

Exploring potential mechanisms of fludioxonil resistance in *Fusarium oxysporum* on Melon

Yan-Fen Wang^{1,2}, Fang-Min Hao³, Huan-Huan Zhou¹, Jiang-Bo Chen¹, Hai-Chuan Su¹, Fang Yang¹, Yuan-Yuan Cai¹, Guan-Long Li¹, Meng Zhang^{2,*} and Feng Zhou^{1,*}

- ¹ Henan Engineering Research Center of Biological Fertilizer Development and Collaborative Application, Henan Institute of Science and Technology, Xinxiang 453003, China; wyfhist@163.com; ZHH6920@163.com; Chen1124@163.com; Suhc2021@163.com; 1879190004@qq.com; 2824881878@qq.com; 3066382948@qq.com
- ² College of Plant Protection, Henan Agricultural University, Zhengzhou 450002, China
- ³ Institute of Vegetables and the Key Lab of Cucurbitaceous Vegetables Breeding in Ningbo City, Ningbo Academy of Agricultural Sciences, Ningbo 315040, China; haofangmin@163.com
- * Correspondence: F. Zhou, E-mail: zfhist@163.com; M. Zhang, E-mail: zm2006@126.com

Abstract: Melon *Fusarium* Wilt (MFW), which is caused by *Fusarium oxysporum* f. sp. *melonis*, is a soil-borne disease that commonly impacts melon cultivation worldwide. In the absence of any disease-resistant melon cultivars, the control of MFW relies heavily on the application of chemical fungicides. Fludioxonil, a phenylpyrrole fungicide, has been shown to have broad-spectrum activity against many crop pathogens. Preliminary experiments have showed that low concentrations of fludioxonil exhibit a strong inhibitory effect on the mycelial growth of *F. oxysporum* isolates. Five pydiflumetofen-resistant *F. oxysporum* mutants were successfully generated by repeated exposure to fludioxonil under the laboratory conditions. Although the mutants exhibited significantly reduced mycelial growth in the presence of the fungicide, and further investigation revealed that the sporulation of the fludioxonil-resistant mutants was affected and exhibiting significantly ($P < 0.05$) reduced growth rates in response to KCl, NaCl, glucose, and mannitol. Meanwhile, molecular analysis of the mutants strongly suggested that the observed fludioxonil resistance was related to changes to the sequence and expression of the *FoOs1* target gene. In addition, the current study found that no evidence cross-resistance between fludioxonil and any of the other fungicides tested, these results indicate that fludioxonil has great potential as an alternative method of control for *F. oxysporum* in melon crops.

Keywords: *Fusarium oxysporum*; fludioxonil; fungicide resistance; resistance mechanism; cross-resistance

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *J. Fungi* **2022**, *8*, x. <https://doi.org/10.3390/xxxxx>

Academic Editor: Firstname Lastname

Received: date

Accepted: date

Published: date

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Melon (*Cucumis melo* L.), of which common varieties include musk melon, cantaloupe, and honeydew, is a popular fruit crop in the Cucurbitaceae family that is cultivated throughout the tropics [1,2]. In addition to being a favorite for consumption due to their flavor, melons have also been noted for their nutritional and medicinal properties, some of which have been investigated as possible treatments for kidney stones, cancer, cardiovascular disorders, and stroke [3,4]. Although the data is incomplete, it has been estimated that over 30 million tons of melon are produced globally each year [5], while a recent report by the Food and Agriculture Organization of the United Nations (FAO) suggested that the total area under melon cultivation in China comprises as much as 427.8 thousand hectares, with a total output of 144 thousand tons (<http://faostat3.fao.org/download/Q/QC/E>). However, diseases have always posed a threat to successful melon production, especially the Melon *Fusarium* Wilt (MFW) caused

by *Fusarium oxysporum* f. sp. *melonis* [6]. Continuous production of melons in monoculture in recent years has led to an increase in the incidence and severity of soilborne fungal diseases [7]. The spread of MFW is of particular concern as it can affect the entire growth cycle of the melon plant, but especially from anthesis through to fruiting, when it can cause yield losses of 50–70% [8]. Although the development of disease-resistant cultivars would be the best option for the control of MFW [9], at present there are no disease-resistant field cultivars available, and consequently the management of melon diseases relies primarily on cultural practices and the application of chemical fungicides such as cupric-ammonium complexation was used by root irrigation [9,10]. However, the inappropriate and chronic overuse of chemical fungicides has caused a series of negative problems including pesticide residues and environmental pollution, as well as emergence of fungicide resistance pathogens [11,12].

Fludioxonil, a phenylpyrrole fungicide, has been shown to have broad-spectrum activity against a wide range of fungal pathogens and has been used to control many diseases in the field [13–18]. Previous mode of action studies have suggested that fludioxonil inhibits fungal growth by overstimulating the high osmolarity glycerol (HOG) stress response signal transduction pathway, which causes the hyphae to swell and burst [19]. The HOG stress response pathway, a branched mitogen activated protein kinase (MAPK) signal transduction system has been well characterized in *Saccharomyces cerevisiae* and is known to mediate osmoregulation in response to environmental factors [20,21]. The HOG1 protein itself is a key enzyme in the HOG1-MAPK signaling cascade [22], and has also been shown to play a role in the initial response to osmotic stress, prior to the activation of the HOG1-MAPK signaling pathway [23]. Although the molecular details are yet to be characterized fully, it is currently thought that the fludioxonil causes hyperactivation of the HOG1-MAPK signaling transduction pathway by targeting group III hybrid histidine kinases (HK) such as *FoOs1* [13–15]. In addition to osmoregulation, the HOG1-MAPK pathway is involved in many fungal processes including responding to heavy metal and oxidative stress, which perhaps explains why fludioxonil exhibits such a broad spectrum of activity against such a wide range of fungal pathogens [13,15,24]. However, the inappropriate and excessive use of fludioxonil has already resulted in the emergence of fludioxonil resistance in many plant pathogens including *Neurospora crassa*, *Alternaria brassicicola*, *Penicillium expansum*, *Sclerotinia sclerotiorum*, *Fusarium* spp, *Aspergillus carbonarius*, and *Botrytis cinerea* [17,25–32]. Although, most previous studies have shown that the primary resistance mechanism appears to be associated with a set of well-characterized amino acid changes in the histidine kinase *FoOs1* [25,26,32], there have also been several reports of fludioxonil-resistant mutants that have no such mutations [33], indicating that the resistance mechanism of fludioxonil is more complicated than initially thought, and could involve multiple biological processes.

To date, the use of fludioxonil for the control of MFW has yet to be approved in China (<http://www.icama.org.cn/hysj/index.jhtml>). However, preliminary experiments conducted in the current study suggested that fludioxonil had a strong inhibitory effect on the mycelial growth of *F. oxysporum* isolates collected from melon-growing regions located in the Zhejiang province of China, even at low concentrations (Figure S1). Further research is therefore required to investigate the biological characteristics and molecular mechanism of fludioxonil-resistance, especially the risk of highly resistant isolates emerging, to determine whether fludioxonil should be registered for the control of MFW in China. The objectives of the current study were to: (1) compare the fitness parameters and physiological characteristics of sensitive and fludioxonil-resistant isolates of *F. oxysporum*; (2) explore potential mechanisms of fludioxonil resistance associated with the *FoOs1* gene; and (3) determine whether there are any patterns of cross-resistance between fludioxonil and other commonly used fungicides for the control of soil-borne crop diseases, including tebuconazole, prochloraz, fluazinam, carbendazim, pyraclostrobin, kresoxim-methyl, and difenoconazole.

2. Materials and Methods

2.1. Fungicides and fungal isolates

Fungicides, commonly used to control of soil-borne crop disease caused by *Fusarium* sp., as candidates for cross-resistance research in this study (Table S1). Technical grade sources of eight fungicides including 96.0% active ingredient (a. i.) fludioxonil (Hubei Jianyuan Chemical Co. Ltd.), 96.2% a. i. tebuconazole (Sheyang Huanghai Pesticide Chemical Co. Ltd.), 97.0% a. i. prochloraz (Hubei Kangbaotai Fine Chemical Co., Ltd), 96.0% a. i. fluazinam (Hubei Jianyuan Chemical Co. Ltd.), 97.7% a. i. pyraclostrobin (Hubei Kangbaotai Fine Chemical Co., Ltd), 97.5% a. i. kresoxim-methyl (Jiangsu Gengyun Chemical Co. Ltd.), 95.0% a. i. difenoconazole (Hubei Jianyuan Chemical Co. Ltd.), and 98.1% a. i. carbendazim (Haili Guixi Chemical Co. Ltd.) were used to prepare 10,000 µg/mL stock solutions, which were dissolved either in acetone for the hydrophobic fungicides, or 0.1 M hydrochloric acid (HCl) in the case of carbendazim. The stock solutions were stored at 4°C, and mycelial growth assays performed to confirm that the solvents had no effect on the growth of *F. oxysporum* at the range of concentrations tested (data not shown).

The *F. oxysporum* isolates used in the current study were initially collected from root and leaf samples of *Cucumis melo* L. plants exhibiting typical symptoms of MFW growing in the melon fields of Taizhou and Ningbo in the Zhejiang province of China during the 2019 growing season. The *Fusarium* strains were isolated and purified by conventional culture methods, and 11 isolates selected on the basis of the sequences of their Internally Transcribed Spacer (ITS) and Translation Elongation Factor 1α (TEF-1α), as well as pathogenicity experiments to confirm the severity of wilt symptoms in the melon host [8]. The fungal cultures were briefly preserved on potato dextrose agar (PDA) at 4°C, and mycelial samples prepared using the protocol of a previous study [18].

2.2. Cross-resistance between fludioxonil and other commonly used fungicides

The mycelial growth assay described above was similarly used to investigate potential cross-resistance between fludioxonil and a range of other commonly used fungicides for the control of soil-borne crop diseases, including tebuconazole, prochloraz, fluazinam, carbendazim, pyraclostrobin, kresoxim-methyl, difenoconazole. The concentrations of a. i. varied, with 0, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 µg/mL being used for prochloraz, and fludioxonil when assessing the fludioxonil-sensitive isolates; 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 µg/mL for tebuconazole, fluazinam, carbendazim, pyraclostrobin, kresoxim-methyl, and difenoconazole; and 0, 1.0, 3.0, 9.0, 27, 81.0, 243.0, 729.0, and 1000.0 µg/mL for fludioxonil when assessing the fludioxonil-resistant mutants, after incubation at 24°C with a 12 h photoperiod for 4d, the diameter of each colony was measured twice at right angles. Each treatment/isolate combination was represented by three separate plates, and the entire experiment performed triplicate. EC50 values were calculated according to Zhou et al. (2014).

2.3. Generation of fludioxonil-resistant mutants of *F. oxysporum* by repeated exposure to fludioxonil, and heritable stability of fludioxonil-resistance

All the wild-type *F. oxysporum* isolates assessed in the current study were found to be highly sensitive to fludioxonil, five of which were chosen for further study including TY-1, TY-3, TY-5, TY-8, and TG-5, which had EC50 values of 0.03, 0.02, 0.03, 0.03, and 0.04 µg/mL, respectively. Repeated exposure to fludioxonil in accordance with the protocol of a previous study [18], resulted in the selection of five highly resistant laboratory mutants including TY-1-fR, TY-3-fR, TY-5-fR, TY-8-fR, and TG-5-fR, which had EC50 values of 225.84, 204.07, 230.42, 264.22, and 232.96 µg/mL, respectively. The hereditary stability of the above five resistant mutants was assessed using a modified version of previously published protocols [16,18]. Briefly, each mutant was subjected to

10 successive rounds of subculture on fludioxonil-free PDA before their EC₅₀ values were re-evaluated on PDA containing fludioxonil at the following concentrations: 0, 1.0, 3.0, 9.0, 27, 81.0, 243.0, 729.0, and 1000.0 µg/mL, after incubation at 24°C with a 12 h photoperiod for 4d, the diameter of each colony was measured twice at right angles. The stability of the resistance was then estimated from the change in EC₅₀ values calculated for the 1st, 4th, 6th, 8th, and 10th subculture. Each mutant was represented by three separate plates, and the entire experiment performed triplicate.

2.4. Mycelial growth and sporulation of fludioxonil-resistant mutants

The mycelial growth of the five laboratory mutants (TY-1-fR, TY-3-fR, TY-5-fR, TY-8-fR, and TG-5-fR) was compared to that of their parental isolates (TY-1, TY-3, TY-5, TY-8, and TG-5) using the protocols of previous studies [18]. Briefly, mycelial plugs (5 mm) were taken from the edge of 3-day-old colonies and transferred to fresh PDA. The plates were incubated at 24°C with a 12 h photoperiod and the resulting colonies observed daily and the diameter of each measured at 24, 48, 72, 96, and 120-h post inoculation (hpi). Each isolate was represented by five separate plates, and the entire experiment performed triplicate. Meanwhile, the macroconidia of the five mutants and the five parental isolates were similarly assessed using the method detailed in a previous study [18], with a few modifications. Briefly, mycelial plugs (5 mm) were taken from the edge of 3-day-old colonies and transferred to fresh PDB medium, after incubation at 24°C with shaking (120 rpm) for 4 days, the resulting macroconidia were harvested and counted using a hemocytometer (Shanghai Qiujiing Biochemical Reagent Instrument Co., Ltd.) after its filtration with a double sterile gauze. Each isolate was represented by five separate flasks, and the entire experiment performed twice. Fisher's least significant difference test in the SPSS software (ver. 17.0; SPSS Inc.) was calculated to determine statistical significance among different mutants (or isolates).

2.5. Sensitivity of fludioxonil-resistant mutants to osmotic stress

The response of the fludioxonil-resistant mutants to osmotic stress was assessed using the methods described in previous studies [18,34], with a few modifications. Briefly, mycelial plugs (5 mm) from 3-day-old PDA cultures of five laboratory mutants (TY-1-fR, TY-3-fR, TY-5-fR, TY-8-fR, and TG-5-fR) and their parental isolates (TY-1, TY-3, TY-5, TY-8, and TG-5) were transferred onto fresh PDA amended with either KCl, NaCl, glucose, or Mannitol at final concentrations of 0.5 or 1 M to induce osmotic stress. The sensitive parental isolates were also assessed for comparison, and identical amendment-free cultures used as a negative control. The diameters of the resulting colonies were measured after 72 h incubation at 24°C with a 12 h photoperiod, and the percentage inhibition of mycelial growth calculated according to the formula detailed in previous studies [18,34]. Each treatment was represented by six replicate plates, and the entire experiment performed triplicate. Fisher's least significant difference test in the SPSS software (ver. 17.0; SPSS Inc.) was calculated to determine statistical significance among different mutants (or isolates).

2.6. Cloning and sequencing analysis of the FoOs1 gene

The full-length open reading frame (ORF) of the FoOs1 gene (FRV6_07880) from *F. oxysporum* was found to be highly homologous to the FgOs1 gene (FGSG_07118) which is the potential action target gene of fludioxonil in *F. graminearum* (data not shown), maybe also related to the fludioxonil resistance [18]. The liquid cultures used for genomic DNA extraction were prepared in accordance with the protocols of previous studies [18,34]. Each isolate was inoculated in PDB medium (Beijing Aoboxing Bio-tech Co. Ltd.) and incubated at 24°C with shaking (130 rpm) for 72 h before the mycelium was harvested using sterile filter paper, washed in sterile water, and flash frozen in liquid nitrogen. The total genomic DNA was then extracted using the Omega bio-tek Fungal DNA Kit

(Omega bio-tek Inc., Guangzhou, China) in accordance with the protocol of the manufacturer. The resulting DNA was then used as a template to amplify the full-length sequence of the *FoOs1* gene using the FoOs1-F1/FoOs1-R1 primer set (Table 1), which was designed using primer premier software (ver.6.0., PREMIER Biosoft, Canada). The polymerase chain reactions (PCR) themselves, as well as the cloning and sequence analysis were conducted using the protocol of a previous study [18], with the sequences from the fludioxonil resistant mutants being compared to those of the sensitive parental isolates using the software DNAMAN (Lynnon Biosoft Inc. CA, USA).

Table 1. Primers were used for amplification of *FoOs1* and analysis its gene expression.

Primers	Sequences (5'-3')	Purpose	Sources
FoOs1-F1	ATGGTTGACGACGCGGCCCTCGCCGCT	Full-length gene amplification	Current study
FoOs1-R1	TTAGTTGGTAAGACTTCGCATATCAGAG		
RT-FoOs1-F1	GGCGTCAAATCTCACAGTCC	Gene expression analysis	Karlsson et al., 2021
RT-FoOs1-R1	AACTCGCTGCACTTCGTAAC		
RT-EF1 α -F	CATCGGCCACGTCGACTCT		Current study
RT-EF1 α -R	AGAACCCAGGCGTACTTGAA		

2.7. Determination of the relative expression levels of the *FoOs1* gene by quantitative real-time PCR

The relative expression levels of the *FoOs1* gene of the five laboratory mutants relative to their respective sensitive parental isolates were assayed by quantitative real-time PCR (qPCR). Total RNA was extracted from mycelial samples prepared as described in the DNA extraction protocol using a fungal RNA kit (Omega bio-tek, Switzerland) in accordance with the instructions of the manufacturer. First-strand cDNA was initially prepared using the PrimeScript RT reagent kit (TaKaRa, Japan), and used as a template for the qPCR analysis, which amplified a 200 bp fragment of the *FoOs1* gene using the RT-FoOs1-F1/ RT-FoOs1-R1 primer set. The real-time PCR amplification was performed using the applied biosystems QuantStudio 6 Flex PCR detection systems (ThermoFisher, USA) in conjunction with SYBR Green I fluorescent dye (TaKaRa, Japan) with the relative expression of the *FoOs1* gene being standardized to the *F. oxysporum* TEF-1 α reference gene, which was amplified using the RT- EF1 α -F/ RT- EF1 α -R primer set (Table 1) in accordance with the method described in a previous study [35]. Procedures for gene expression analysis of these study with the following program: an initial denaturation at 95°C for 10 s; following program by 40 cycles of 95°C for 5 s, 60°C for 20s; and dissociation protocol as 95°C for 10 s, 60°C for 40s and 95°C for 15 s. Three biological replicates were assessed for each isolate and mutant, and the entire experimented conducted three times, with the resulting values being used to calculate the mean expression and standard error (SE). The relative expression of each gene was calculated with the $2^{-\Delta\Delta CT}$ method [36], and the data collected in this study were calculated by analysis of variance using SPSS software (ver. 17.0; SPSS Inc.) by the methods of Fisher's least significant difference test ($P = 0.05$).

3. Results

3.1. Hereditary stability, mycelial growth, sporulation and osmotic sensitivity of fludioxonil-resistant *F. oxysporum* mutants

Repeated exposure to fludioxonil resulted in the generation of five highly resistant mutants, TY-1-fR, TY-3-fR, TY-5-fR, TY-8-fR, and TG-5-fR, which had EC₅₀ values of 225.84, 204.07, 230.42, 264.22, and 232.96 µg/mL, respectively. After ten successive rounds of subculture, it was found that the EC₅₀ values of the five mutants had not changed significantly (Figure S2), indicating that the mutations were genetically stable. Initial exploration of the biological characteristics of the resistant mutants revealed that their rate of mycelial growth was unaffected and did not differ significantly compared to the sensitive parental isolates, whether at 12, 24, 48, 72, 96, or 120 hpi (Figure 1; Figure S1). However, further investigation indicated that several of the mutants had reduced fitness with regard to sporulation, with four of the resistant mutants (TY-1-fR, TY-3-fR, TY-5-fR, and TG-5-fR) exhibiting significantly reduced levels of sporulation ($P < 0.05$) compared to their parental isolates (Figure 2), while the fifth, TY-8-fR, actually had significantly increased levels of the macroconidia compared to its parental isolate, TY-8. Taken together, these results not only demonstrate that there may be some fitness cost associated with fludioxonil resistance, but that the biological characteristics associated with fludioxonil resistance can vary, possibly suggesting different mechanisms of resistance.

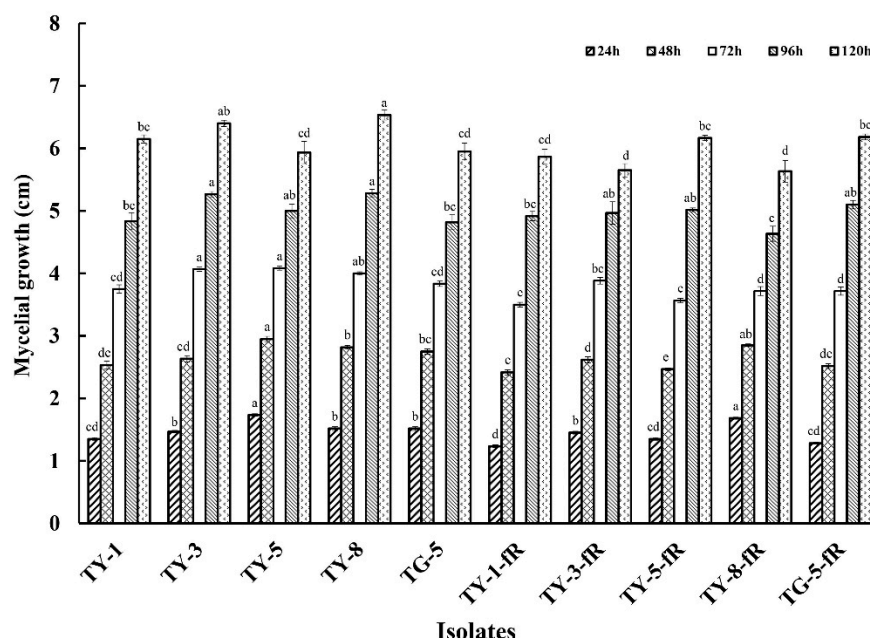


Figure 1. Mycelial growth of five fludioxonil-resistant mutants of *F. oxysporum* in comparison to their sensitive parental isolates when grown on PDA medium at 24°C. Different letters above columns indicate significant differences according to Fisher's least significant difference test ($P \leq 0.05$).

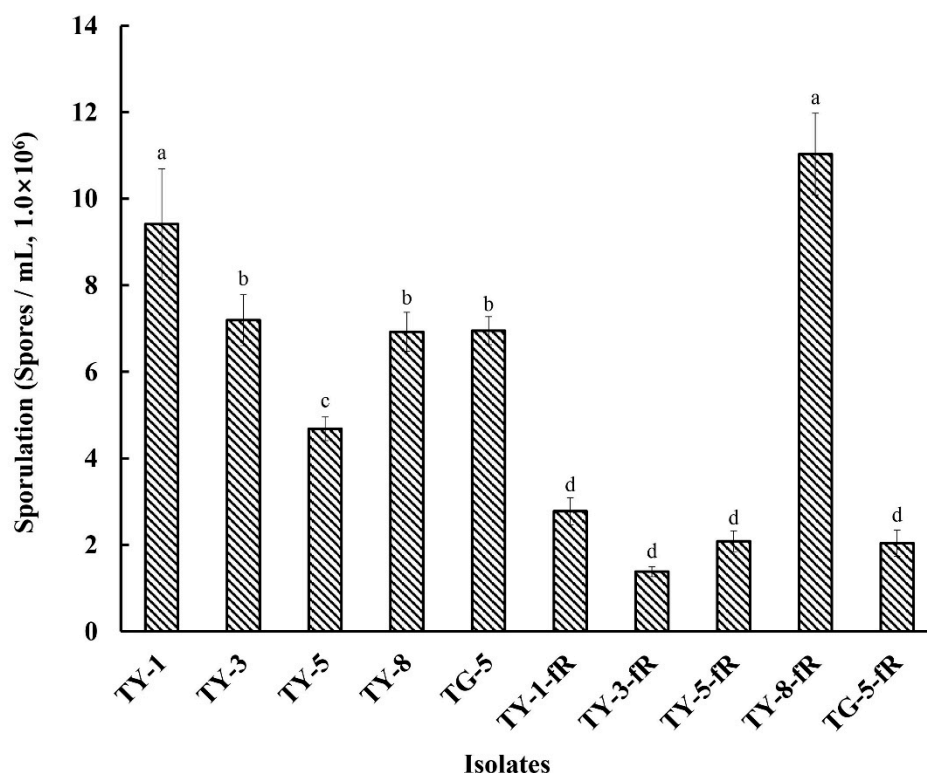


Figure 2. Sporulation of five fludioxonil-resistant mutants of *F. oxysporum* in comparison to their sensitive parental isolates. Different letters above columns indicate significant differences according to Fisher's least significant difference test ($P \leq 0.05$).

Meanwhile, the fludioxonil-resistant mutants were also found to be more susceptible to osmotic stress, exhibiting significantly ($P < 0.05$) reduced growth rates on PDA amended with KCl (Figure 3A), NaCl (Figure 3B), glucose (Figure 3C), and Mannitol (Figure 3D), whether at the higher concentration of 1 M or the lower concentration of 0.5 M. Taken together, these results provide further confirmation of a fitness cost associated with fludioxonil molecular resistance, particularly with regard to their response to osmotic stress, which is consistent with the established fludioxonil mode of action as well as known resistance mechanisms.

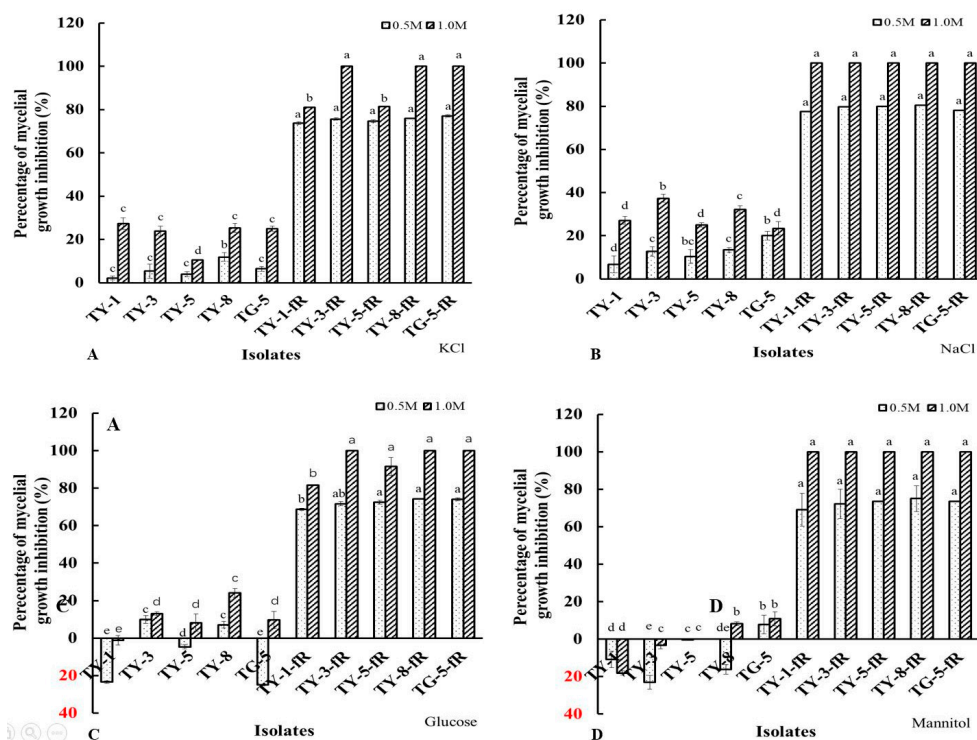


Figure 3. Mycelial growth inhibition of five fludioxonil-resistant mutants of *F. oxysporum* and their sensitive parental isolates in response to osmotic stress induced by (A) KCl, (B) NaCl, (C) Glucose, (D) Mannitol at final concentrations of either 0.5 or 1 M. Error bars indicate one standard error of the mean (SE) as calculated from the mean of two separate experiments, while different letters above columns indicate significant differences according to Fisher's least significant difference test ($P \leq 0.05$).

3.2. Sequence analysis of the FoOs1 gene from fludioxonil-resistant mutants of *F. oxysporum*

Previous studies have shown that several specific amino acid changes in the FoOs1 sequence of *F. graminearum* and other fungal species are linked to fludioxonil resistance [18], so it was not surprising to find that the predicted FoOs1 sequences of the fludioxonil-resistant mutants of *F. oxysporum* assessed in the current study were found to contain similar changes, none of which were present in the parental wild-type isolates (Table 2). For example, the fludioxonil-resistant mutants TY-1-fR, TY-3-fR, TY-5-fR, TG-5-fR contained two mutations, and although one mutation, E702Q, was common in all four, the second differed with TY-3-fR and TY-5-fR both having a mutation that resulted in the A896T substitution, while TY-1-fR and TG-5-fR had changes at S564P and N537T, respectively. The fifth mutant, TY-8-fR, was found to have three mutations, again the common mutation E702Q, as well as the N537T mutation found in TG-5-fR, and the R66A substitution that was not found in any of the other mutants. The five mutants were also found to contain a range of other nucleotide point mutations that did not affect the amino acid sequence of the predicted FoOs1 protein, including two in the intron region, G835A and C841T, and several synonymous mutations in the coding region including A1294G, G1916C, T2015C, C2108T, A2471G, T3533C, G3745A, and G4063A.

Table 2. Mutations in the predicted FoOs1 protein sequence of five fludioxonil-resistant mutants of *F. oxysporum*.

Strains	Mutation type and location	
	Nucleotide	Amino acid

TY-1-fR	A1294G ^A , T1863C ^B , G1916C, T2015C, C2108T, S564P and E702Q A2330G, A2471G, T3533C, G3745A, and G4063A
TY-3-fR	A1294G, G1916C, C2108T, A2330G, A2471G, E702Q and A896T G2910A, T3533C, G3745A, and G4063A
TY-5-fR	A1294G, G1916C, C2108T, A2330G, A2471G, E702Q and A896T G2910A, T3533C, G3745A, and G4063A
TY-8-fR	T66C, A1722C, G1916C, C2108T, A2330G, R66A, N537T, and A2471G, T3533C, G3745A, and G4063A E702Q
TG-5-fR	A1722C, G1916C, A2330G, T3533C, G3745A, and N537T and E702Q G4063A

^A Several of the nucleotide point mutations were found to have no effect on the amino acid sequence of the predicted protein, including G835A and C841T, which were located in the intron region, and A1294G, G1916C, T2015C, C2108T, A2471G, T3533C, G3745A, and G4063A, which were synonymous mutations.

^B The remaining point mutation did cause amino acid changes, which are detailed as follows: T1863C (S564P), A2330G (E702Q), G2910A (A896T), T66C (R66A), and A1722C (N537T).

3.3. Relative expression of the *FoOs1* gene in fludioxonil-resistant mutants of *F. oxysporum*

The qPCR analysis conducted in the current study found that the *FoOs1* gene was differentially expressed in all of the fludioxonil-resistant mutants (Figure 4). For example, in the absence of fludioxonil the expression of *FoOs1* was significantly down-regulated ($P < 0.05$) in four of the resistant mutants (TY-1fR, TY-3fR, TY-5fR, and TY-8fR) compared to the sensitive parental isolates (TY-1, TY-3, TY-5, and TY-8), while the expression of *FoOs1* was significantly up-regulated ($P < 0.05$) in the fifth mutant (TG-5fR). However, in the presence of fludioxonil (0.1 µg/mL), *FoOs1* expression was found to be significantly lower ($P < 0.05$) than that of the sensitive isolates in all of the mutants assessed, even though expression was slightly elevated compared to the control treatment in TY-1fR, TY-3fR, and TY-8fR, and slightly depressed in TY-5fR and TG-5fR (Fig. 4). In contrast, the addition of fludioxonil had a dramatic effect on the wild-type isolates significantly increasing *FoOs1* expression ($P < 0.05$) by more than 100% in some cases. Taken together these results not only suggest that the *FoOs1* gene plays an important role in the response of wild-type *F. oxysporum* isolates to fludioxonil, but also that the depressed levels of *FoOs1* expression observed in the mutants could contribute to the fludioxonil resistance mechanism resulting in reduced sensitivity to the fungicide treatment.

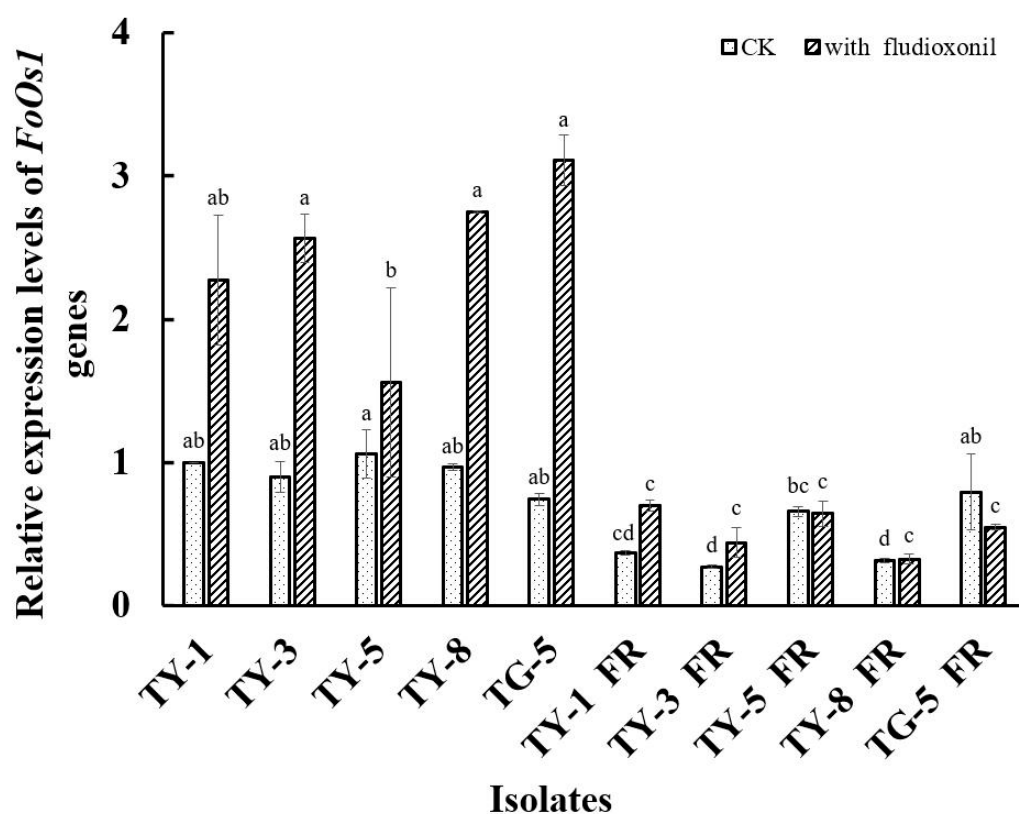


Figure 4. Relative expression of the *FoOs1* gene in five fludioxonil-resistant mutants of *F. oxysporum* and their sensitive parental isolates in the absence and presence of fludioxonil. Error bars indicate one standard error of the mean (SE) as calculated from the mean of two separate experiments, while different letters above columns indicate significant differences according to Fisher's least significant difference test ($P \leq 0.05$). CK indicates the expression level in the absence of fludioxonil.

3.4. Cross-resistance with other fungicides in fludioxonil-resistant mutants of *F. oxysporum*

The current study found no evidence of cross-resistance between fludioxonil and any of the other fungicides tested, including tebuconazole, prochloraz, fluazinam, carbendazim, pyraclostrobin, kresoxim-methyl, and difenoconazole (Table 3). This result indicates that fludioxonil could provide an alternative method of MFW control in areas for which resistance has already emerged to other fungicides, and that alternate or combined application of these fungicides could be used to reduce the risk of resistance developing to fludioxonil itself.

Table 3. Cross-resistance between fludioxonil and other commonly used fungicides.

Fungicides	Fludioxonil-sensitive isolates					Fludioxonil-resistant isolates				
	(EC50, µg/mL)					(EC50, µg/mL)				
	TY-1	TY-3	TY-5	TY-8	TG-5	TY-1-f R	TY-3-f R	TY-5-f R	TY-8-f R	TG-5-f R
Fludioxonil	0.03 ^c	0.02	0.03	0.03	0.04	225.84	204.07	230.42	264.22	232.96
Tebuconazole	0.33	0.23	0.22	0.38	0.31	0.39	0.32	0.34	0.43	0.40
Prochloraz	0.003	0.003	0.003	0.002	0.001	0.007	0.002	0.006	0.003	0.003

Fluzainam	0.051	0.033	0.04	0.041	0.02	0.049	0.035	0.053	0.016	0.058
Carbendazim	1.094	0.793	0.748	0.706	0.633	0.536	0.467	0.403	0.484	0.506
Pyraclostrobin	0.114	0.097	0.104	0.130	0.106	0.687	0.646	0.206	0.518	0.369
Kresoxim-methyl	0.392	0.581	0.528	0.520	0.119	0.569	0.295	0.599	0.238	0.764
Difenoconazole	0.303	0.212	0.256	0.207	0.167	0.222	0.119	0.232	0.119	0.800

^c Values indicate the EC50 (50% effective concentration) for each isolate/fungicide combination.

4. Discussion

Melon (*Cucumis melo* L.) is an important vine crop belonging to the Cucurbitaceae family, and is one of the most widely cultivated fruits globally [1,2]. In recent years, the MFW caused by *F. oxysporum* f. sp. *melonis* has become an increasing threat to melon production [6,9]. In the absence of MFW-resistant melon varieties, damage from MFW can have a significant impact on the yield and quality of melon fruits [5,9,10]. Furthermore, although chemical fungicides can provide effective control of *Fusarium* pathogens, relatively few fungicides have high efficacy against MFW. Fludioxonil has been found to have broad-spectrum activity against both basidiomycete and ascomycete pathogens [13,37], and although fludioxonil has not yet been registered for the control MFW in China (<http://www.icama.org.cn/hysj/index.jhtml>), our preliminary investigation found that fludioxonil exhibited high efficacy against *F. oxysporum* (Figure S1), exhibiting both preventative and curative activity. The current study assessed 11 *F. oxysporum* isolates collected from plants exhibiting typical symptoms of MFW growing in the melon fields of Taizhou and Ningbo in the Zhejiang province of China, and found an average EC50 value of 0.03 µg/mL.

Previous studies regarding the biological characteristics of fludioxonil-resistant mutants of plant pathogenic fungi have consistently found fitness cost associated with fludioxonil resistance [17,18,28]. For example, nearly all the reports investigating laboratory mutants of *S. sclerotiorum*, *B. cinerea*, and *F. graminearum* have documented reduced levels of growth, sporulation and pathogenicity as well as increased sensitivity to osmotic stress [17,18,28]. The current study found that although the fludioxonil-resistant mutants of *F. oxysporum* did not appear to exhibit impaired growth, their sporulation was significantly affected ($P < 0.05$) with four of the mutants (TY-1-fR, TY-3-fR, TY-5-fR, and TG-5-fR) having reduced levels of sporulation, while the fifth (TY-8-fR) had elevated levels compared to their respective parental isolates. Reproduction via spores is a common reproductive method in fungal pathogens, and sporulation is an essential phase in the life cycle of many plant pathogenic fungi [38]. In general, a reduction in sporulation is considered a factor that can limit the potential of a pathogen to reproduce and spread to new hosts, and therefore this fitness cost is associated with lower levels of risk according to the Fungicide Resistance Action committee (<https://www.frac.info/docs/default-source/publications/monographs/monograph-2.pdf>), whilst the increased levels of sporulation observed in TY-8-fR would suggest increased risk.

Although there are currently few reports of fludioxonil-resistant mutants emerging in crop production in the field [16–18,37], there have been numerous studies of laboratory mutants that can easily be induced through continual exposure to fludioxonil under laboratory conditions in species as diverse as *Cochliobolus heterostrophus*, *B. cinerea*, *Ustilago maydis*, *S. sclerotiorum*, *Sclerotinia homoeocarpa*, and *F. graminearum* [16–18,28,32,39,40]. Many of these reports have provided clues as to the resistance mechanism, as changes in the expression and amino acid sequences of candidate target genes such as *FoOS1*, which is an important component of the HOG1-MAPK signaling

pathway, are associated with fludioxonil resistance [26,32,41]. It is therefore of great interest that the current study also found similar changes associated with the *FoOs1* gene of the fludioxonil-resistant mutants of *F. oxysporum melonis*. For example, four of the mutants (TY-1-fR, TY-3-fR, TY-5-fR, and TG-5-fR) exhibited two amino acid changes including S564P and E702Q, E702Q and A896T, E702Q and A896T, N537T and E702Q, while the fifth (TY-8-fR) had three: R66A, N537T, and E702Q (Table 2). Furthermore, it is particularly noteworthy that the E702Q mutation was common to all of the resistant mutants, perhaps indicating a critical role of this substitution in the fludioxonil resistance of *F. oxysporum*. Further bioinformatics analysis revealed that the E702Q mutation was located between the HAMP domain (646-698) and the HisKA domain (714-777), which are located in the flexible region that is closely linked to the potential binding site of fludioxonil within the FoOs1 protein. Despite this, it is interesting to note that this particular amino acid mutation has not been documented in fludioxonil-resistant mutants of other plant pathogenic fungi. Indeed, none of the point mutations identified in the current study (Table S2) have been documented in previous studies of other *Fusarium* species that have investigated the MAP kinase proteins of *Fusarium asiaticum* and *F. graminearum* [18,34]. Further investigation, including verification through reverse genetics, is therefore required to determine the importance of the E702Q mutation in fludioxonil resistance in *F. oxysporum*, as well as its potential role in other plant pathogenic fungi.

Similarly, the qPCR analysis conducted in the current study found that the altered expression of the *FoOs1* gene might be linked to the observed fludioxonil resistance of the *F. oxysporum* mutants. In this case all of the mutants exhibited altered *FoOs1* expression in the absence of fludioxonil, but perhaps of more interest was their relatively low *FoOs1* expression in the presence of fludioxonil compared to the dramatically increased expression observed in the wild-type isolates when exposed to fludioxonil. These results are consistent not only with the increased sensitivity of the fludioxonil-resistant mutants to osmotic stress, but also with the established fludioxonil mode of action that is thought to target the HOG1-MAPK signaling pathway, which is closely associated with osmoregulation in fungi. To date it has generally been assumed that fludioxonil resistance is linked to amino acid changes in various MAPK proteins (Table S1); however, the expression data from the current study and the discovery of at least one resistant mutant of *F. asiaticum* that lacks any mutations in its *FgOs1* gene [33,34] indicate that the fludioxonil resistance mechanism could be more complex than previously thought, and might result from multiple resistance mechanisms. Further investigation, including transcriptome, metabolome, and proteome analysis are therefore required to identify key genes and fully characterize the mechanisms by which fludioxonil resistance can emerge in plant pathogen fungi. Such data would not only be extremely useful in managing the risk of fludioxonil resistance, but could also assist in the development of novel phenylpyrrole fungicides designed to target the HOG1-MAPK signaling pathway more effectively.

In conclusion, the results of the current study provide strong evidence that the use of fludioxonil could be an effective fungicide for control of MFW, especially in areas where resistance to other fungicides has already emerged, while the fitness cost associated with fludioxonil resistance observed in the *F. oxysporum* mutants, with regard to both sporulation and increased osmotic sensitivity, could indicate a low to moderate risk of fungicide resistance emerging in the field. However, the lack of any cross-resistance between fludioxonil and other fungicides such as tebuconazole, prochloraz, fluazinam, carbendazim, pyraclostrobin, kresoxim-methyl, and difenoconazole (Table 3) could mitigate such risk if fludioxonil were applied alternately or in combination with these other compounds, and under such circumstances it is likely that the appropriate use of fludioxonil within an integrated pest management (IPM) program could provide effective control of MFW and safeguard the future of melon production in China and worldwide.

Supplementary Materials: The following are available online at: www.mdpi.com/xxx/s1, Figure S1: Mycelial growth of five fludioxonil-resistant mutants of *F. oxysporum* and their sensitive parental isolates; Figure S2: Heritable stability of five fludioxonil-resistance mutants of *F. oxysporum* assessed according to the variation in their fludioxonil EC50 values; Table S1: Fungicides used in the current study; Table S2: Mutations in the MAP kinase protein sequence of fludioxonil-resistant strains of *F. graminearum*, *F. asiaticum*, and *F. oxysporum* were reported in China.

Author Contributions: Conceptualization, F.Z. and M.Z.; methodology, F.-M.H. and Y.-F.W.; software, H.-H.Z. and J.-B.C.; formal analysis, H.-C.S. and Y.-F.W.; investigation, F.Y. and Y.-Y.C.; resources, F.Z.; data curation, G.-L.L.; writing—original draft preparation, Y.-F.W.; writing—review and editing, Y.-F.W., F.Z. and M.Z.; visualization, Y.-Y.C.; supervision, F.Z. and M.Z.; project administration, F.Z.; funding acquisition, F.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This study was sponsored by the Key Scientific and Technological Research Project of Henan Province (No. 222102110027), the National Natural Science Foundation of China (No. 32001860), the Key Scientific Research Projects of Colleges and Universities in Henan Province (No. 22A210017).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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

References

- Hu, Z.C.; Shi, X.Y.; Chen, X.M.; Zheng, J.; Zhang, A.A.; Wang, H.S.; Fu, Q.S. Fine-mapping and identification of a candidate gene controlling seed coat color in melon (*Cucumis melo* L. var. *chinensis* Pangalo). *Theor. Appl. Genet.* **2022**, *135*, 803–815.
- Vidya, R.; Kalaivani, K.; Amudha, P. Therapeutic potential of *Cucumis melo* (L.) fruit extract and its silver nanoparticles against DEN-Induced hepatocellular cancer in rats. *Appl. Biochem. Biotech.* **2022**, *194*, 368–381.
- Ritschel, P.S.; Lins, T.C.; Tristan, R.L.; Buso, G.S.; Buso, J.A.; Ferreira, M.E. Development of microsatellite markers from an enriched genomic library for genetic analysis of melon (*Cucumis melo* L.). *BMC Plant Biol.* **2004**, *4*, 9.
- Lester, G.E.; Jifon, J.L.; Crosby, K.M. Superoxide dismutase activity in mesocarp tissue from divergent *Cucumis melo* L. genotypes. *Plant Food. Hum. Nutr.* **2009**, *64*, 205–211.
- Branham, S.E.; Levi, A.; Katawczik, M.; Fei, Z.; Wechter, W.P. Construction of a genome-anchored, high-density genetic map for melon (*Cucumis melo* L.) and identification of *Fusarium oxysporum* f. sp. *melonis* race 1 resistance QTL. *Theor. Appl. Genet.* **2018**, *131*, 829–837.
- Li, J.M.; Fokkens, L.; van Dam, P.; Rep, M. Related mobile pathogenicity chromosomes in *Fusarium oxysporum* determine host range on cucurbits. *Mol. Plant Pathol.* **2020**, *21*, 761–776.
- Zhang, Z.H.; Ren, W.C.; Wang, J.; Chen, C.J.; Chen, W.C.; Sang, C.W. Resistance risk assessment of *Fusarium oxysporum* f. sp. *melonis* against phenamacril, a myosin inhibitor. *Pestic. Biochem. Phys.* **2018**, *147*, 127.
- Hao, F.M.; Yan, L.Y.; Zang, Q.Y.; Ma, E.L.; Deng, W.H.; Huang, Y.P.; Wang, L.H. Identification of *Fusarium* from roots and leaves of melon in Zhejiang province. *Chinese Cucurbits and Vegetables* **2020**, *33*, 13–17.
- Khastini, R.O.; Ogawara, T.; Sato, Y.; Narisawa, K. Control of Fusarium wilt in melon by the fungal endophyte, *Cadophora* sp. *Eur. J. Plant Pathol.* **2014**, *139*, 339–348.
- Keane, J.F.; Brown, J. F. Disease management: resistant cultivars. *Plant pathogens and plant disease*. J F Brown and HJ Ogle, eds. University of New England, Armidale, Australia **1997**, 405–426.
- Cal, A.D.; Sztejnberg, A.; Sabuquillo, P.; Melgarejo, P. Management Fusarium wilt on melon and watermelon by *Penicillium oxalicum*. *Biol. Control.* **2009**, 480–486.
- Zhang, N.; Xu, H.; Xie, J.C.; Cui, J.Y.; Yang, J.; Zhao, J.; Tong, Y.J.; Jiang, J.C. Screening of cucumber Fusarium wilt bio-inhibitor: high sporulation *Trichoderma harzianum* mutant cultured on Moso bamboo medium. *Front. Microbiol.* **2021**, *12*, 763006.
- Yoshimi, A.; Tsuda, M.; Tanaka, C. Cloning and characterization of the histidine kinase gene Dic1 from *Cochliobolus heterostrophus* that confers dicarboximide resistance and osmotic adaptation. *Mol. Gen. Genomics.* **2004**, *271*, 228–236.
- Furukawa, K.; Randhawa, A.; Kaur, H. Fungal fludioxonil sensitivity is diminished by a constitutively active form of the group III histidine kinase. *FEBS Lett.* **2012**, *586*, 2417–2422.
- Brandhorst, T.T.; Kean, I.R.L.; Lawry, S.M.; Wiesner, D.L.; Klein, B.S. Phenylpyrrole fungicides act on triosephosphate isomerase to induce methylglyoxal stress and alter hybrid histidine kinase activity. *Sci. Rep.* **2019**, *9*, 5047.

16. Hu, J.; Zhou, Y.X.; Gao, T.; Geng, J.M.; Dai, Y.; Ren, H.Y.; Lamour, K.; Liu, X.L. Resistance risk assessment for fludioxonil in *Sclerotinia homoeocarpa* in China. *Pestici. Biochem. Phys.* **2019**, *156*, 123–128. 489
17. Zhou, F.; Hu, H.Y.; Song, Y.L.; Gao, Y.Q.; Liu, Q.L.; Song, P.W.; Chen, E.Y.; Yu, Y.A.; Li, D.X.; Li, C.W. Biological characteristics and molecular mechanism of fludioxonil resistance in *Botrytis cinerea* from Henan province of China. *Plant Dis.* **2020a**, *104*, 1041–1047. 490
18. Zhou, F.; Li, D.X.; Hu, H.Y.; Song, Y.L.; Fan, Y.C.; Guan, Y.Y.; Song, P.W.; Wei, Q.C.; Yan, H.F.; Li, C.W. Biological characteristics and molecular mechanism of fludioxonil resistance in *Fusarium graminearum* in China. *Plant Dis.* **2020b**, *104*, 2426–2433. 491
19. Corran, A.; Knauf-Beiter, G.; Zeun, R. Fungicides acting on signal transduction. In: *modern crop protection compounds. 2nd ed. Germany: Wiley-VCH.* **2012**, 715–737. 492
20. Hohmann, S. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol. Biol. Rev.* **2002**, *66*, 300–372. 493
21. Hohmann, S. Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **2009**, *583*, 4025–4029. 494
22. Brewster, J.L.; de Valoir, T.; Dwyer, N.D.; Winter, E.; Gustin, M.C. An osmosensing signal transduction pathway in yeast. *Science* **1993**, *259*, 1760–1763. 495
23. Miller, T.K.; Renault, S.; Selitrennikoff, C.P. Molecular dissection of alleles of the osmotic-1 locus of *Neurospora crassa*. *Fungal Genet. Biol.* **2002**, *35*, 147–155. 496
24. Koch, E.; Leadbeater, A.J. Phenylpyrroles-a new class of fungicides for seed treatment. Brighton Crop Protection Conference, Pests and Diseases. *Brit. Crop Prot. Coun.* **1992**, *3*, 1137–1146. 497
25. Ochiai, N.; Fujimura, M.; Motoyama, T.; Ichiishi, A.; Usami, R.; Horikoshi, K.; Yamaguchi, I. Characterization of mutations in the two-component histidine kinase gene that confer fludioxonil resistance and osmotic sensitivity in the os-1 mutants of *Neurospora crassa*. *Pest Manag. Sci.* **2001**, *57*, 437–442. 498
26. Avenot, H.; Simoneau, P. Characterization of mutations in the two-component histidine kinase gene AbNIK1 from *Alternaria brassicicola* that confer high dicarboximide and phenylpyrrole resistance. *Curr. Genet.* **2005**, *47*, 234–243. 499
27. Li, H.X.; Xiao, C.L. Characterization of fludioxonil-resistant and pyrimethanil-resistant phenotypes of *Penicillium expansum* from apple. *Phytopathol.* **2008**, *98*, 427–435. 500
28. Kuang, J.; Hou, Y.P.; Wang, J. X.; Zhou, M.G. Sensitivity of *Sclerotinia sclerotiorum* to fludioxonil: in vitro determination of baseline sensitivity and resistance risk. *Crop Prot.* **2011**, *30*, 876–882. 501
29. Gachango, E.; Kirk, W.; Hanson, L. First report of in vitro fludioxonil-resistant isolates of *Fusarium* spp. causing potato dry rot in Michigan. *Plant Dis.* **2011**, *95*, 228. 502
30. Peters, R.D.; Platt, H.W.; Drake, K.A. First report of fludioxonil-resistant isolates of *Fusarium* spp. causing potato seed-piece decay. *Plant Dis.* **2008**, *92*, 172. 503
31. Malandrakis, A.A.; Vattis, K.N.; Doukas, E.G. Effect of phenylpyrrole-resistance on fitness parameters and ochratoxin production in *Aspergillus carbonarius*. *Int. J. Food Microbiol.* **2013**, *165*, 287–294. 504
32. Ren, W.C.; Shao, W.Y.; Han, X.; Zhou, M.G.; Chen, C.J. Molecular and biochemical characterization of laboratory and field mutants of *Botrytis cinerea* resistant to fludioxonil. *Plant Dis.* **2016**, *100*, 1414–1423. 505
33. Catlett, N.L.; Yoder, O.C.; Turgeon, B.G. Whole-genome analysis of two-component signal transduction genes in fungal pathogens. *Eukaryot. Cell* **2003**, *2*, 1151–1161. 506
34. Qiu, J.B.; Yu, M.Z.; Yin, Q.; Xu, J.H.; Shi, J.R. Molecular and characterization, fitness and mycotoxin production of *Fusarium asiaticum* strains resistant to fludioxonil. *Plant Dis.* **2018**, *102*, 1759–1765. 507
35. Karlsson, I.; Persson, P.; Friberg, H. *Fusarium* head blight from a microbiome perspective. *Front. Microbiol.* **2021**, *12*, 628373. 508
36. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods* **2001**, *25*, 402–408. 509
37. Kilani, J.; Fillinger, S. Phenylpyrroles: 30 years, two molecules (nearly) no resistance. *Front. Microbiol.* **2016**, *7*, 2014. 510
38. Wang, H.; Lin, A.; Gu, W.; Huan, L.; Gao, S.; Wang, G. The sporulation of the green alga *Ulva prolifera* is controlled by changes in photosynthetic electron transport chain. *Sci. Rep.* **2016**, *6*, 24923. 511
39. Yoshimi, A.; Imanishi, J.; Gafur, A.; Tanaka, C.; Tsuda, M. Characterization and genetic analysis of laboratory mutants of *Cochliobolus heterostrophus* resistant to dicarboximide and phenylpyrrole fungicides. *J. Gen. Plant Pathol.* **2003**, *69*, 101–108. 512
40. Ziogas, B.N.; Markoglou, A.N.; Spyropoulou, V. Effect of phenylpyrrole-resistance mutations on ecological fitness of *Botrytis cinerea* and their genetical basis in *Ustilago maydis*. *Eur. J. Plant Pathol.* **2005**, *113*, 83–100. 513
41. Taiwo, A.O.; Harper, L.A.; Derbyshire, M.C. Impacts of fludioxonil resistance on global gene expression in the necrotrophic fungal plant pathogen *Sclerotinia sclerotiorum*. *BMC Genomics* **2021**, *22*, 91. 514