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Molecular Characterization, Fitness, and Mycotoxin Production of *Fusarium asiaticum* Strains Resistant to Fludioxonil

J. B. Qiu and **M. Z. Yu,** Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences; Key Lab of Food Quality and Safety of Jiangsu Province-State Key Laboratory Breeding Base; Key Laboratory of Control Technology and Standard for Agro-Product Quality and Safety, Ministry of Agriculture; Key Laboratory of Agro-Product Safety Risk Evaluation, Ministry of Agriculture; Collaborative Innovation Center for Modern Grain Circulation and Safety, Nanjing, 210014, China; **Q. Yin,** Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing Botanical Garden Mem, Sun Yat-sen, Nanjing, 210014, China; and **J. H. Xu**† and **J. R. Shi,**† Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences; Key Lab of Food Quality and Safety of Jiangsu Province-State Key Laboratory Breeding Base; Key Laboratory of Control Technology and Standard for Agro-Product Quality and Safety, Ministry of Agriculture; Key Laboratory of Agro-Product Safety Risk Evaluation, Ministry of Agriculture; Collaborative Innovation Center for Modern Grain Circulation and Safety, Nanjing, 210014, China

Abstract

Fludioxonil is used in seedborne disease management of various fungal pathogens, including *Fusarium asiaticum*, the predominant causal agent of Fusarium head blight in China. In this study, we screened resistant strains from a large number of *F. asiaticum* strains collected from 2012 to 2016 and found that 4 of 1,000 field strains were highly resistant to fludioxonil. The 50% effective concentration values of the resistant strains and induced mutants ranged from 80 to >400 μg/ml. Compared with field-sensitive strains, all field-collected and laboratory-induced resistant strains exhibited fitness defects in traits including mycelial growth, conidial production, pathogenicity, and sensitivity to osmotic conditions. In the presence of fludioxonil, significantly higher glycerol accumulation was found in sensitive strains but not in resistant individuals. The fludioxonil-resistant strains produced lower amounts of glycerol in liquid culture and lower amounts of trichothecene mycotoxins in rice culture and inoculated wheat spikelets than the fludioxonil-sensitive strains. Sequence analyses of the key genes of the two-component histidine kinase signaling pathway showed various amino acid substitutions in the *Os1*, *Os4*, and *Os5* genes between field-sensitive and resistant strains or mutants. The results of this study suggest a potential risk of fludioxonil resistance development and a possible influence of resistance mutations on fitness parameters and toxin production in *F. asiaticum*.

Fusarium asiaticum, lineage 6 of the *F. graminearum* species complex (O'Donnell et al. 2004), is the predominant pathogen causing Fusarium head blight (FHB) in certain parts of Asia (Lee et al. 2009; Qiu and Shi 2014; Qiu et al. 2016b; Suga et al. 2008; Zhang et al. 2012). This fungus is a cause of continuing concern not only because of the FHB epidemic and the subsequent yield losses but also due to its ability to synthesize mycotoxins (Goswami and Kistler 2004). Mycotoxins, including the chemical deoxynivalenol (DON) and its derivatives, nivalenol (NIV) and zearalenone (ZEN), have been shown to correlate with toxic effects and some mycotoxicoses in humans and animals (D'Mello et al. 1999). Considering the potential threat posed by toxin-contaminated food and feed, many countries have established regulatory limits and guidelines for food and feedstuffs. Recently, China has updated the regulatory limit of mycotoxins in food. For cereals and their products, the maximum permissible levels of DON and ZEN are now 1,000 and 60 μg/kg, respectively (GB 2761-2017). In *Fusarium* spp.-infected plants, DON could be glycosylated to deoxynivalenol-3-glucoside (D3G), which was considered to be one form of masked mycotoxin and transferred to DON in the course of cereals processing (Berthiller et al. 2013; De Angelis et al. 2013; Zachariasova et al. 2012).

Multiple methods have been used to control disease incidence and prevent mycotoxin contamination in crops from field to postharvest. The selection and breeding of cereal varieties resistant to FHB should be a priority for these purposes (Gunnaiah and Kushalappa 2014). Some progress has been made in the breeding of FHB-resistant wheat but wheat varieties with high FHB resistance are not yet available. Some agricultural practices such as crop rotation (Dill-Macky and Jones 2000; Qiu et al. 2016a), soil cultivation (Obst et al. 1997), and fertilization (Martin et al. 1991) are effective in controlling disease or toxins but further long-term research and monitoring the effect of the agronomic measures are required. In addition, some microbes, including bacteria, and fungi, have been effective in controlling FHB (Hue et al. 2009; Schisler et al. 2002) but their effectiveness needs to be further confirmed under field conditions.

Currently, the most efficient way to reduce FHB and mycotoxin accumulation in grains remains the spraying of appropriate fungicides such as carbendazim, triazoles, and phenamacril at wheat anthesis (Scarpino et al. 2015; Zhang et al. 2010). However, the long-term use of a single class of fungicide in a location may trigger the development of fungicide resistance that greatly reduces the efficacy of control (Brent and Hollomon 1998). The usefulness of several fungicides such as carbendazim, phenamacril, and tebuconazole has become limited against FHB due to their resistance or aggravated toxin contamination (Becher et al. 2010; Qiu et al. 2011; Zhang et al. 2009; Zhang et al. 2017).

Fludioxonil is a synthetic analog of pyrrolnitrin, which is a natural antifungal compound produced by *Pseudomonas pyrocinia* (Nishida et al. 1965). Although its mode of action has not been fully revealed, fludioxonil is believed to target the osmotic-regulatory signal transduction pathway (Furukawa et al. 2012). Because this pathway is involved in many fungal activities, fludioxonil exhibits a broad spectrum of activity against various fungal pathogens (Koch and Leadbeater 1992), and inhibits the mycelial growth of *F. graminearum* strains derived from maize and soybean at low concentrations in vitro (Broders et al. 2007; Munkvold and O'Mara 2002).

Fludioxonil resistance has been reported in *Botrytis cinerea* (Ren et al. 2016), *Sclerotinia sclerotiorum* (Kuang et al. 2011), *Penicillium expansum* (Li and Xiao 2008), *Aspergillus carbonarius* (Malandrakis et al. 2013b), *Neurospora crassa* (Ochiai et al. 2001),

†Corresponding authors: J. H. Xu; E-mail: xujianhongnj@126.com; and J. R. Shi; E-mail: shiji@jaas.ac.cn

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Alternaria brassicicola (Avenot et al. 2005), and *Fusarium* spp. (Gachango et al. 2011; Peters et al. 2008), and its resistance mechanism has been associated with mutation in the histidine kinase *Os1* (Avenot et al. 2005; Ochiai et al. 2001; Ren et al. 2016). However, there were several reports that no *Os* gene mutation was found in fludioxonil-resistant strains of some plant pathogens and the authors suggested that mutations in other *Os*-like genes might explain fungicide resistance (Catlett et al. 2003). Furthermore, The ATP-binding cassette transporter may be important in modulating the pathogen-specific effects of phenylpyrrole fungicides (Vermeulen et al. 2001). In China, fludioxonil had been registered on wheat, rice, cotton, and peanut (ICAMA, Institute for the Control of Agrochemicals, MOA). Although fludioxonil has not been widely applied in the direct control of FHB, wheat seed are usually treated with Beret Gold (active ingredient: fludioxonil at 24.3 μg/ml; Syngenta) before being sown to prevent soilborne diseases (Bailey et al. 2005). Fludioxonil-resistant isolates of *F. graminearum* have been found in corn and soybean seedling samples (Broders et al. 2007) but there was no report about resistant strains of this species from other crops. In this study, we surveyed the occurrence of fludioxonil resistance from a large population of *F. asiaticum* isolates from wheat fields in China to determine the possible use of fludioxonil to control FHB.

The occurrence and development of fungicide resistance affects not only the longevity of fungicide effectiveness but also the toxin production of resistant strains of some mycotoxigenic fungi. The relationship between mycotoxin production and fludioxonil resistance has been studied in *Aspergillus carbonarius* and *A. parasiticus* (Malandrakis et al. 2013b; Markoglou et al. 2008) but little information is available about the effect of resistance on toxin production in *F. asiaticum*, a well-known mycotoxigenic fungus of wheat in China. In addition, strains resistant to fludioxonil showed high sensitivity to hyperosmotic environments triggered by the addition of certain chemicals (Motoyama et al. 2005; Oshima et al. 2002; Ren et al. 2016). Several previous studies have indicated that fludioxonil stimulates glycerol biosynthesis; thus, it is believed that fludioxonil could imitate the osmotic stress response because glycerol production is induced by increased environmental osmolarity (Pillonel and Meyer 1997; Zhang et al. 2002).

Thus, the main aims of this study were to (i) determine the resistance frequency of *F. asiaticum* isolates from the wheat-growing areas of China; (ii) compare the fitness parameters and mycotoxin production levels of laboratory mutant, field-resistant, and sensitive strains; and (iii) analyze molecular characteristics of resistant strains. This study assesses the possible risk of occurrence of fludioxonil-resistant strains of *F. asiaticum* and their mycotoxin-producing abilities. These results may also contribute to our understanding of the resistance mechanism of *F. asiaticum* to fludioxonil.

Materials and Methods

Fungal strains and media. Wheat grains were collected in areas of FHB occurrence from 2012 to 2016, including Anhui, Sichuan, Shandong, Jiangsu, and Hubei provinces, and *F. asiaticum* strains were isolated from wheat grains showing FHB symptoms. Single-spore isolation of the strains was performed by the previously described method (Zhang et al. 2012). All strains were grown on potato dextrose agar (PDA) as the regular culture medium and stored in 20% glycerol solution at −80°C. Mung bean broth (MBB) was used for conidial production of *F. asiaticum* strains. To determine intracellular glycerol accumulation, each strain was incubated in a conical flask containing 100 ml of potato dextrose broth (PDB) (Ren et al. 2016). To determine mycotoxin production, each strain was inoculated to autoclaved rice grains (20 g) and incubated for 30 days at 25°C (Qiu and Shi 2014).

Determination of sensitivity to fludioxonil. In a preliminary experiment, we determined the growth of 20 strains on PDA amended with fludioxonil at various concentrations and found that they were completely inhibited by fludioxonil at 0.5 μg/ml. Thus, a concentration of fludioxonil at 0.5 μg/ml was used to screen resistant field strains in vitro. Of 3,000 strains of *F. asiaticum*, 1,000 strains were randomly chosen to screen resistant strains.

Growth of all strains at varied concentrations of fludioxonil was measured after growing on PDA for 3 days at 25°C, and the 50% effective concentration (EC_{50}) was calculated, along with the inhibitory rate at the corresponding concentration. Sensitive strains were grown on PDA plates containing fludioxonil at 0, 0.01, 0.02, 0.04, 0.08, and 0.16 μg/ml, and resistant strains were grown on PDA plates containing fludioxonil at 50, 100, 200, 400, and 800 μg/ml.

Generation of fludioxonil-resistant mutants. Fludioxonil-resistant mutants were obtained through a previously published method, with minor modifications (Ren et al. 2016). Two sensitive strains (Fa1657 and Fa1666) were randomly chosen and placed on PDA plates amended with fludioxonil at 0.1 μg/ml. Fast-growing sectors were transferred to new PDA plates containing fludioxonil at 10 and 50 μg/ml. Those colonies that grew normally on the plates were kept for resistance identification. Two mutants of each strain were selected for further trials.

Mycelial growth and conidial production. To determine mycelial growth rate, a mycelial plug was transferred onto the center of fungicide-free PDA plates, and the plates were incubated at 25°C for 72 h. At least two independent strains were performed for each test, and each strain was tested in three replicates.

Conidial production was measured by counting the number of conidia after incubating 10 mycelial plugs from PDA in 100 ml of MBB under a 12-h photoperiod for 7 days at 25°C on a rotary shaker (170 rpm). The number of conidia was counted with a hemocytometer.

Determination of glycerol content. For intracellular glycerol accumulation analysis, 10 mycelial plugs of each strain were incubated in a conical flask containing 100 ml of PDB for 3 days at 25°C with shaking at 170 rpm. The mycelia were collected, ground with a grinder in liquid nitrogen, and transferred to a glycerol extraction buffer. Then, glycerol content was measured with a glycerol assay kit (Applygen) according to the manufacturer's instructions.

Stress responses. To determine the response to stress factors, mycelial plugs were transferred to PDA plates amended with 1 M NaCl, 1 M KCl, 1 M glucose, 1 M sorbitol, sodium dodecyl sulfate (SDS) at 0.1 g/liter, Congo red at 0.05 g/liter, and caffeine at 0.05 g/liter. PDA plates without any amendment chemicals were used as a control. After incubation for 72 h at 25°C, the colony diameter of each strain was measured in three replicates. The percentage of inhibition of mycelial growth was calculated.

Pathogenicity test and toxin analysis. The pathogenicity of all strains was determined on wheat variety Yangmai 16 cultivated in Luhe Science Base, Jiangsu Academy of Agricultural Sciences. Conidial suspension (10 μl at 10^5 conidia/ml) of each strain was injected into the center spikelet at anthesis, as previously described (Wu et al. 2005), and 100 replicate wheat heads were utilized for each strain. Disease severity was assessed 20 days after inoculation with the previously described method (Zhang et al. 2015). A disease index (DI) was then calculated for each replicate using the formula $DI = (\sum n_s S / 7N) \times 100$, where S is the scale value of each plant, n_s is the number of plants in the category, and N is the total number of plants assessed per replicate.

Wheat grains in the pathogenicity trial were sampled at harvest time for mycotoxin analysis in vivo, and a 1-kg representative sample from each plot was used to analyze the toxin content in the grain. The extraction and detection were performed as described in a previous study, with a minor modification (Dong et al. 2017; Ji et al. 2014; Qiu and Shi 2014). Wheat flour was extracted with acetonitrile-water (90:10, vol/vol) and purified with a solid-phase extraction column (SampliQ Amino; Agilent Technologies). A high-pressure liquid chromatography/electrospray ionization-tandem mass spectrometry system was used to simultaneously quantify multi-mycotoxin. The composition of the platform, mobile phases, and analysis program was introduced in detail by Dong et al. (2017). DON, 3-acetyl-DON (3ADON), D3G, and ZEN contents in wheat samples were analyzed.

Autoclaved rice grains were inoculated with 20 agar plugs of each strain to qualify mycotoxin production in vitro. Colonized rice cultures were ground into flour after drying, and 5-g samples were used

to extract and detect mycotoxins. The detailed procedure was described above. DON, 3ADON, and ZEN contents in rice culture were analyzed.

Sequence analysis of *OS1*, *OS2*, *OS4*, and *OS5*. The genomic DNA of each strain was extracted with a previously described method (Leslie and Summerell 2006). Putative kinase genes were identified in previous studies (Ochiai et al. 2007; Ramamoorthy et al. 2007). The protein sequences of these genes were obtained from the Munich Information Center for Protein Sequences *F. graminearum* database (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_p13839_Fus_grami_v32) (Wong et al. 2011). Specific primers were designed to amplify four genes from each strain (Table 1). Fifteen mutants of each strain were generated and used for the analysis of *Os* genes. All of the polymerase chain reaction (PCR) products were sequenced by Sangon Biotech Co., Ltd.. BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to calculate the amino acid sequence differences between resistant and sensitive strains.

Statistical analysis. EC_{50} values were calculated by transforming the relative inhibition of mycelial growth against the $\log_{10}$ fungicide concentrations. Statistical comparisons of EC_{50} values and fitness parameters were made with a *t* test. All statistical analyses were performed with the SigmaStat statistical software package (SPSS, version 11).

Results

Sensitivity of field strains and laboratory mutants to fludioxonil. We isolated 3,000 strains of *F. asiaticum* from wheat grains and confirmed their identification by PCR and sequencing analyses of translation elongation factor 1α (Qiu and Shi 2014; Qiu et al. 2014). Of 1,000 strains of *F. asiaticum*, only 4 strains (Fa16101, Fa1252, Fa12213, and Fa12610) were found to have normal growth on medium amended with fludioxonil, even at 50 μg/ml. Their accurate EC_{50} values in response to the fungicide were determined through further analysis and are listed in Table 2. The EC_{50} values of the sensitive strains were <0.01 μg/ml, significantly ($P < 0.05$) lower than those of the field-resistant strains (ranging from 80 to >400 μg/ml). Several resistant mutants induced from two sensitive strains (Fa1657 and Fa1666) were able to grow on PDA plates containing fludioxonil at 50 μg/ml. Their EC_{50} values were >400 μg/ml, higher than those of field-resistant strains. Resistance factor values for the induced mutants were all >10,000 (Table 2).

Intracellular glycerol accumulation. Under control conditions, glycerol accumulation in the liquid PDB was low, and no significant differences were present between the parental strains and their corresponding derived mutants (Fig. 1A and B). The field-resistant strains appeared not to produce higher amounts of glycerol compared with the field-sensitive strains. (Fig. 1C). When treated with fludioxonil at 0.5 μg/ml, the glycerol content of the sensitive strains significantly increased, whereas there were no significant changes in the glycerol production of the resistant strains and mutants (Fig. 1).

Mycelial growth and conidial production. After 72 h of culture, the growth rates of all resistant strains or mutants were significantly lower than those of the sensitive strains (Table 3). After 7 days of culture, significant declines in the sporulation capacity of the resistant strains and mutants were observed in comparison with the sensitive populations. More severe reduction of conidial production occurred in field-resistant strains than in induced mutants (Table 3).

Sensitivity to stress factors. Upon the addition of four osmotic agents, the mycelial growth of all strains was significantly inhibited, especially for the induced mutants. The induced mutants were more sensitive to NaCl, KCl, glucose, and sorbitol than field-resistant strains were, although they showed similar trends in fludioxonil EC_{50} values, except Fa1657R1 (Table 3).

Cell wall and cell membrane integrity. Congo red and caffeine are usually applied to verify the integrity of the cell wall, because they specifically bind to cellulose and chitin, respectively, in the cell wall. Because SDS can solubilize lipids and proteins, SDS was used to test the membrane integrity. All strains showed similar sensitivity to these three inhibitors (Table 3).

Pathogenicity. The pathogenicity of all strains was determined by point inoculation of conidial suspensions onto flowering wheat heads. Twenty days later, more wheat-head spikelets inoculated with the field-sensitive strains exhibited typical scab symptoms than did the spikelets inoculated with resistant mutants and strains, in which FHB symptoms were restricted to regions near the inoculation sites (Table 3).

Table 2. Origin and sensitivity of *Fusarium asiaticum* strains to fludioxonil

Strain	Origin	Sensitivity[x]	EC_{50}[y]	Resistance factor[z]
Fa16122	Wheat (Shandong)	S	0.006	…
Fa12636	Wheat (Hubei)	S	0.004	…
Fa1657	Wheat (Jiangsu)	S	0.008	…
Fa1666	Wheat (Jiangsu)	S	0.005	…
Fa1657R1	Laboratory induction from Fa1657	R	>400	>10,000
Fa1657R3	Laboratory induction from Fa1657	R	>400	>10,000
Fa1666R1	Laboratory induction from Fa1666	R	>400	>10,000
Fa1666R3	Laboratory induction from Fa1666	R	>400	>10,000
Fa16101	Wheat (Jiangsu)	R	80	>10,000
Fa1252	Wheat (Jiangsu)	R	334	>10,000
Fa12213	Wheat (Henan)	R	>400	>10,000
Fa12610	Wheat (Anhui)	R	396	>10,000

[x] S = sensitive and R = resistant.
[y] Effective concentration (μg/ml) for 50% inhibition of mycelial growth.
[z] EC_{50} in the resistant mutants is represented as a ratio relative to the EC_{50} of the sensitive strain.

Table 1. Primers used in this study

Locus	Description of gene product	Primer	Sequence (5′–3′)	Size (bp)
FGSG_16781	Related to NIK-1 nonidentical kinase-1 (*Os1*)	Os1F1	ACCCACCCGTTCAAACTACAC	2,339
	…	Os1R1	ATCTCGCCTGATGCCTCTAC	…
	…	Os1F2	CAAGCCTGAACACGAACAAC	2,446
	…	Os1R2	AGCAACGAATAACCAGAGCC	…
FGSG_09612	Osmotic sensitive-2 protein (*Os2*)	Os2F	ACCACACCTATCAAACCACTGC	2,368
	…	Os2R	TTCCCTTATCTCCCCAACG	…
FGSG_00408	Related to MAPK[z] Wis4 (*Os4*)	Os4F1	GCAGCCACAGCAAGACGAA	2,442
	…	Os4R1	CGGGGACGCAATCACATAGA	…
	…	Os4F2	CGACTGAAATGAGCAAACGC	2,843
	…	Os4R2	AGAAGAAAGAGGAAGTGAAAG	…
FGSG_08691	Related to tyrosine protein kinase of the MAPK (*Os5*) family	Os5F	TTACCGTCCCTGGGATTCTAC	2,867
	…	Os5R	CCTGCCTTCCTTATCTTGTCTT	…

[z] MAPK = mitogen-activated protein kinase.

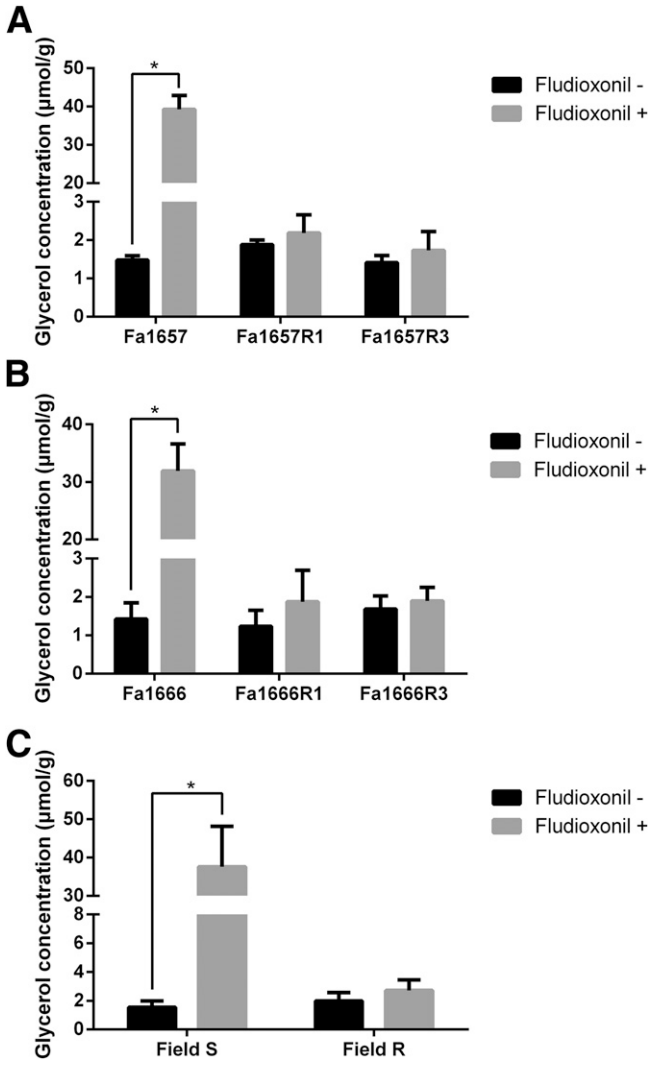

Fig. 1. Intracellular glycerol contents of *Fusarium asiaticum* strains in the absence (black column) or presence (gray column) of fludioxonil. An asterisk indicates that mean values are significantly different ($P < 0.05$) between the treatment and the control. **A,** Field-sensitive strain Fa1657 and its induced mutants; **B,** field-sensitive strain Fa1666 and its induced mutants; and **C,** field-sensitive populations and field-resistant populations.

Toxin production. After wheat harvest, the inoculated samples were ground, and the mycotoxin levels in the resulting wheat flour were analyzed by high-performance liquid chromatography mass spectrometry. All induced mutants produced lower amounts of 3ADON, D3G, and DON compared with the parent strains, whereas no significant reduction was found in ZEN production among strains (Fig. 2A and B). DON differed between the field groups, unlike 3ADON, D3G, and ZEN, although the latter compounds were slightly reduced in resistant strains (Fig. 2C).

All strains grown on rice produced more toxins than those in wheat samples (Fig. 3). A notable decrease in DON and ZEN levels was found in two mutants of Fa1657, and 3ADON level varied only in Fa1657R1 (Fig. 3A). However, two mutants of Fa1666 showed a different pattern of toxin production compared with those of Fa1657 (Fig. 3B). Interestingly, all toxin accumulation was reduced in field-resistant strains (Fig. 3C).

Sequence analysis of *Os1*, *Os2*, *Os4*, and *Os5*. In the present study, we sequenced the open reading frame sequences of four genes in all strains. The whole sequences of the four *Os* genes were identical for four sensitive strains. Fa12610 and Fa12213 had single-point mutations R753STOP and Q927STOP, respectively, in *Os1*, while the other two strains carried no mutations. For *Os2*, *Os4*, and *Os5*, there was no mutation site in all field-resistant strains. For the laboratory-resistant mutants, Fa1666R3 and Fa1657R15 had point mutations in P1109S and P1161H, respectively, in *Os1*. Fa1657R6 carried one point mutation in *Os4*, C928Y. Fa1666R10 and Fa1657R2 shared the same point mutation in *Os5*, S116T. The mutants Fa1657R11 and Fa1666R14 carried two point mutations in *Os5*, R598T and R602G. Fa1657R5, Fa1666R12, and Fa1657R4 had point mutations S39T, S90T, and L350P, respectively (Table 4).

Discussion

Fungicide resistance is the result of increased selection pressure on a pathogen population due to the excessive use of a particular chemical (Brent and Hollomon 1998). This problem is becoming more and more serious in the control of FHB, and the application of several effective fungicides such as carbendazim, phenamacril, and tebuconazole is under threat (Becher et al. 2010; Zhang et al. 2009; Zhang et al. 2017). In China, fludioxonil has not been registered to control FHB and has mainly been used in seed-coating agents such as Beret Gold to prevent seedborne diseases. However, this drug has been shown to have strong inhibitory effects on mycelial growth in *Fusarium* spp. and can control plant diseases, including those affected by *F. graminearum* (Broders et al. 2007; Munkvold and O'Mara 2002). In this study, we monitored fludioxonil resistance in the field and found 4 resistant individuals among 1,000 *F. asiaticum* strains. We hypothesized that these resistant strains might have come from soil

Table 3. Comparison of *Fusarium asiaticum* fludioxonil-resistant mutants or strains with field-sensitive strains with respect to ecological fitness parameters[u]

				Sensitivity[v]						
Strain[w]	Growth[x]	Spor[y]	DI[z]	NaCl	KCl	Glucose	Sorbitol	SDS	Congo red	Caffeine
Fa16122 (S)	82.0 a	14.1 a	12.3 a	36.0 i	27.1 i	2.9 h	1.9 h	3.3 a	7.2 bc	40.8 bc
Fa12636 (S)	82.7 a	5.4 c	11.5 a	40.7 h	38.7 g	9.7 g	4.9 g	1.9 bc	7.2 bc	40.2 bc
Fa1657 (S)	81.5 a	9.9 b	9.0 b	40.3 h	31.9 h	9.6 g	3.9 gh	3.1 a	7.0 cd	40.2 bc
Fa1666 (S)	82.9 a	9.3 b	11.8 a	43.4 g	24.7 i	8.3 g	2.7 gh	3.1 a	7.6 a	35.2 cd
Fa1657R1 (IR)	76.1 b	1.8 e	4.5 c	68.9 e	68.5 e	63.3 e	61.5 e	3.2 a	7.2 bc	36.7 cd
Fa1657R3 (IR)	65.2 c	2.0 de	3.0 d	100.0 a	100.0 a	100.0 a	100.0 a	1.7 bc	7.2 bc	36.7 cd
Fa1666R1 (IR)	66.3 c	5.1 c	2.8 d	100.0 a	100.0 a	100.0 a	100.0 a	2.9 a	7.4 ab	32.5 d
Fa1666R3 (IR)	62.7 c	4.9 cd	3.5 cd	91.2 b	91.4 b	85.6 b	82.8 b	3.0 a	7.2 bc	39.5 bc
Fa16101 (R)	65.3 c	0.3 e	3.0 d	87.4 c	87.3 c	76.7 c	73.7 c	3.0 a	6.9 d	44.8 a
Fa1252 (R)	77.2 b	0.3 e	2.8 d	55.4 f	54.4 f	38.7 f	33.7 f	2.4 ab	7.1 cd	44.9 a
Fa12213 (R)	62.6 c	0.3 e	3.3 d	84.0 d	81.8 d	69.9 d	62.9 e	2.6 ab	6.3 e	23.0 e
Fa12610 (R)	61.3 c	0.3 e	3.0 d	82.4 d	82.7 d	71.0 d	66.2 d	2.8 ab	7.5 a	43.4 ab

[u] Data followed by the same letter in each column are not significantly different ($P < 0.05$).
[v] Growth inhibitory rate (%) in the presence of osmotic or cell wall damaging agents. SDS = sodium dodecyl sulfate.
[w] S = field-sensitive strains, IR = induced resistant mutants, and R = field-resistant strains.
[x] Colony diameter (mm) after 3 days of incubation.
[y] Sporulation (Spor) = number ($\times 10^6$ CFU/ml) of conidia after 7 days of incubation.
[z] Pathogenicity (DI = disease index) after 20 days of inoculation.

where fludioxonil had been applied. Although few field-resistant strains have been recorded to date, it is important to accept that fludioxonil may give selection pressure in the field. Drug resistance requires attention, because fludioxonil-containing seed-coating agents remain in common use.

In contrast to the results of previous studies, which found that mutations in the N-terminal region of *Os1* resulted in fludioxonil resistance in various fungal species (Avenot et al. 2005; Ochiai et al. 2001; Ren et al. 2016), no amino acid substitution was found in *Os1* in field-resistant strains in our study. Similar results were reported in *B. cinerea* and *P. digitatum* strains with varied resistance to this fungicide (Fernández-Ortuño et al. 2014; Kanetis et al. 2008; Li et al. 2014; Ren et al. 2016), suggesting that the sequences of other genes in the mitogen-activated protein kinase cascade might be involved in resistance. Several osmotic sensitivity loci (*Os1*, *Os2*, *Os4*, and *Os5*) participated in fludioxonil and osmotic sensitivity in *F. graminearum* (Ochiai et al. 2007; Ramamoorthy et al. 2007). Further analysis of the rest these genes confirmed that no mutations existed in the field-resistant strains. However, in some filamentous fungi, several histidine kinases have been found (Catlett et al. 2003); therefore, mutations in other *Os*-like genes may be possible causes of fungicide and osmotic sensitivity. The molecular mechanism of resistance in the field *F. asiaticum* strains to fludioxonil requires further studies.

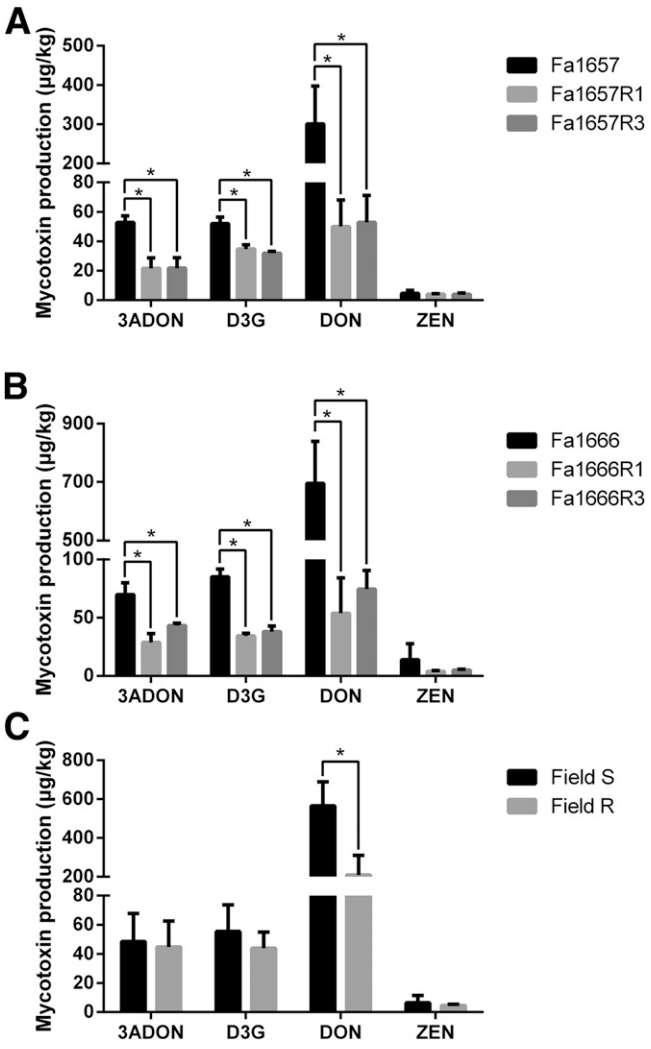

Fig. 2. Mycotoxin (3-acetyl-deoxynivalenol [3ADON], deoxynivalenol-3-glucoside [D3G], deoxynivalenol [DON], and zearalenone [ZEN]) production by *Fusarium asiaticum* strains in infected wheat heads 20 days after inoculation. An asterisk indicates that mean values are significantly different ($P < 0.05$) between strains or populations. **A,** Field-sensitive strain Fa1657 and its induced mutants; **B,** field-sensitive strain Fa1666 and its induced mutants; and **C,** field-sensitive populations and field-resistant populations.

In our study, we also found several amino acid sites detected in *Os1*, *Os4*, and *Os5* between field-sensitive and induced-resistant strains. However, different genotypes showed similar resistance levels because all mutants were highly resistant to this fungicide. In addition, most mutation sites were located in just one or two mutants and we did not find a common mutation type. Whether these mutations directly led to fludioxonil resistance still requires further studies.

To evaluate the potential risk of fludioxonil resistance in *F. asiaticum*, we created several mutants. Compared with the field-sensitive strains, both the induced mutants and the field-resistant strains showed reductions in mycelial growth, sporulation ability, and pathogenicity,

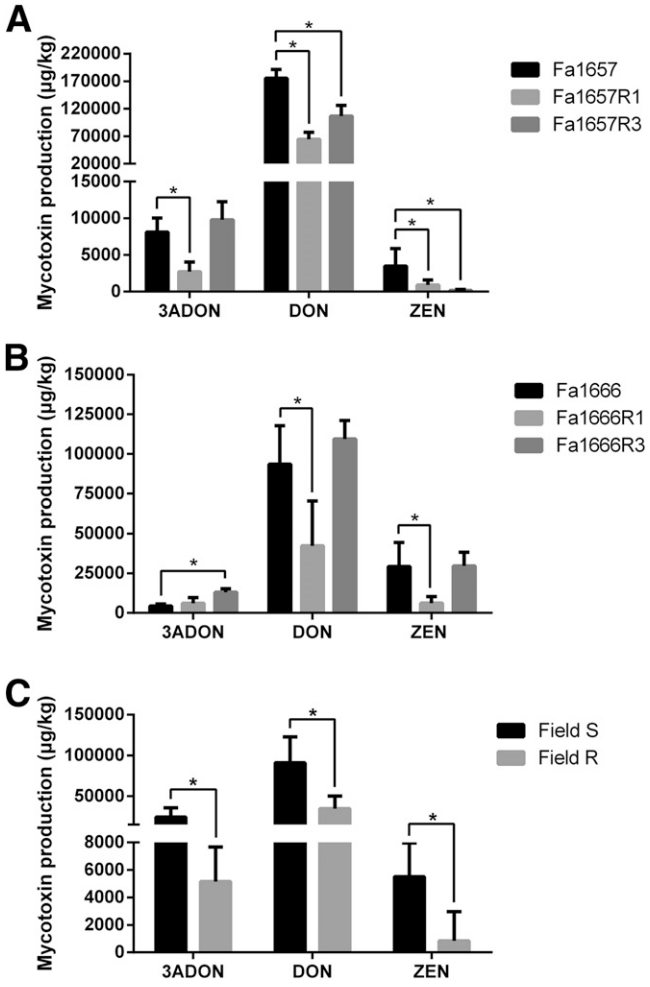

Fig. 3. Mycotoxin (3-acetyl-deoxynivalenol [3ADON], deoxynivalenol [DON], and zearalenone [ZEN]) production by *Fusarium asiaticum* strains in rice culture. An asterisk indicates that mean values are significantly different ($P < 0.05$) between strains or populations. **A,** Field-sensitive strain Fa1657 and its induced mutants; **B,** field-sensitive strain Fa1666 and its induced mutants; and **C,** field-sensitive populations and resistant populations.

Table 4. Mutations in *Os* sequences in laboratory-resistant mutants

Mutation type	Location	Strain or mutant
P1109S	*Os1*	Fa1666R3
P1161H	*Os1*	Fa1657R15
C928Y	*Os4*	Fa1657R6
S39T	*Os5*	Fa1657R5
S90T	*Os5*	Fa1666R12
S116T	*Os5*	Fa1666R10, Fa1657R2
L350P	*Os5*	Fa1657R4
R598T	*Os5*	Fa1657R11, Fa1666R14
R602G	*Os5*	Fa1657R11, Fa1666R14

which suggested that fludioxonil resistance was associated with fitness defects, perhaps explaining why the frequency of resistant strains in the field was relatively low. Similar findings have been reported in *P. expansum* (Li and Xiao 2008), *S. sclerotiorum* (Kuang et al. 2011), and *B. cinerea* (Ren et al. 2016). However, carbendazim-resistant strains and site-directed mutants of *F. graminearum* shared similar fitness parameters and virulence (Chen et al. 2007; Qiu et al. 2011). When *F. graminearum* strains were adapted to tebuconazole and phenamacril in vitro, mutants exhibiting various phenotypes were obtained (Becher et al. 2010; Zhang et al. 2017). These results indicated that the influence of fungicide resistance on fitness parameters differed by resistance mechanism and target genes, rather than by fungus.

Fludioxonil is currently considered to affect the fungal osmotic signal transduction cascade (Bahn et al. 2006), and resistant strains of various fungal pathogens were more sensitive to osmotic stress (Kanetis et al. 2008; Ren et al. 2016). Here, both resistant mutants and field strains exhibited hypersensitivity to adverse osmotic conditions such as those induced by NaCl, KCl, glucose, and sorbitol. Although significant differences were found among strains and mutants, no correlation was apparent between osmotic stress tolerance and response to fludioxonil. This result was in contrast to several reports in which fludioxonil-resistant mutants and field strains with a decreased sensitivity to the fungicide presented an increased sensitivity to osmotic conditions (Oshima et al. 2002; Zhang et al. 2002). However, other findings suggested no close correlation between sensitivity to osmotic stress and sensitivity to fludioxonil. Laboratory and field strains of *Alternaria brassicicola* and *N. crassa* were classified into two groups based on different sensitivities to the osmotic environment or fludioxonil (Avenot et al. 2005; Fujimura et al. 2000). The present findings and the previous results indicated that osmotic stress tolerance was not solely associated with sensitivity to fludioxonil in pathogenic fungi.

Because the development of fludioxonil resistance is accompanied by the inhibition of glycerol synthesis, and a reduced glycerol content is correlated with adaptation to osmotic conditions (Fujimura et al. 2000; Zhang et al. 2002), we determined intracellular glycerol accumulation among the sensitive and resistant groups. In the absence of fludioxonil, all strains produced similar, relatively low amounts of glycerol. Although glycerol production varied among individuals, no significant difference existed between the groups. This result was consistent with that in *B. cinerea* (Ren et al. 2016), because we adopted the same analysis method, but contradicted previous findings in *P. digitatum*, in which the glycerol level in sensitive strains was higher (Kanetis et al. 2008). Under fludioxonil treatment, glycerol accumulation significantly increased in sensitive strains but not in resistant mutants or strains. As previously reported, fludioxonil could stimulate glycerol synthesis in various fungi (Kanetis et al. 2008; Okada et al. 2005; Pillonel and Meyer 1997), which was thought to reflect the overexpression of *Os2* (Zhang et al. 2002). In an immunodetection study with *P. digitatum*, sensitive strains expressed more phosphorylated *Os2* than resistant strains did (Kanetis et al. 2008). In addition, the expression levels of *Os2* in the sensitive strains were significantly higher than those in the resistant individuals (Ren et al. 2016). These results can explain the small changes in glycerol content in resistant groups after exposure to fludioxonil, and we will investigate the expression of *Os* genes with or without this fungicide in the future.

In addition to the fitness parameters, the effects of fludioxonil resistance on toxin production merit more attention; a variety of studies have revealed positive correlations between fungicide resistance and toxin levels in other mycotoxigenic species such as *Fusarium* spp. (D'Mello et al. 2000; Markoglou et al. 2009; Qiu et al. 2014; Zhang et al. 2009), *Penicillium* spp. (Karaoglanidis et al. 2011; Malandrakis et al. 2013a), and *Aspergillus* spp. (Markoglou et al. 2008). In the present study, all induced mutants on wheat produced significantly reduced levels of 3ADON, D3G, and DON compared with the field strains from which they were derived. A similar trend in toxin production was found in rice culture between most laboratory mutants and their parent strains. Schmidt-Heydt et al. (2012) considered that ochratoxin accumulation in *Penicillium* spp. was induced under high osmotic stress caused by high NaCl concentration. Thus, we consider that resistance mutations compromising the osmotic response might explain the observed reductions in toxin production. In *F. graminearum*, mycotoxin accumulation is influenced by various environmental factors and transcriptional regulatory factors; therefore, we will focus on the possible relationship between osmotic pressure and toxin production in future studies.

The risk of resistance for fungicides such as mutation frequency in the field, cross resistance, fitness of resistant mutants, and mechanism of action was determined by plant pathogens and the fungicides. The possibility of the occurrence of the fungicide resistance and the predominance of field-resistant populations was assessed with the virulence, genetic stability, competitive ability of resistant strains, and so on. In our study, we found a very low frequency of fludioxonil resistance among *F. asiaticum* strains from wheat fields in China and the resistant strains showed fitness defects compared with the fungicide-sensitive strains. Based on these findings, we can preliminarily suggest that the risk of *F. asiaticum* to develop resistance to fludioxonil was low to medium. This fungicide could be used as an alternative agent for controlling crop diseases. If fludioxonil was more frequently sprayed to control FHB in wheat, there might be a risk of high occurrence of fludioxonil-resistant strains in the field.

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