Research Article

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Triazole resistance in *Aspergillus fumigatus* exposed to new chiral fungicide mefentrifluconazole

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Abstract

BACKGROUND: Triazole resistance in the human fungal pathogen *Aspergillus fumigatus* has been a growing challenge in clinic treatment with triazole drugs such as itraconazole. The fast evolvement of triazole resistance in *A. fumigatus* in the ecosystem has drawn great attention, and there has been a possible link between the application of triazole fungicides in agriculture and triazole resistance in *A. fumigatus*. The change in susceptibility of *A. fumigatus* exposed to the new chiral triazole fungicide mefentrifluconazole was investigated in this study.

RESULTS: The results indicated that triazole resistance in *A. fumigatus* was acquired with exposure to mefentrifluconazole at a level of greater than or equal to 2 mg L^{-1} in liquid medium and soil (not at 0.4 nor 1 mg L^{-1}). Interestingly, stereoselectivity was found in the acquisition of triazole resistance in *A. fumigatus* when exposed to mefentrifluconazole. R-mefentrifluconazole, which is very active on plant pathogens, exhibited stronger possibility in the development of the resistance in *A. fumigatus* than its antipode. Overexpression of *cyp51A*, *AtrF*, *AfuMDR1* and *AfuMDR4* were associated with the acquired resistance in *A. fumigatus* with hereditary stability.

CONCLUSION: The results suggest that triazole resistance in *A. fumigatus* could be resulted from the selection of mefentrifluconazole at concentrations larger than 2 mg L^{-1}. Mefentrifluconazole should be applied within the dosage recommended by good agricultural practice to avoid the resistance in *A. fumigatus* in soil. This also may be applicable to other triazole fungicides.

Supporting information may be found in the online version of this article.

Keywords: *Aspergillus fumigatus*; mefentrifluconazole; triazole resistance; chirality; efflux pump genes

1 INTRODUCTION

Mefentrifluconazole, [(2*RS*)-2-(4-(4-chlorophenoxy)-α,α,α-trifluoro-o-tolyl)-1-(1H-1,2,4-triazol-1-yl)propan-2-ol, is a novel chiral triazole fungicide launched at 2019 by BASF. It has been applied in the United States, Australia, the European Union, Canada, Korea and China, and shows excellent bioactivity for controlling many fungal diseases, such as powdery mildew, leaf spot, anthracnose, sheath blight, brown rot, gray mold, rust, and downy mildew, on over 60 crops.[1] Mefentrifluconazole has a unique isopropanol molecular structure that contributes to a high biological efficacy and also can help to control shifted strains, such as *Zymoseptoria tritici*.[2] A previous study regarding usage safety has demonstrated that mefentrifluconazole has low human CYP19 inhibition, and no embryofetal toxicity or teratogenicity in rats or rabbits.[3] However, broad application of triazole fungicide in agriculture may result in unexpected modification of soil microbe. For example, Hart and Brooks illustrated that the soil fungal ergosterol content was decreased by about 30% after 7 days of incubation in the soil samples treated with epoxiconazole at a concentration of 0.25 mg kg^{-1}.[4] The half-life of mefentrifluconazole in soil is about 100–200 days, which may cause long-term residue and thus may have negative effects on soil microorganisms.[5]

Aspergillus fumigatus is a ubiquitous saprophytic fungus in soil and the major opportunistic fungal pathogen that causes invasive aspergillosis (IA) in immunocompromised patients.[6,7] To date, azoles including itraconazole (ITZ), voriconazole (VRC) and posaconazole (POS), are recommended for prophylaxis and treatment of aspergillosis.[8] Since the first emergence of azole-resistant isolate in 1997, the triazole resistance in *A. fumigatus* has been reported increasingly worldwide.[9,10] For the time being, mounting evidences have demonstrated that

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the emergence of triazole resistant *A. fumigatus* could be selected by the application of triazole fungicide, such as tebuconazole, difenoconazole and propiconazole.[11,12] Although *A. fumigatus* is not a target for triazole fungicides, triazole resistance in *A. fumigatus* in soil might be selected by residual triazole fungicides because one important hypothesis regarding the development of triazole resistance in *A. fumigatus* in environment is that azole fungicides and triazole medicines share the same mode-of-action with similar molecular structure that acting as 14α-demethylase (CYP51) inhibitors to prevent ergosterol biosynthesis and then inhibit the growth of fungus.[13] Thus, residual mefentrifluconazole resulting from its wide application in agriculture may drive the development of the resistance in *A. fumigatus* triazole medicines.

Therefore, this study was aimed to explore whether the triazole resistance in *A. fumigatus* could be selected by mefentrifluconazole. In addition, considering that mefentrifluconazole is a chiral fungicide, we wanted to determine whether there is one enantiomer of mefentrifluconazole with high potency against phytopathogens but low risk in the development of triazole resistance in *A. fumigatus*. The results will be helpful for risk assessment of mefentrifluconazole in agriculture.

2 METHODS AND MATERIALS

2.1 Chemical and materials

The rac-mefentrifluconazole (≥95%) was purchased from Bioberry Inc. (Dover, DE, USA). The two mefentrifluconazole enantiomers (≥98.5%) were prepared by Chiralway Biotech Co., Ltd. (Shanghai, China). The absolute configuration and optical rotation of two enantiomers of mefentrifluconazole was verified in our previous study.[14] Voriconazole (VRC) (>99.9%) and itraconazole (ITZ) (>98%) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Posaconazole (POS) (>99.9%) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Hygromycin B and chloramphenicol were purchased from Sangon Biotech (Shanghai, China). Other chemicals used in this experiment were all of analytical grade.

Surface soil samples (0–10 cm) were collected from Huajiachi campus at Zhejiang University, Hangzhou, China. This location had never been treated with mefentrifluconazole. The physical and chemical properties of the soil samples were as follows: organic matter content (OMC), 3.13%; total N, 1.49%; cation exchange capacity (CEC), 3.3 cmol kg^{-1}; sand, 37.2%; silt, 15.2%; clay, 47.7%; and pH, 5.14. The soil was air-dried and passed through a 2-mm sieve before the experiment.

One susceptible strain (S17) of *A. fumigatus* was labeled with hygromycin gene. The pBC-Hygro plasmid (GeneBio Biotech, Hangzhou, China) was first linearized by *Hin*dIII enzyme and then electroporated into the recipient S17 as described by Sánchez and Aguirre.[15] The transformant (WX) was selected with a potato dextrose agar (PDA) plate supplemented with 100 mg L^{-1} hygromycin B and verified by amplifying the hygromycin gene (Supporting Information, Fig. S1). WX was sensitive to ITZ, VRC and POS with minimum inhibitory concentrations (MICs) of 0.125 (0.125), 0.25 (0.125–0.25) and 0.03125 (<0.03125–0.03125) mg L^{-1}, respectively. Meanwhile, the MICs of mefentrifluconazole, R-mefentrifluconazole and S-mefentrifluconazole to WX were tested to be, 2 mg L^{-1} (2 mg L^{-1}), 1 mg L^{-1} (1–2 mg L^{-1}) and 8 mg L^{-1} (8–16 mg L^{-1}), respectively. The MICs of all tested substances to S17 were the same as the MIC of WX, indicating that the transformation did not have impact on the susceptibility of *A. fumigatus*.

2.2 Selection of triazole resistance in *A. fumigatus* in liquid media by mefentrifluconazole

Mefentrifluconazole racemate and two enantiomers of mefentrifluconazole were dissolved in acetonitrile. The solution of all three tested substances were diluted with Sabouraud's dextrose broth medium (SDBM) in 10-mL glass vessels aseptically and sequentially. The concentration of acetonitrile in SDBM did not exceed 0.1%. The strain of *A. fumigatus* (WX) was cultured on PDA for 5 days at 35 °C, and the conidia were collected in 0.85% sodium chloride (NaCl) solution. Half a milliliter of spore suspension was added into 1.5 mL SDBM fortified with one of the tested substances. The final concentration of spore in all vessels containing SDBM was 10^7 colony forming units (CFU) per mL. The final concentrations were 0.2, 0.4, 1.0, 2.0 and 4.0 mg L^{-1} for mefentrifluconazole racemate, and 0.1, 0.2, 0.5, 1.0 and 2.0 mg L^{-1} for each of two enantiomers of mefentrifluconazole. The mixtures were incubated in the dark at 37 °C for 7 days. Afterwards, 0.5 mL of the mixture was transferred to 1.5 mL fresh SDBM supplemented with each tested substances at the same concentration described above and incubated for another 7 days at 37 °C in the dark. The same process was repeated ten times with once a week (70 days in total). A group amended with the same amount of acetonitrile was served as the control. Each treatment was conducted in quintuplicate.

2.3 Resistant isolate screen and susceptibility testing of *A. fumigatus*

After 10 weeks exposure, 100 μL of suspension in each tube was spread onto PDA plate containing hygromycin B (100 mg L^{-1}) together with ITZ (2 mg L^{-1}), VRC (2 mg L^{-1}) or POS (0.5 mg L^{-1}), respectively, and cultured at 37 °C for 5 days to screen for resistant isolate. The visible fungal colonies were selected and cultured at PDA plate for another 5 days. The susceptibility of *A. fumigatus* isolate was performed according to the EUCAST E. DEF 9.3.2 method.[16] The VRC, ITZ, POS, mefentrifluconazole and mefentrifluconazole enantiomer solutions were serially diluted in a 96-well microtiter plate (Corning Incorporated, Corning, NY, USA) using RPMI 1640 media (Sigma Aldrich) supplemented with 2% glucose. The tested concentration for the MIC determination of ITZ, VRC, mefentrifluconazole and mefentrifluconazole enantiomers ranged from 0.125 to 16 mg L^{-1}, and the concentration of POS ranged from 0.03125 to 4 mg L^{-1} at 1:2 dilutions. *A. fumigatus* ATCC 204305 was used as quality control. The MIC was defined as the antifungal concentration at which visual growth was completely inhibited after 48 h incubation at 37 °C. The susceptibility test was conducted in triplicate for each strain. The resistance of *A. fumigatus* against triazole drugs is defined as MIC > 2 mg L^{-1} for VRC and ITZ, and MIC > 0.25 mg L^{-1} for POS.[7]

2.4 Influence of mefentrifluconazole on triazole resistance in *A. fumigatus* in soil

The standard solution of mefentrifluconazole racemate and its two enantiomers were diluted with acetone. Subsamples of sieved soil (100 g) were mixed with the desired amounts of tested substances, and equivalent 400 g of unamended soil was added into spiked soil after evaporation of the solvent to form the concentration of 0.4, 2.0 and 4.0 mg kg^{-1} for mefentrifluconazole racemate, and 0.2, 1.0 and 2.0 mg kg^{-1} for two enantiomers,

respectively. According to the documentation for plant protection containing mefentrifluconazole (China pesticide information network, http://www.chinapesticide.org.cn/), the real application doses are 90–150 g ha^{-1} and mefentrifluconazole would be applied no more than three times for a growing season of crops such as cucumber, potato and peanut. The maximum application dose of mefentrifluconazole is calculated to be 0.4 mg kg^{-1} (recommendation rate), and five- or ten-fold dosage was set for simulating the worst scenario of pesticide residue in the field.[14] Soil treated with the same amount acetone was set as control. Subsequently, the treated soil was added with conidia solution (WX) and fully stirred thoroughly to achieve a final spore density of 10^7 CFU g^{-1}. The treated soil was transferred into a plastic basin (160 × 120 × 100 mm). Soil moisture content was adjusted to 60% of the maximum water-holding capacity with distilled water. The perforated aluminum foil was sealed on the plastic basin to guarantee sufficient ventilation. The plastic pots were placed in an incubator and cultivated at 30 ± 1 °C in the dark. Distilled water was added during the whole experiment if necessary. After different exposure periods (0, 14, 28, 42, 54, 70, 84 and 112 days), approximately 25 g of soil sample was taken for the isolation of triazole resistant *A. fumigatus* and the determination of mefentrifluconazole and its enantiomers. Each treatment was performed in triplicate.

2.5 Isolation and identification of triazole-resistant *A. fumigatus* from soil

Approximately 5 g soil was suspended in a 50-mL Erlenmeyer flask containing 30 mL aseptic 0.85% NaCl solution and shaken on a horizontal shaker (150 rpm) at 37 °C for 2 h. Then 100 μL of the suspension was spread onto a PDA plate containing 100 mg L^{-1} hygromycin B, 100 mg L^{-1} chloramphenicol or 2 mg L^{-1} itraconazole. Each treatment was distributed onto three plates, sealed and placed in a 37 °C incubator for 120 h. When visible colony was observed on the plate, it was transferred onto a PDA plate for purification. The identification of *A. fumigatus* was conducted by their microscopic and morphological characteristics together with hygromycin gene amplification and microsatellite analysis.[17,18] The MICs of purified isolates to medical triazole drugs and mefentrifluconazole and mefentrifluconazole enantiomers were determined according to the methods described above.

2.6 Hereditary stability of triazole-resistant *A. fumigatus*

For the determination of the stability of obtained triazole resistance in *A. fumigatus*, each strain was inoculated onto a PDA plate without mefentrifluconazole or its enantiomers. Each strain was cultured at 37 °C in the dark, and transferred onto another blank PDA plate (without any tested substances) every 5 days. The MIC values were measured each five transfers to determine whether the acquired triazole resistance of each strain was retained.

2.7 *cyp51A* gene sequence of *A. fumigatus*

Genomic DNA of triazole-resistant *A. fumigatus* was extracted by a Column Fungi Genomic DNA Purification Kit (Sangon Biotech) according to the manufacturer's instructions, and then *cyp51A* gene of each strain was amplified by the primers of A7 (5′-TCATATGTTGCTCAGCGG-3′) and P450-A2 (5′-CTGTCTCACTTGGATGTG-3′).[11] The sequence was compared with that from the sensitive *A. fumigatus* (GenBank accession no. AF338659) using DNAMAN (version 9.0) software.

2.8 Expression of *cyp51* genes and drug efflux transporter

The total RNA of triazole-resistant *A. fumigatus* was extracted by Spin Column Fungal Total RNA Purification Kit (Sangon Biotech). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted to quantify the expression of five efflux pump genes (*AtrF, AfuMDR1, AfuMDR2, AfuMDR3, AfuMDR4*), *cyp51A* and *cyp51B* following the method of Cui *et al.* (2019).[17] Glycerol-3-phosphate dehydrogenase (GAPDH) was set as an internal control. The expression of *cdr1B* was performed according to method described by Fraczek *et al.*[19] Expression levels of each gene were measured in triplicate for each strain, and the results were analyzed according to the method described by Livak and Schmittgen.[20]

2.9 Determination of mefentrifluconazole in soil

The extraction procedure for rac-mefentrifluconazole and its enantiomers was conducted according to the method described in our previous study with a little modification.[14] Five grams of soil (dry weight equivalent) were thoroughly mixed with 10 mL acetonitrile and 10 mL distilled water in a 50-mL polypropylene tube. After that, 1 g NaCl and 4 g anhydrous $MgSO_4$ were added to the tube, shaken for 5 min and ultrasonicated for 20 min followed by centrifugation at 6000 rpm for 5 min. For the extract of the soil collected from mefentrifluconazole at a concentration of 0.4 mg kg^{-1} and the two enantiomers at a concentration of 0.2 mg kg^{-1}, 8 mL of the acetonitrile layer was concentrated into 1 mL (re-dissolved with acetonitrile) under nitrogen (N_2) gas. The supernatant was transferred to a 2-mL centrifuge tube containing 50 mg of Primary Secondary Amine (PSA) and 150 mg $MgSO_4$. After vortexing for 30 s, the tube was centrifuged and the supernatant was filtered for high performance liquid chromatography (HPLC) analysis.

The detection of mefentrifluconazole and its enantiomers was performed using an Agilent 1260 series HPLC system (Agilent Technologies Co. Ltd, Beijing, China) equipped with a diode array detector (DAD). The column used was a Superchiral R-AG column (150 mm × 4.6 mm, 3 μm) (Shanghai Chiralway Biotech Co., Ltd., Shanghai, China). The mobile phase was acetonitrile/water/formic acid (65:35:0.01, v/v/v) with a flow rate of 1 mL min^{-1}. The column temperature was kept at 30 °C and the detection wavelength was 240 nm.

2.10 Statistical analysis

All of the statistical analysis was conducted with the software SPSS 22.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) with Student's *t*-test was used to assess the significant differences between isolated strains and the initial strain (WX), and $P < 0.05$ was considered to be significant. The MIC range of three repetitions is shown in parentheses next to the final MIC value of the *A. fumigatus* strain in section 3.1 and section 3.2.

3 RESULTS

3.1 Triazole resistance in *A. fumigatus* exposed to mefentrifluconazole in liquid medium

This experiment was set to determine whether the cross-resistance in *A. fumigatus* could be generated by rac-mefentrifluconazole or its enantiomers under continuous and stable selecting pressure in liquid medium. MIC values of the triazole medicals against the isolated strains of *A. fumigatus* are listed in Table 1. The microsatellite data of these strains were identical to that of the initial strains

Table 1. Susceptibility of *A. fumigatus* to triazole drugs (MIC) after selection with mefentrifluconazole racemate and its enantiomers in liquid medium

Test substance	Concentration (mg L^{-1})	Strain no.	MIC after induction		
			ITZ	VRC	POS
Rac-M	0.4	Rac-0.4-1	2	0.25	0.0625
	1	Rac-1-1	2	0.5	0.0625
	2	Rac-2-1	2	0.5	0.125
		Rac-2-2	>16	2	0.125
		Rac-2-3	>16	2	0.0625
	4	Rac-4-1	2	1	0.25
		Rac-4-2	2	0.5	0.125
		Rac-4-3	4	2	0.5
		Rac-4-4	4	1	0.25
		Rac-4-5	>16	1	0.25
		Rac-4-6	>16	2	0.5
		Rac-4-7	>16	1	0.5
R-M	0.2	R-0.2-1	2	1	0.125
		R-0.2-2	2	0.5	0.0625
	0.5	R-0.5-1	2	0.25	0.0625
	1	R-1-1	>16	1	0.25
		R-1-2	>16	1	0.125
	2	R-2-1	>16	1	0.125
		R-2-2	>16	2	0.25
		R-2-3	>16	2	0.5
		R-2-4	>16	2	0.0625
S-M	2	S-2-1	2	1	0.0625

(WX) (Supporting information, Table S1). It demonstrated that no contamination was observed and that the triazole resistance in these strains was only caused by fungicide exposure.

No resistant strain was screened from the control, indicating acetonitrile and the experimental process did not affect the susceptibility of *A. fumigatus* to medical triazoles. In the case of rac-mefentrifluconazole at 0.4 and 1.0 mg L^{-1} (Rac-M), the screened strains Rac-0.4–1 and Rac-1-1 slightly decreased their sensibility to the ITZ with a MIC of 2 mg L^{-1} (1–2 mg L^{-1}, $P < 0.05$). With the increase of the exposure level, there were two ITZ-resistant strains (Rac-2-2, Rac-2-3) with a MIC of >16 mg L^{-1} isolated in the case of rac-mefentrifluconazole at 2.0 mg L^{-1} (16- > 16 mg L^{-1}, $P < 0.05$). Furthermore, two strains (Rac-4-3, Rac-4-4) with a MIC of 4 mg L^{-1} (2–4 mg L^{-1}, $P < 0.05$) and three strains (Rac-4-5, Rac-4-6 and Rac-4-7) with a MIC of >16 mg L^{-1} (>16 mg L^{-1}, $P < 0.05$) were moderately and highly resistant to ITZ after exposure to rac-mefentrifluconazole at 4.0 mg L^{-1}, respectively. Among these five strains from the treatment with rac-mefentrifluconazole at 4.0 mg L^{-1}, three screened strains (Rac-4-3, Rac-4-6 and Rac-4-7) also were resistant to POS with a MIC of 0.5 mg L^{-1} (0.25–0.5 mg L^{-1}, $P < 0.05$). These data suggest that rac-mefentrifluconazole may generate triazole resistance in *A. fumigatus*. It is likely that the probability of occurrence of the resistance was dependent on the concentration of rac-mefentrifluconazole that *A. fumigatus* was exposed to.

In the R-(−)-mefentrifluconazole-treated group (R-M), there were three strains (R-0.2-1, R-0.2-2 and R-0.5-1) exhibiting tolerance to ITZ with a MIC of 2 mg L^{-1} (2 mg L^{-1}, $P < 0.05$) after exposure at 0.2 and 0.5 mg L^{-1}. In the treatment with R-(−)-mefentrifluconazole at 1.0 mg L^{-1}, there were two ITZ-resistant strains R-1-1 and R-1-2 with a MIC of >16 mg L^{-1} (16- > 16 mg L^{-1}, $P < 0.05$). ITZ-resistance with a MIC of >16 mg L^{-1} (16- > 16 mg L^{-1}, $P < 0.05$) was observed in four strains R-2-1, R-2-2, R-2-3 and R-2-4 after selection by R-(−)-mefentrifluconazole at a level of 2.0 mg L^{-1}. One of these four strains R-2-3 also was resistant to POS with a MIC of 0.5 mg L^{-1} (0.5 mg L^{-1}, $P < 0.05$). However, there were no resistant strain found in the S-(+)-mefentrifluconazole-treated group (S-M). Meanwhile, MICs of mefentrifluconazole and its enantiomers for these strains were calculated with the data presented in Table S2. These showed that these strains have significantly lower sensitivity to those tested substances than the start strain WX, and that triazole resistance in *A. fumigatus* was only formed after its exposure to rac-mefentrifluconazole and R-(−)-mefentrifluconazole.

3.2 Triazole resistance in *A. fumigatus* exposed to mefentrifluconazole in soil

The dissipation of mefentrifluconazole and its enantiomers in the tested soil as a function of time is illustrated in Fig. 1. Kinetic data for dissipation were subjected to the first-order function with the detailed parameter shown in Table S3. Dissipation rates of rac-mefentrifluconazole were 58.7%, 47.4% and 42.1% at concnetrations of 0.4, 2.0 and 4.0 mg kg^{-1}, with corresponding half-lives of 74.2, 112.9 and 132.3 days, respectively. The dissipation proportions of R-(−)-mefentrifluconazole were 55.4%, 42.7% and 39.9% at concentrations of 0.2, 1.0 and 2.0 mg kg^{-1}, respectively, with corresponding calculated half-lives of 72.8, 120.5 and 140.3 days, respectively. The dissipation rates of S-(+)-mefentrifluconazole were 59.5%, 46.7% and 40.5% at concentrations of 0.2, 1.0 and 2.0 mg kg^{-1}, respectively, with DT_{50} values of 69.7, 124.2 and 131.3 days, respectively. No antipode transformation was observed in the single enantiomer treatment group.

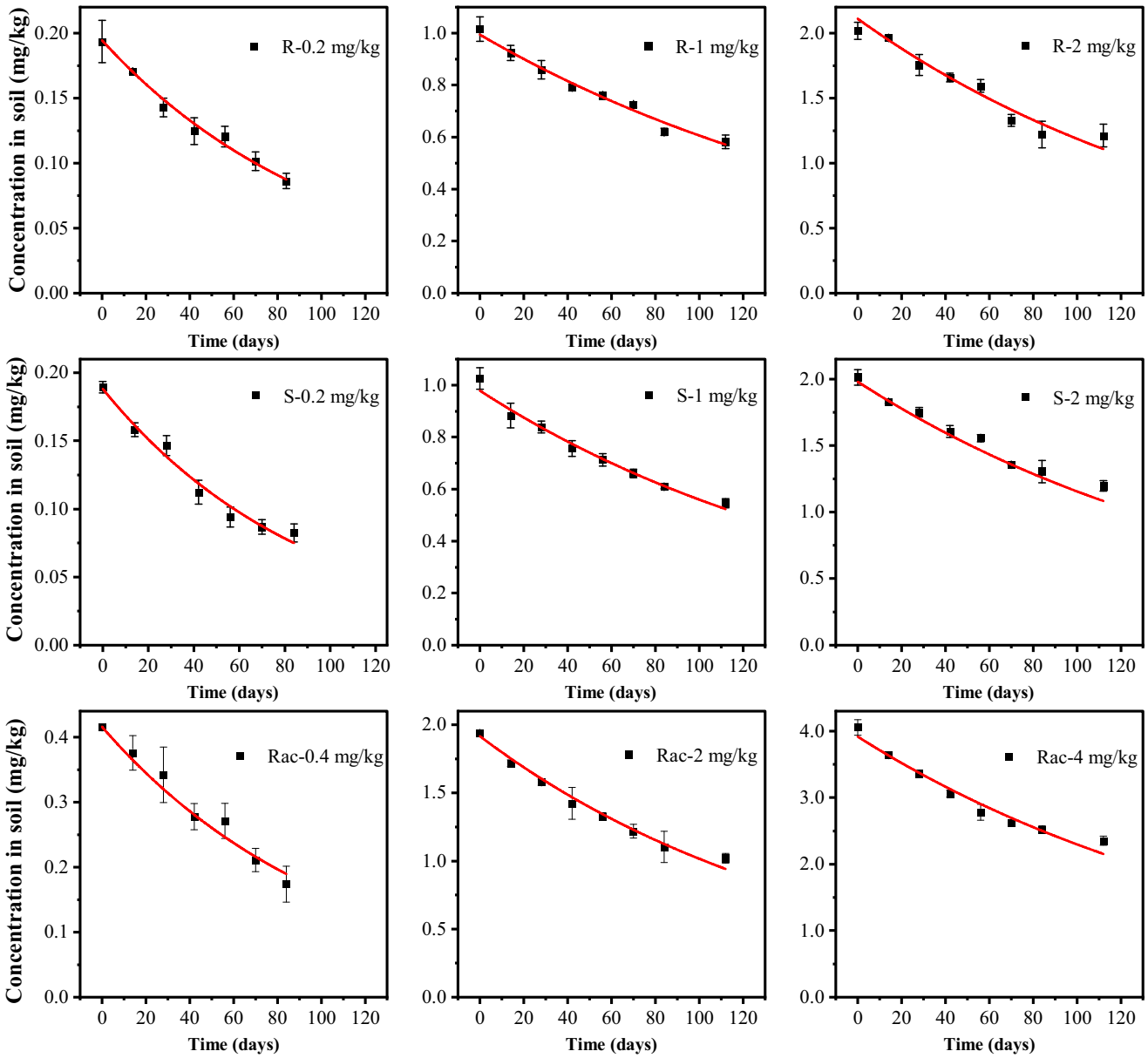

Figure 1. Dissipation of mefentrifluconazole and its enantiomers in the tested soil under different treated concentrations.

All resistant strains isolated from soil are listed in Table 2. No resistant strain was isolated in the control soil without test substance throughout the experiment. A total of 18 strains of WX were screened from the rac-mefentrifluconazole-treated soil samples with corresponding MICs shown in Table S4. No resistant strain was isolated in the case of rac-mefentrifluconazole at 0.4 mg kg^{-1}. In the treatment of rac-mefentrifluconazole at 2.0 mg kg^{-1} (Soil-Rac-2), a strain Rac-6 displaying resistance to ITZ with a MIC of 8 mg L^{-1} (8 mg L^{-1}, $P < 0.05$) was isolated 56 days post-treatment. Higher resistance to ITZ with a MIC of >16 mg L^{-1} (16- > 16 mg L^{-1}, $P < 0.05$) was observed in the strain Rac-10 isolated on Day (D)84. As the concentration increased to 4 mg kg^{-1} (Soil-Rac-4), more resistant strains were isolated from the soil. There were three resistant strains (Rac-14, Rac-16 and Rac-18) screened on D56, D70 and D84, respectively.

In the R-(−)-mefentrifluconazole-treated soil, a total of 14 strains were isolated (Table S5). No resistant strain was observed in the R-(−)-mefentrifluconazole treatment at level of 0.2 mg kg^{-1}. One ITZ-resistant strain R-3 with a MIC of >16 mg L^{-1} (16- > 16 mg L^{-1}, $P < 0.05$) was isolated in the case of R-(−)-mefentrifluconazole at 1.0 mg L^{-1} after 70 days of exposure (Soil-R-1). In the 2 mg kg^{-1} treatment group (Soil-R-2), two triazole-resistant strains (R-9 and R-13) were isolated. The strain R-9 collected on D42 was moderately resistant against ITZ with a MIC of 4 mg L^{-1} (2–4 mg L^{-1}, $P < 0.05$), whereas the other one R-13 harvested on D84 showed high resistance to ITZ with a MIC of >16 mg L^{-1} (>16 mg L^{-1}, $P < 0.05$) and resistance to POS with a MIC of 0.5 mg L^{-1} (0.25–0.5 mg L^{-1}, $P < 0.05$). However, there was no resistant strain isolated from the soil treated with S-(+)-mefentrifluconazole (Table S6). Besides, the MICs of rac-mefentrifluconazole and its enantiomers against triazole resistant strains isolated from soil are presented in Table S7, indicating that resistance of these strains to those tested compounds was increased.

Table 2. MICs of ITZ, VRC and POS against triazole resistant strains isolated from soil under the treatment of rac-mefentrifluconazole and its enantiomers

Treatment	Time (days)	Strain no.	MIC after induction		
			ITZ	VRC	POS
Soil-Rac-4	56	Rac-14	16	1	0.25
	70	Rac-16	>16	1	0.25
	84	Rac-18	>16	2	0.125
Soil-Rac-2	56	Rac-6	8	2	0.25
	84	Rac-10	>16	2	0.25
Soil-R-2	42	R-9	4	1	0.125
	84	R-13	>16	2	0.5
Soil-R-1	70	R-3	>16	1	0.25

3.3 Stability of triazole resistance in *A. fumigatus*

In order to verify the hereditary stability of resistant *A. fumigatus* evolved by mefentrifluconazole, all resistant strains isolated from liquid medium and soil (21 in total) were inoculated onto the blank PDA plate without any tested substance and serially transferred every 5 days for 15 times. The MIC changes of ITZ, VRC and POS of these strains after every five transfers are shown in Tables S8 and S9. Six of them had hereditary stability for resistance to ITZ and were further investigated with respect to *cyp51* and transporter overexpression (Table 3). Sensitivity to triazole medical was recovered by 15 transfers for other strains.

3.4 Resistance mechanisms of the strains with stable resistance

In order to find the resistance mechanism involved in these six strains with stable resistance, the *cyp51A* gene and promoter regions were amplified and sequenced to find out the possible amino-acid substitutions or repeated fragments. No difference in *cyp51A* and its promoter was found between the selected resistant strains and start strain (WX). Hence, expression levels of the drug efflux transporter and *cyp51* genes were assessed by qRT-PCR with data presented in Table 3. The expression leveld of *cyp51A* in six resistant strains were 2.46–7.51-fold ($P < 0.05$) higher than that in the start strain WX. For the efflux pump gene, the expression levels of *AtrF* in these six strains were significantly increased 2.05–5.73-fold that in strain WX. The level of *AfuMDR1* in these six strains were significantly upregulated by 2.21–6.79-fold compared to that in control. Meanwhile, the expression level of *AfuMDR4* in strains Rac-4-7 and R-13 were 4.97- and 3.92-fold higher than that in the parental strain WX, respectively ($P < 0.05$).

4 DISCUSSION

Triazole-resistant *A. fumigatus* has been isolated not only from IA patients treated with triazole drugs, but also in environmental samples such as soil, air, plant seeds, leaves, water and compost.[21] Chen *et al*. have found that 10.2% *A.fumigatus* (206 isolates in total) was triazole resistant isolated from agricultural farms located in 8 cities of China.[22] Up to now, researchers have established a possible link between the application of triazole fungicide in agriculture and the development of triazole-resistant *A. fumigatus*.[23] Nevertheless, there has been a long-running debate about the environmental route for the evolvement of triazole resistance in *A. fumigatus*.[24–26] Specifically, there are rising concerns that the exposure to triazole fungicide is an implausible route for the development of triazole resistance in *A. fumigatus* in agricultural soil, due to the significantly lower concentration in agricultural soil than that in the blood serum (approximately 11 mg L^{-1}) of patients receiving daily oral treatment for aspergillosis.[27] In this study, we found that exposure to 2 mg L^{-1} rac-mefentrifluconazole in liquid medium can result in the emergence of triazole resistance in *A. fumigatus* with significantly increased MICs to ITZ ($P < 0.05$). Likewise, a previous study found that triazole resistance could be observed after exposure to difenoconazole at a level of 0.5 mg L^{-1} in liquid medium.[28] In addition, the emergence of triazole-resistant *A. fumigatus* can be observed in soil supplemented with rac-mefentrifluconazole at concentrations of 2 and 4 mg kg^{-1}, whereas no resistant strain was found in cases of less than 2 mg kg^{-1}. It seems that the emergence of resistance in *A. fumigatus* exposed to the fungicide relies upon the exposure level. Cao *et al*. found that the prevalence of resistant *A. fumigatus* is positively ($P < 0.0001$) correlated with the total concentration of azole fungicides in soils.[29]

Table 3. *Cyp51* and efflux transporter gene expression levels in the resistant isolates by qRT-PCR

Strain no.	*cyp51A*	*cyp51B*	*AtrF*	*cdr1B*	*AfuMDR1*	*AfuMDR2*	*AfuMDR3*	*AfuMDR4*
CK	1.00 ± 0.01	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.01
Rac-2-3	2.46 ± 0.07	1.47 ± 0.12	2.05 ± 0.14	1.80 ± 0.07	6.37 ± 0.26	0.84 ± 0.03	1.34 ± 0.11	1.61 ± 0.05
Rac-4-7	3.49 ± 0.19	0.95 ± 0.14	3.75 ± 0.09	0.92 ± 0.05	5.04 ± 0.47	1.25 ± 0.07	0.81 ± 0.04	4.97 ± 0.38
R-2-1	3.86 ± 0.37	1.17 ± 0.11	3.96 ± 0.32	1.46 ± 0.02	2.35 ± 0.05	0.82 ± 0.01	1.41 ± 0.16	1.57 ± 0.08
R-2-3	7.51 ± 0.69	1.26 ± 0.17	2.24 ± 0.04	0.91 ± 0.06	4.25 ± 0.04	1.30 ± 0.14	1.41 ± 0.04	1.17 ± 0.03
Rac-10	3.17 ± 0.14	0.91 ± 0.08	5.09 ± 0.27	1.06 ± 0.03	2.21 ± 0.09	0.70 ± 0.03	1.43 ± 0.13	1.04 ± 0.05
R-13	6.16 ± 0.89	1.32 ± 0.11	5.73 ± 0.45	1.31 ± 0.01	6.79 ± 0.17	1.87 ± 0.09	1.24 ± 0.12	3.92 ± 0.55

Genetic stability test revealed that the triazole resistance in a total of 15 strains of *A. fumigatus* cannot be maintained (Tables S6 and S7). Recovered susceptibility to triazole medicines in these strains could be observed in the absence of mefentrifluconazole. This indicates that the emergence of triazole resistance is a result of the adaptation of environmental stress in these strains and can be reversible. Likewise, Faria-Ramos *et al*. reported that the MIC of the triazole-resistant strain LMF11 selected by prochloraz against VRC decreased from 16 to 2 mg L^{-1} after 30 days subcultured in the absence of prochloraz.[30] Harish *et al*. also reported that *A. fumigatus* Af293 exposed to sublethal levels of tebuconazole generated conidia that had transiently increased germination and growth against VRC, and this determinant was disappeared after one passage on drug-free plates.[31] In terms of the triazole resistance mechanism, mutations in the *cyp51A* gene have been regarded as an important factor in the development of triazole resistance in *A. fumigatus*.[32] No mutation was identified in *cyp51A* of six resistant strains with hereditary stability in this study. Likewise, Bueid *et al*. have reported that 43% of resistant strains of *A. fumigatus* submitted to the Mycology Reference Centre Manchester in 2008 and 2009 did not carry a *cyp51A* mutation.[33] Over-expression of *cyp51A* gene in these six resistant strains in comparison with the control (by 2.46–7.51-fold) was observed (Table 3). A previous study indicated that triazole resistance in *A. fumigatus* without point mutation in *cyp51A* was associated with increased *cyp51A* gene expression.[34] A reduced intracellular concentration of triazole medicals has been identified as another direct cause of resistance, which is related to the over-expression of genes encoding membrane transporters (*AfuMDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4* and *AtrF*).[19,35] In the present study, we found that there were significant upregulations by several-fold in *AtrF* and *AfuMDR1* of all six resistant strains (Table 3). Significant over-expression of the *AfuMDR4* gene was observed for two isolates (Rac-4-7 and R-13). This result implied that the mechanism of the acquired resistance in *A. fumigatus* generated by mefentrifluconazole could rely on the over-expression of *cyp51A* gene and efflux transporter genes (*AtrF, AfuMRR1* and *AfuMDR4*). Likewise, Wang *et al*. found the expression levels of *cyp51A*, *AfuMDR1* and *AfuMDR4* in a triazole-resistant strain without point mutation in *cyp51A* generated by difenoconazole and propiconazole were 4.93-, 5.01- and 2.91-fold higher than that of the parental strain, respectively.[28] Moazeni *et al*. reported that the overexpression of *cyp51A, AtrF, AfuMDR2* and *AfuMDR4* genes were observed in resistant *A. fumigatus* without azole-resistance-related mutations.[36]

Interestingly, the acquired triazole resistance in *A. fumigatus* generated by mefentrifluconazole was found to be enantioselective and concentration-dependent. After exposure to rac-mefentrifluconazole in liquid medium at levels of 2 and 4 mg L^{-1} for 70 days, we found seven strains with moderate-to-high resistance to ITZ. In the case of R-(−)-mefentrifluconazole at concentrations of 1 and 2 mg L^{-1}, there were six strains identified to be highly resistant to ITZ. Likewise, in the soil experiment, there were five and three resistant strains isolated from the treatments of rac-mefentrifluconazole and R-(−)-mefentrifluconazole, respectively. However, no resistant strain was identified in the case of S-(+)-mefentrifluconazole in liquid medium or soil. These demonstrated that triazole-resistant *A. fumigatus* only emerged in the treatment with rac-mefentrifluconazole and its R-isomer at the tested concentrations. This is mainly ascribed to the different activity of mefentrifluconazole and its two enantiomers against *A. fumigatus*. We found that the R-enantiomer of mefentrifluconazole had higher activity against *A. fumigatus* than the S-isomer. In our previous study, we found that the R-enantiomer of mefentrifluconazole exhibited higher bioactivity than the S-enantiomer against four plant pathogens (*Botryitis cinerea*, *Rhizoctonia solani*, *Alternaria solani* and *Colletotrichum gloeosporioides*).[14] A previous study demonstrated that R-(−)-mefentrifluconazole had a stronger binding ability to CYP51 in *B. cinerea* than its antipode through homology modeling and docking.[37] Meanwhile, it is worth noting that we also conducted a MIC test for the ATCC strain, and the MICs of mefentrifluconazole, R-mefentrifluconazole and S-mefentrifluconazole to the ATCC 204305 strain were 16, 8 and >16 mg L^{-1}, respectively. This result was in accordance with the result in the paper of Jorgensen *et al*.[38] However, the MIC of mefentrifluconazole against WX (MIC = 2) and acquired resistant strains (MIC = 4– >16 mg L^{-1}) was below the range of MIC results of sensitive strains and resistant strains in the paper of Jorgensen *et al*. (MIC >32).[38] In fact, genetic and phenotypic diversity of *A. fumigatus* is abundant in the environment.[18,39] Faria-Ramos *et al*. reported that the MIC of strain LMF05 against prochloraz was 0.125, which was below the MIC results (0.25–0.5 mg L^{-1}) in that paper.[30] Cui *et al*. reported that MIC of strain S5 against tebuconazole was 0.5 mg L^{-1}, which was below the MIC results (2–4 mg L^{-1}) in that paper.[17] Cao *et al*. found that azole-resistant strains collected from an environment have significant geographical and genetic differences.[29] We believe the results reported in our manuscript may provide some reference to the triazole resistance monitoring in the agricultural environment. Thus, combined with the results given in this study, it is suggested that the R-enantiomer of mefentrifluconazole performed higher selective pressure than the S-isomer leading to the emergence of the resistance in *A. fumigatus*.

5 CONCLUSION

In conclusion, the obtained data in this study demonstrated that acquired resistance of *A. fumigatus* against triazole medicals can be generated by mefentrifluconazole in liquid medium and soil. The fungicidal inconsistency between the enantiomers of mefentrifluconazole would result in the difference of the emergence of triazole resistance in *A. fumigatus*. It is very likely that R-mefentrifluconazole, which has higher activity against plant pathogens, has a better chance in generating the triazole resistance in *A. fumigatus* than its S-isomer. It is noteworthy that no triazole resistance was acquired in *A. fumigatus* exposed to mefentrifluconazole at a concentration up to the calculated maximum residues (0.4 mg kg^{-1}) in soil as published in the official document for plant protection in China. Our results suggest that mefentrifluconazole should be applied according to the label (not higher), for avoiding the emergence of triazole resistance in *A. fumigatus* in practical agriculture: overdosing must be strictly avoided. This dictum also may be applicable to other triazole fungicides.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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