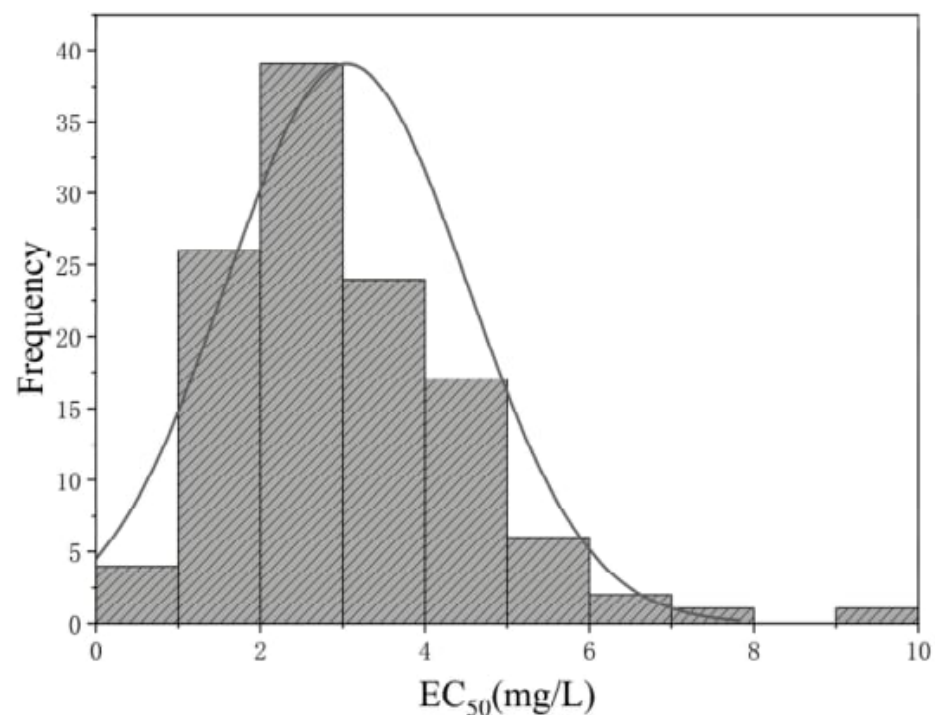


Figure 2. Frequency distribution of EC₅₀ of the FGSC to pyrifenoxy. The y-axis indicates the number of isolates.

3.3. Biological Fitness of Resistant Variants

The following fitness assays were performed using tenth-generation variants. Because these variants had already lost most of their resistance (Table 1), the observed changes in mycelial growth, sporulation, pathogenicity, and DON production cannot be attributed directly to resistance and should be interpreted as phenotypic characteristics of the variant lines. Measurements of the mean mycelial growth rate and sporulation capacity revealed that WF-2X, XT-2X, and HA-4X exhibited reduced growth rates compared with their parental isolates, whereas WF-2XX displayed a significantly higher growth rate than its parent (0.83 vs. 0.70 cm/d, $p < 0.05$). All resistant variants produced significantly fewer spores than their corresponding parental isolates. With regard to sporulation capacity, all resistant variants produced significantly fewer spores than their corresponding parental isolates. As indicated by the lesion length on coleoptiles, the mean lesion length caused by all variants was significantly shorter than that caused by the parental isolates, indicating a decline in pathogenicity (Figure 3). Specifically, the pathogenicity levels of WF-2X, WF-2XX, and XT-2X decreased to moderate, and that of HA-4X decreased to low (Table 4). It should be noted that the lesion length of XT-2X (0.50 cm) lies at the boundary between the low (0–0.5 cm, excluding 0.5 cm) and moderate (0.5–1.0 cm) pathogenicity classes; according to our classification criteria, it was assigned to the moderate category. The DON content analysis revealed significant differences in DON production between the parental isolates and their respective variants ($p < 0.05$). In the WF-2 and XT-2 groups, the variants produced significantly less DON than their parental isolates, whereas in the HA-4 group, the mutant produced significantly more DON than its parental isolate. These results suggest that the acquisition of the resistant phenotype was associated with changes in DON biosynthesis, and the effect varied among different parental backgrounds (Figure 4). However, because a

UV-only control was not included and the variants had already lost most of their resistance by the tenth generation, these changes cannot be attributed solely to resistance, and the possible contribution of UV mutagenesis cannot be excluded. In addition, the differences in DON production were relatively small (approximately 39–46 ng/mL), and their biological relevance should be interpreted with caution (Figure 4).



Figure 3. Pathogenicity of the pyrifenoX-resistant mutant and parental strains on wheat coleoptile. Coleoptiles were inoculated with spore suspensions (1×10^6 conidia/mL) of the parental isolates (WF-2, XT-2, HA-4) and their corresponding resistant variants (WF-2X, WF-2XX, XT-2X, HA-4X), with sterile water as the control (CK). Lesions were measured 3–5 days after inoculation.

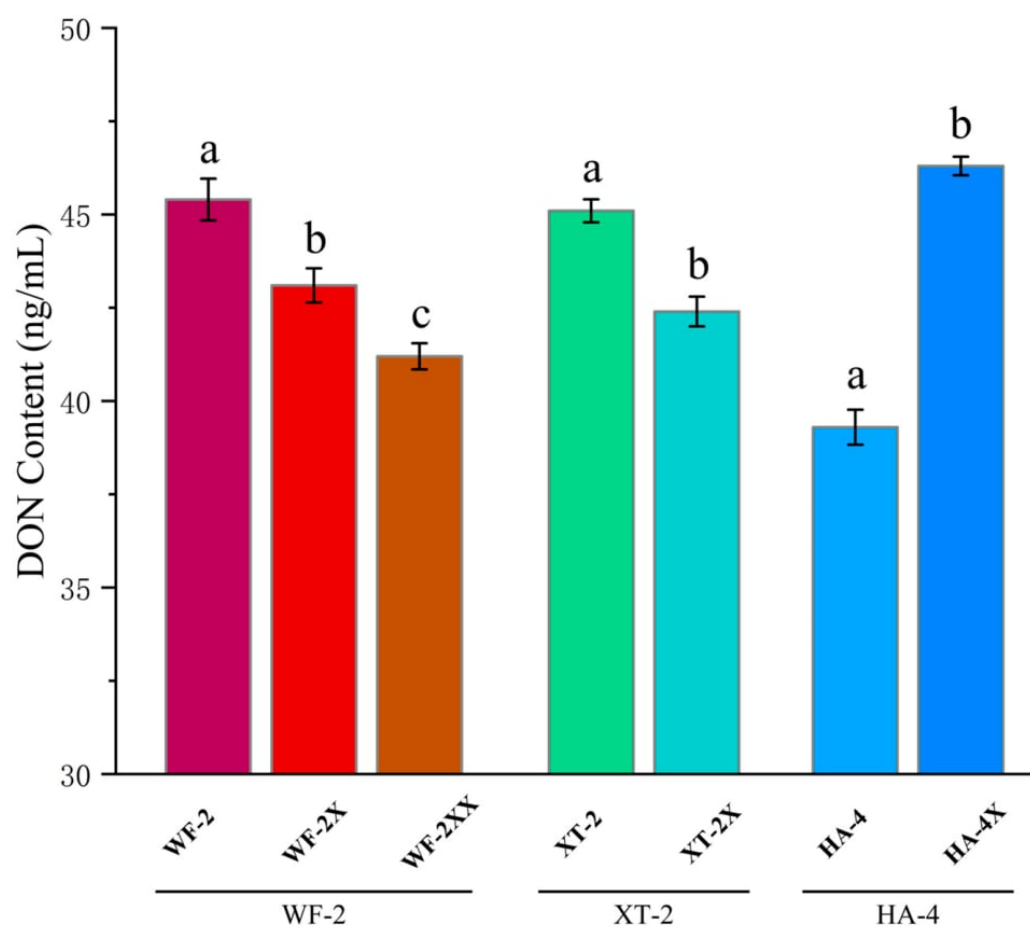


Figure 4. Mycelial DON content of pyrifenoX-resistant variants and their parental isolates. DON production was determined for each parental isolate (WF-2, XT-2, and HA-4) and its corresponding pyrifenoX-resistant variant (WF-2X, WF-2XX, XT-2X, and HA-4X). Bars represent the mean DON content (ng/mL) $\pm$ standard error. Within each parental group, different lowercase letters (a, b, and c) above the bars indicate significant differences among isolates according to Fisher's least significant difference (LSD) test at $p < 0.05$; bars sharing the same letter are not significantly different. The letters are only comparable within the same parental group and not between groups.

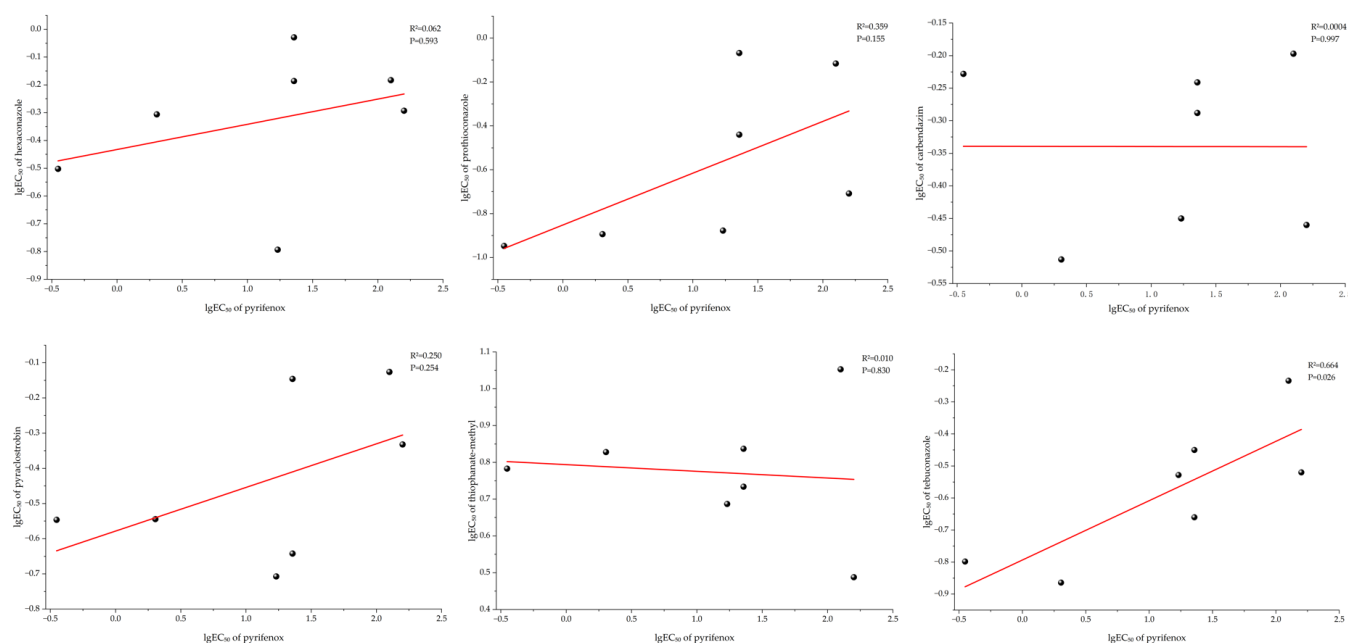
Table 4. Lesion length following infection with pyrifeno-resistant variants and their parental strains.

Strain Number	Average of Growth Rate $\pm$ SE (cm/d)	Spore Production $\pm$ SE (10^6 /mL)	Average Lesion Length $\pm$ SE (cm)	Pathogenicity Level
WF-2	0.70 ± 0.08 a	69.20 ± 3.87 a	1.50 ± 0.09 a	High
WF-2X	0.46 ± 0.03 b	42.80 ± 2.85 b	0.74 ± 0.06 b	Medium
WF-2XX	0.83 ± 0.03 c	26.40 ± 2.38 c	0.86 ± 0.05 b	Medium
XT-2	0.97 ± 0.01 a	46.00 ± 2.55 a	0.93 ± 0.07 a	Medium
XT-2X	0.65 ± 0.01 b	35.20 ± 1.67 b	0.50 ± 0.04 b	Medium
HA-4	0.82 ± 0.02 a	31.60 ± 2.10 a	1.43 ± 0.08 a	High
HA-4X	0.73 ± 0.02 b	9.60 ± 1.06 b	0.45 ± 0.03 b	Low

Note: Values are means $\pm$ standard error. Within each column, different lowercase letters (a, b, and c) indicate significant differences among isolates according to Fisher's least significant difference (LSD) test at $p < 0.05$; values sharing the same letter are not significantly different. Pathogenicity levels were classified as low (0–0.5 cm), moderate (0.5–1.0 cm), and high (>1.0 cm) based on lesion length.

3.4. Cross-Resistance Between Pyrifeno and Other Fungicides in the FGSC

As shown in Figure 5 and Table 2, pairwise correlation analysis of the sensitivity of the four variants and their three parental isolates (seven isolates in total) to pyrifeno and six other fungicides was conducted. The results showed that, except for a certain degree of cross-resistance between pyrifeno and tebuconazole, no significant cross-resistance was observed between pyrifeno and the remaining five fungicides.

**Figure 5.** Cross-resistance of the FGSC to pyrifeno and six different types of fungicides by $\log EC_{50}$ values.

3.5. Nucleotide Sequence Analysis of CYP51 Genes in Resistant Variants of the FGSC and Their Parental Isolates

Gene sequencing was performed to compare the amino acid sequences of the CYP51A, CYP51B, and CYP51C genes between the resistant variants and their parental isolates. The results revealed no amino acid mutations in any of the resistant variants compared with their respective parental isolates, indicating that the resistance of the variants was not conferred by target-site mutations (Supplementary Figure S1).

4. Discussion

In this study, the sensitivity of 122 FGSC isolates collected from major wheat-producing regions in China to pyrifenoxy was determined using the mycelial growth rate method. After excluding two outlier isolates based on normality testing, the sensitivity of the remaining 120 isolates displayed a continuous unimodal distribution, and the baseline sensitivity was established at 3.300 mg/L. These results define the sensitivity level of field isolates in China and provide a reference for resistance monitoring, resistance classification, and the establishment of early warning thresholds. All tested isolates exhibited a certain degree of sensitivity to pyrifenoxy in vitro. However, the mean EC_{50} value of pyrifenoxy (3.300 mg/L) was approximately 10–20 times higher than those of tebuconazole (0.137–0.583 mg/L) and prothioconazole (0.113–0.856 mg/L) in this study. This indicates that pyrifenoxy has moderate in vitro inhibitory activity against the FGSC compared with these commonly used DMI fungicides. Two naturally tolerant isolates were identified in Jiangsu Province; although their frequency in the field is currently low, they may evolve into resistant inoculum sources under sustained fungicide application, underscoring the necessity of long-term resistance monitoring [38,39].

To assess the resistance risk of the FGSC to pyrifenoxy, three parental isolates representing high, moderate, and low sensitivity levels were selected, and four resistant variants were obtained through a combination of fungicide adaptation and UV mutagenesis. The resistance factors ranged from 5.53 to 450.26, covering high, moderate, and low resistance levels, indicating that the pathogen possesses the evolutionary potential to develop resistance [27,40] and that the resistance level is influenced by the genetic background of the isolate. After ten successive subcultures on fungicide-free medium, the resistance of the variants declined substantially, with highly and moderately resistant isolates reverting to sensitive or low-resistance types, demonstrating that the resistance could not be stably inherited. Fitness measurements showed that the mycelial growth rate, sporulation capacity, and pathogenicity of the tenth-generation variants were significantly lower than those of the parental isolates. However, because these variants had already lost most of their resistance (Table 1), these changes cannot be directly attributed to resistance. They should instead be interpreted as phenotypic alterations in the variant lines, and the possibility that they resulted from the mutagenesis and selection procedure rather than from resistance itself cannot be excluded. Numerous studies have confirmed that resistant variants of plant pathogens often exhibit reduced environmental fitness compared with their parental isolates. For example, resistant variants of *Botrytis cinerea* to fenhexamid exhibited significantly slower mycelial growth rates compared with their wild-type counterparts [41]. Similarly, fludioxonil-resistant isolates of the FGSC showed significantly reduced mycelial growth rates, sporulation capacity, and pathogenicity to wheat compared with fludioxonil-sensitive isolates [16]. In light of these previous findings, the reduced physiological fitness and pathogenicity of the resistant isolates would likely place them at a competitive disadvantage in the field, making it difficult for them to become dominant populations. However, two major limitations of this study should be acknowledged.

A further limitation of this study is that species identity within the FGSC was not resolved. ITS sequencing was used to confirm that the 21 representative isolates belonged to the FGSC, but this marker cannot reliably distinguish the FGSC *sensu stricto* from *F. asiaticum* or other closely related species within the complex, and *Fusarium* species are known to carry non-orthologous ITS2 copies. Only 21 of the 122 isolates were subjected to molecular analysis, and neither TEF1- α sequencing nor species-specific PCR was performed. This is particularly relevant because *F. asiaticum* is known to predominate in Jiangsu and Anhui provinces, from which part of our isolates were collected. Therefore, the isolates used in this study should be regarded as members of the FGSC rather than the FGSC *sensu*

stricto, and the baseline sensitivity and resistance risk reported here should be interpreted as referring to the FGSC as a whole. Future studies should use TEF1- α sequencing or species-specific PCR to determine species composition within the FGSC and to assess whether sensitivity to pyrifenoxy differs among FGSC species.

Second, single-spore purification was not performed. Because the FGSC hyphae are multinucleate, the resistant phenotype observed after UV irradiation of mycelial plugs may reflect physiological adaptation or segregation of heterokaryotic mycelium rather than stable genetic mutation. This is supported by the observation that the EC₅₀ values of all four variants decreased substantially after ten successive transfers on fungicide-free medium. Therefore, the resistant isolates described here should be regarded as resistant variants rather than stable mutants, and the resistance risk inferred from these variants should be interpreted with caution.

Third, a UV-only control (parental isolates exposed to UV irradiation without fungicide selection) was not included. It therefore cannot be fully excluded that part of the phenotypic changes observed in the resistant variants were caused by UV exposure rather than by fungicide adaptation alone. Future studies should include this control to separate the effects of UV mutagenesis from those of fungicide selection.

Although these limitations should be considered, the variants exhibited reduced fitness and unstable resistance, and the overall resistance risk of pyrifenoxy is therefore considered relatively low.

Cross-resistance patterns directly influence the design of fungicide rotation and mixture strategies in the field [42]. The correlation analysis in this study revealed that pyrifenoxy exhibited positive cross-resistance only with tebuconazole, another DMI fungicide, but showed no cross-resistance with the other five commonly used fungicides. Both substances, tebuconazole and pyrifenoxy, inhibit the enzyme rather than acting on the gene. Adaptive modifications developed by the pathogen to tolerate pyrifenoxy, such as upregulation of CYP51 gene expression or enhanced degradation by metabolic enzymes, may also confer similar tolerance to tebuconazole, thereby resulting in a certain degree of cross-resistance [43–45]. Among the remaining fungicides, no cross-resistance was observed with pyraclostrobin, thiophanate-methyl, or carbendazim, which possess different modes of action and target sites. Although a positive correlation was detected between pyrifenoxy and tebuconazole, the magnitude of the change in tebuconazole sensitivity was small. In the resistant variants, the EC₅₀ values of tebuconazole increased by only approximately 1.3- to 1.9-fold relative to their parental isolates, which is considerably lower than the resistance factors observed for pyrifenoxy (5.53–450.26). This suggests that the cross-resistance between pyrifenoxy and tebuconazole is weak and may not be of practical significance in the field. It should also be noted that this correlation was based on a small number of isolates and on tenth-generation variants that had already lost most of their pyrifenoxy resistance, and the biological relevance of this weak cross-resistance therefore requires further validation. In contrast, hexaconazole and prothioconazole are also DMI fungicides, yet no cross-resistance was detected between pyrifenoxy and these two compounds in this study. This suggests that cross-resistance among DMI fungicides is not necessarily universal and may depend on the specific DMI compound and the underlying resistance mechanism. The absence of cross-resistance with hexaconazole and prothioconazole is noteworthy, given that all three compounds belong to FRAC group 3. This may indicate that the resistance phenotype in these variants is not mediated by a common target-site alteration shared by all DMIs, but rather by mechanism(s) that affect only certain DMI compounds. Alternatively, the lack of cross-resistance may reflect the limited number of isolates and the instability of the resistance phenotype. These possibilities warrant further investigation. This result is consistent with findings from many previous studies. For example, among 3714 FGSC isolates

collected from eight provinces in China, 11 exhibited dual resistance to tebuconazole and prothioconazole [46]. Similarly, Fonseka and Gudmestad identified individuals of *Alternaria solani* exhibiting cross-resistance to difenoconazole and metconazole [47]. As a pyridine DMI fungicide, pyrifenox may complement existing triazole-based fungicide strategies for FHB management, optimize rotation strategies, and delay the overall development of fungicide resistance. Pyrifenox was first introduced in Switzerland in 1986 and is no longer approved in several markets, including the European Union. In China, it is not currently registered for FHB control. Evaluating its activity against the FGSC therefore provides fundamental data for resistance risk assessment and for assessing its potential role in fungicide rotation programs.

Resistance to DMI fungicides is commonly associated with mutations in the target genes *CYP51A*, *CYP51B*, and *CYP51C* [48–50]. In this study, sequencing and alignment of these three genes in the four resistant variants revealed nucleotide and amino acid sequences identical to those of the sensitive parental isolates, with no base substitutions, insertions, or deletions detected. This is consistent with previous reports that some DMI-resistant isolates do not harbor *CYP51* mutations [49]. Resistance to DMI fungicides is commonly associated with mutations in the target genes *CYP51A*, *CYP51B*, and *CYP51C* [46–48]. In this study, sequencing and alignment of these three genes in the four resistant variants revealed nucleotide and amino acid sequences identical to those of the sensitive parental isolates, with no base substitutions, insertions, or deletions detected. This is consistent with previous reports that some DMI-resistant isolates do not harbor *CYP51* mutations [47]. Because the sequenced variants were tenth-generation material that had already lost most of their resistance, the absence of *CYP51* mutations at this generation cannot be used to infer the resistance mechanism. No conclusion can therefore be drawn regarding the involvement of target-site mutations in pyrifenox resistance from these data. However, this does not exclude other target-site mechanisms, such as alterations in the promoter regions or changes in *CYP51* expression, which were not examined in this study. Non-target-site mechanisms, including enhanced efflux or metabolic detoxification, may also be involved, but were not investigated here. Therefore, the involvement of non-target-site mechanisms in pyrifenox resistance remains a hypothesis that requires further experimental validation, including promoter analysis, qRT-PCR-based expression analysis of *CYP51* genes, and expression profiling of ABC and MFS transporters. Given the instability of the resistance phenotype after serial transfer, this hypothesis should be interpreted with great caution [51]. These findings provide a direction for further investigation into the molecular mechanisms underlying pyrifenox resistance, including promoter analysis, *CYP51* expression analysis, and characterization of efflux transporters. However, given the limited evidence and the instability of the resistance phenotype, the mechanism proposed here should be regarded as a hypothesis rather than a confirmed conclusion [51].

In addition, several limitations of this study should be acknowledged. First, the mycelial growth assay was performed with two replicates per isolate and was not independently repeated; future studies should include more replicates to strengthen the reliability of the results. Second, the number of irradiated mycelial plugs was limited, and the sporulation assay was not independently repeated; these factors may have influenced the recovery of resistant variants and the reliability of the sporulation data. Third, DON production was expressed per milliliter of culture rather than per unit of mycelial dry weight. Because the variants differed in growth rate, this may have influenced the comparison of DON production among isolates. Although this approach is consistent with previous studies of DON production in liquid culture [33,34], the lack of normalization to mycelial dry weight remains a limitation, and future studies should normalize DON values to mycelial dry weight. Finally, the trichothecene chemotype of the isolates was not determined, and the

ELISA kit cross-reacts with 3-ADON and 15-ADON; therefore, the measured values may represent the sum of DON and its acetylated derivatives rather than DON alone. Future studies should include more replicates, increase the number of irradiated plugs, normalize DON values to mycelial dry weight, and determine the trichothecene chemotype of the isolates. In addition, pathogenicity was assessed only using the wheat coleoptile assay, and spike inoculation was not performed. Because coleoptile inoculation may not fully reflect the ability of the pathogen to infect wheat spikes under field conditions, the pathogenicity results should be interpreted with caution. Future studies should include spike inoculation to confirm the pathogenicity of the variants.

5. Conclusions

To our knowledge, this study is the first to determine and establish the baseline sensitivity of the FGSC to pyrifenoxy in China. Pyrifenoxy exhibited moderate in vitro antifungal activity against the FGSC. The resistant variants obtained in this study showed unstable resistance and reduced fitness; however, because the fitness, cross-resistance, and sequencing assays were performed on tenth-generation variants that had already lost most of their resistance, these results cannot be directly attributed to resistance. The resistance risk of the FGSC to pyrifenoxy should therefore be regarded as preliminary and interpreted with caution. Although pyrifenoxy showed moderate in vitro activity against the FGSC and a relatively low resistance risk in this study, its field efficacy against FHB remains to be evaluated. Therefore, its potential as an alternative fungicide for FHB management should be interpreted with caution.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture16202253/s1>, Figure S1: Amino acid comparison of the *CYP51A*, *CYP51B* and *CYP51C* gene between the resistant mutant and its parental strain; Table S1: Collection information on 122 strains of *Fusarium graminearum*; Table S2: Sensitivity of 122 *Fusarium graminearum* isolates to pyrifenoxy; Text S1: The sequences are primary data from the authors' own isolates and allow independent verification of the main molecular conclusion.

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