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Aspergillus fumigatus Cross-Resistance between Clinical and Demethylase Inhibitor Azole Drugs

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ABSTRACT Drug resistance poses a serious threat to human health and agricultural production. Azole drugs are the largest group of 14-α sterol demethylation inhibitor fungicides that are used both in agriculture and in clinical practice. As plant-pathogenic molds share their natural environment with fungi that cause opportunistic infections in humans, both are exposed to a strong and persistent pressure of demethylase inhibitor (DMI) fungicides, including imidazole and triazole drugs. As a result, a loss of efficacy has occurred for this drug class in several species. In the clinical setting, *Aspergillus fumigatus* azole resistance is a growing public health problem, and finding the source of this resistance has gained much attention. It is urgent to determine if there is a direct link between the agricultural use of azole compounds and the different *A. fumigatus* resistance mechanisms described for clinical triazoles. In this study, we performed *A. fumigatus* susceptibility testing against clinical triazoles and crop protection DMIs using a collection of azole-susceptible and -resistant strains which harbor most of the described azole resistance mechanisms. Various DMI susceptibility profiles have been found in the different *A. fumigatus* population groups based on their azole resistance mechanism and previous whole-genome sequencing (WGS) analysis, which suggests that the different resistance mechanisms have different origins and are specifically associated with the local use of a particular DMI.

IMPORTANCE Due to the worldwide emergence of *A. fumigatus* azole resistance, this opportunistic pathogen poses a serious health threat, and therefore, it has been included in the watch list in the CDC publication *Antibiotic Resistance Threats in the United States, 2019* (CDC, 2019). Azoles play a critical role in the control and management of fungal diseases, not only in the clinical setting but also in agriculture. Thus, azole resistance leads to a limited therapeutic arsenal which reduces the treatment options for aspergillosis patients, increasing their mortality risk. Evidence is needed to understand whether *A. fumigatus* azole resistance is emerging from an agricultural source due to the extended use of demethylase inhibitors as fungicides or whether it is coming from somewhere else, such as the clinical setting. If the environmental route is demonstrated, the current use and management of azole antifungal compounds might be forced to change in the coming years.

KEYWORDS *Aspergillus fumigatus*, azole resistance, azole drugs, DMIs, plant pathogens

Aspergillus fumigatus is responsible for the increased incidence of invasive aspergillosis, with high mortality rates in some immunocompromised hosts (1). In this context, azole drugs play a major role in the prevention and treatment of these infections (2). Generally, these drugs are called demethylation inhibitors (DMIs) and are widely used because of their high efficiency and broad-spectrum activity; in fact, azoles are

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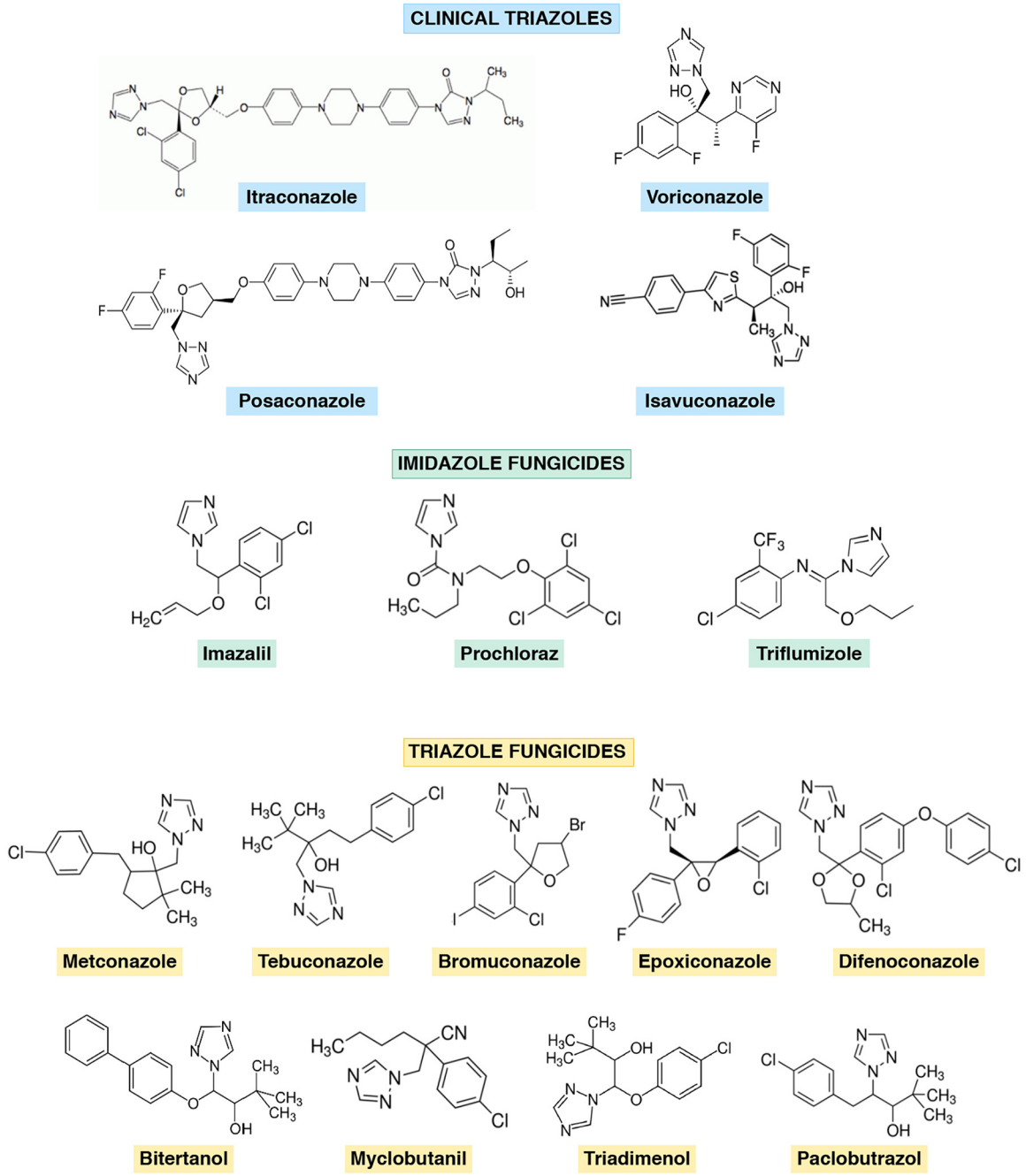

FIG 1 Chemical structures of clinical triazoles and demethylation inhibitor compounds used in this study, grouped as imidazole or triazole fungicides based on the number of nitrogen atoms in the azole aromatic ring.

the only class of compounds that are used in both agriculture and clinical management (3, 4).

Azole drugs have dominated the agricultural fungicide market since they were approved in the 1970s; however, their capacity to induce resistance in the target pathogens is weaker than that of other agricultural fungicides. Chemically, azoles are divided into imidazoles and triazoles (5). Several azole drugs used in crop protection have a molecular structure similar to that of medical triazoles (Fig. 1), and cross-resistance between them has been demonstrated through lab evolution under selective pressure of agricultural azoles (6, 7). In the clinical setting, the introduction of azole drugs initiated a new era in therapy for systemic fungal diseases. Nowadays, the treatment of invasive aspergillosis mainly relies on triazole drugs approved in the late 1990s to

2000s, such as itraconazole (ITZ), voriconazole (VRZ), posaconazole (PSZ), and, more recently, isavuconazole (ISZ) (8).

Along with the increased use of DMI fungicides globally, a rise in the number of *A. fumigatus* azole-resistant isolates has been reported (2). This is especially worrisome due to the critical role that these drugs play in the control and management of fungal diseases. Azole resistance is directly associated with treatment failure; in fact, there is a subset of patients on azole prophylaxis who develop breakthrough aspergillosis that are theoretically untreatable because the use of azole is precluded, which leads to high mortality rates (9). Due to the worldwide emergence of azole resistance, *A. fumigatus* has been included in the watch list in the CDC publication *Antibiotic Resistance Threats in the United States, 2019* (10).

Azole drugs act inhibiting the activity of Cyp51 enzymes, the azole target. Many filamentous fungi, particularly ascomycetes, harbor one, two, or even three *cyp51* paralogous genes encoding these enzymes (11). In *A. fumigatus*, the azole target 14-α sterol demethylase is encoded by two paralogous genes (*cyp51A* and *cyp51B*) (12). In general, *cyp51* mutations resulting in acquired azole resistance are usually restricted to just one paralog, most often *cyp51A*; thus, any cost associated with a change in the protein might be eluded by the other wild-type paralogs with an unchanged enzyme activity (13).

Multiple studies of human and plant pathogens have identified two main mechanisms of azole resistance, which are quite common in both scenarios: (i) mutations in the Cyp51 target resulting in decreased enzyme affinity for inhibitors and (ii) overexpression of the *cyp51* target gene caused by insertions in the predicted promoter regions. Both azole resistance mechanisms can also appear in different Cyp51 combinations resulting in various azole susceptibility profiles (2, 14).

In plant pathogens, the variety of DMIs used for crop protection is high and sometimes the use of various compounds is the rule, which makes it more difficult to link a particular Cyp51 mutation to the specific use of a DMI. In addition, the number of resistance mechanisms and plant pathogens under investigation is quite diverse too (Table 1). However, some Cyp51 point mutations and promoter modifications are consistently found, independently or in combination, in several species of fungi (2, 15–22).

In *A. fumigatus*, the different susceptibility profiles depend on the specific Cyp51A amino acid substitution (Fig. 2). Such is the case of G54 and P216 mutations in the *A. fumigatus* Cyp51A enzyme, responsible for cross-resistance to the long-tailed azole drugs ITZ and PSZ but with unaffected MICs to short-tailed azoles such as VRZ and ISZ (23, 24). Mutation M220 leads to ITZ resistance and variable MIC values to VRZ, PSZ, and ISZ (25), while point mutation G448S yields resistance to VRZ and ISZ and variable MIC values to ITZ and PSZ (26, 27). On the other hand, *A. fumigatus* strains with promoter integrations (tandem repeat [TR]) and *cyp51A* point mutations (TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, TR_{46}/Y121F/T289A, and TR_{53}) normally show a multiazole resistance phenotype (28–30).

Given the similarity among clinical azoles and those used in crop protection, cross-resistance among DMIs and clinical azoles is common. This suggests an association between the azole susceptibility phenotypes and the resistance mechanism shown by both class of fungal pathogens. Moreover, some Cyp51 alterations at equivalent positions in both human and plant pathogens have been found (2).

In this study, a collection of azole-resistant and -susceptible *A. fumigatus* strains were tested against the most commonly used DMIs to analyze whether the susceptibility phenotypes provide enough evidence to ultimately point toward the pathway involved in the *A. fumigatus* environmental source of azole resistance. Different patterns of azole cross-resistance were observed depending on the azole resistance mechanism.

RESULTS AND DISCUSSION

The worldwide emergence of *A. fumigatus* azole-resistant isolates poses a significant threat to the management of these infections (2, 31). The environmental use of azole drugs as agricultural fungicides is believed to be one of the driving forces of the *A. fumigatus* azole resistance emergence, although solid evidence is still lacking (32).

TABLE 1 Main Cyp51 resistance mechanisms to DMIs found in plant pathogens from 2000 to 2020[a]

Plant pathogen	DMI resistance	Cyp51 modification(s)	Promoter alteration	Overexpression	Cyp51 gene	Reference
Penicillium digitatum	TFZ, FNM, BTN	Absent	126-bp TR	Yes	Cyp51	2
	IMZ	Absent	199-bp TR	Yes	Cyp51B	2
Blumeriella jaapii	FBZ	Absent	Truncated retrotransposon	Yes	Cyp51	2
Venturia inaequalis	MCB	Absent	553-bp insertion	Yes	Cyp51A	2
	DFZ	Absent	EL3,1,2 repeated element	Yes	Cyp51A	2
Monilinia fructicola	PPZ	Absent	Mona genetic element	Yes	Cyp51B	2
Ustilaginoidea virens	PPZ	Absent	CC insertion	Yes	Cyp51	2
Pyrenopeziza brassicae	TBZ, MTZ, FSZ, PTZ, PRZ	G460S, S508T	151-bp insertion	Yes	Cyp51	2
Erysiphe necator	MCB, TBZ, FNM	Y136F	ND	Yes	Cyp51B	2
	MCB	Y136F	ND	Yes	Cyp51	2
Puccinia triticina	EPZ	Y134F	ND	Yes	Cyp51B	2
Villosiclava virens	TBZ	Y137H	ND	Yes	Cyp51B	15
Pyrenophora teres	TBZ, MTZ, TRZ, DFZ, PRZ	F489L	ND	Yes	Cyp51A	2
Uncinula necator	TDM	Y136F	ND	No	Cyp51B	2
Erysiphe graminis f. sp. *hordei* (*Blumeria graminis* f. sp. *hordei*)	TDM, TBZ	Y136F, S509T	ND	ND	Cyp51B	2
	BZZ	Y136F	ND	ND	Cyp51B	2
	TDM	Y136F, K147Q	ND	ND	Cyp51	2
Mycosphaerella graminicola (*Zymoseptoria tritici*)	TDM, TBZ, PRZ, TBZ, EPZ	Y137F, I381V, V136A, ΔY459, ΔG460	ND	No	Cyp51	2
	TBZ, DFZ	I381V	ND	ND	Cyp51	2
	TBZ, EPZ	Y461S, Y137F	ND	ND	Cyp51	16
	PTZ, EPZ	S524T	ND	ND	Cyp51	16
Fusarium graminearum	TBZ	Y137H	ND	ND	Cyp51B	17
Penicillium digitatum	PRZ	Y136H, Q309H, G459S, F506I	ND	ND	Cyp51B	18
Ustilago maydis	PPZ	G464S	ND	ND	Cyp51	2
Mycosphaerella fijiensis	TDM, FSZ, PPZ	Y136F, A313G, Y461D, Y463D/N/H	ND	No	Cyp51A	19
Cercospora beticola	TTZ	Absent	Absent	Yes	Cyp51B	20
	EPZ	Absent	Absent	Yes	Cyp51	21
Sclerotinia homoeocarpa	PPZ	Absent	Absent	Yes	Cyp51	22

[a]ND, not determined or not described; IMZ, imazalil; PRZ, prochloraz; TFZ, triflumizole; MTZ, metconazole; TBZ, tebuconazole; EPZ, epoxiconazole; BRZ, bromuconazole; DFZ, difenoconazole; BTN, bitertanol; MCB, myclobutanil; TDM, triadimenol; PPZ, propiconazole; FNM, fenarimol; FBZ, fenbuconazole; FSZ, flusilazole; PTZ, prothioconazole; BZZ, benzimidazole; TTZ, tetraconazole; TRZ, triticonazole.

The *A. fumigatus* strain collection represents a heterogeneous population. Several authors have demonstrated the huge genetic diversity among *A. fumigatus* strains using data from various typing techniques and whole-genome sequencing (WGS) (33–36). All the strains used in this study were identified as *A. fumigatus sensu stricto*. Their azole resistance mechanism was analyzed by PCR amplification and sequencing of the *cyp51A* gene, including its promoter. Since both genetic background and phenotypic features, such as antifungal resistance, may influence susceptibility testing results, the isolates included in this study were distributed in different groups according to their Cyp51A modifications, susceptibility to clinical azole drugs, and WGS cluster based on a previous *A. fumigatus* study performed in our group (33).

A description of each group, resistance mechanism, and number of strains within it is provided in Table 2. The strains used in this work belonged to what we called cluster I, i.e., azole-susceptible *cyp51A* wild-type (WT) strains together with azole-resistant *cyp51A* single-point mutation strains, cluster II, i.e., azole-susceptible and -resistant strains with both *cyp51A* single point mutations combined with TR promoter integrations mechanisms, cluster III, i.e., strains with five particular *cyp51A* modifications (F46Y, M172V, N248T, D255E, and E427K), and cluster IV, i.e., strains with three particular *cyp51A* modifications (F46Y, M172V, and E427K) (33).

Antifungal susceptibility testing (AFST). (i) Clinical azole drugs. Following the European Committee on Antifungal Susceptibility Testing (EUCAST) guidelines (see

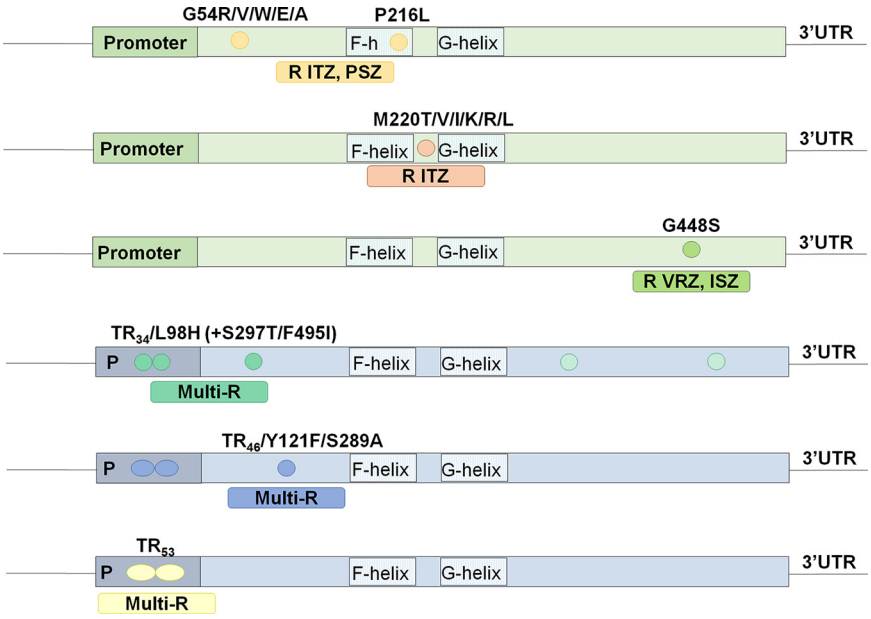

FIG 2 The most common azole resistance mechanisms in *A. fumigatus* and susceptibility profiles to clinical azoles associated with each Cyp51A modification. UTR, untranslated region.

Materials and Methods), the analyzed strains showed a wide range of MIC values to all four clinical antifungals tested—itraconazole (ITZ), voriconazole (VRZ), posaconazole (PSZ), and isavuconazole (ISZ). These differences were based on the specific genetic background (WGS cluster) and azole resistance mechanism. *In vitro* susceptibility testing showed ranges within one or two 2-fold MICs for each strain, which suggests stable and reliable results. However, MIC ranges per group may be broader since several isolates are included in a group. MIC ranges for each clinical azole and group of strains are shown in Table 2. There was no relevant difference in MIC values among the Cyp51A WT strains (from cluster I or II) to the clinical azoles tested. All the *A. fumigatus* azole-resistant strains with G54 mutation were resistant to ITZ and PSZ, while the strains with M220 were resistant to ITZ but variable to VRZ, ISZ, and PSZ. Strains harboring the G448S mutation were resistant to VRZ and ISZ but variable to ITZ and PSZ. Finally, the isolates with the combined resistance mechanism which includes a TR insertion in the *cyp51A* promoter showed a multiazole resistance profile to all clinical azoles tested. No differences in susceptibility to amphotericin B or echinocandin drugs were seen among all the strains tested (see Table S1 in the supplemental material).

(ii) DMIs. Susceptibility testing to eight DMI fungicides used for crop protection, consisting of three imidazole drugs (imazalil [IMZ], prochloraz [PRZ], and triflumizole [TFZ]) and five triazole drugs (metconazole [MTZ], tebuconazole [TBZ], epoxiconazole [EPZ], bromuconazole [BRZ], and difenoconazole [DFZ]), was performed using the *A. fumigatus* strain collection. Again, *in vitro* susceptibility testing showed ranges within one or two 2-fold MICs for each strain. MIC ranges for each DMI and group of strains are shown in Table 2.

There were no remarkable differences in the MIC values to DMI drugs among the isolates that formed the azole-susceptible group (Cyp51A-WT, Cyp51A-3SNPS, and Cyp-5SNPs from clusters I, II, III, and IV), showing that their different genomic backgrounds do not influence their DMI susceptibility profiles (Table 2). However, there were several relevant differences depending on the azole resistance mechanism groups (Table 2 and Fig. 3). In general, most *A. fumigatus* azole-resistant strains showed high MICs to all DMIs tested except for the strains with the Cyp51A-G54 mutation, which exhibited a hypersusceptible phenotype to all the agricultural fungicides tested. Moreover, strains that harbored the resistance mechanisms TR_{46}/Y121F/T289A and TR_{34}/L98H/S297T/

TABLE 2 Ranges of MICs to clinical and agricultural azole antifungals[a]

Cyp51A modification (no. of isolates)	WGS cluster	Range of MICs to clinical azoles (mg/liter)				Range of MICs to agricultural DMIs (mg/liter)							
		ITZ	VRZ	PSZ	ISZ	IMZ	PRZ	TFZ	MTZ	TBZ	EPZ	BRZ	DFZ
AZL-S													
WT (20)	I-II	0.25 to 0.5	0.125 to 0.5	0.06 to 0.125	0.25 to 1	0.125 to 0.5	0.125 to 0.5	8 to 16	0.125 to 0.5	1 to 4	2 to 4	1 to 4	0.5 to 2
5SNPs[b] (6)	III	0.5 to 1	1 to 2	0.125 to 0.5	1	0.25 to 0.5	0.25 to 0.5	16 to >32	0.25 to 1	2 to 8	8 to 16	4 to 16	2 to 8
3SNPs[c] (11)	IV	0.25 to 1	0.5 to 1	0.06 to 0.125	1 to 2	0.125 to 0.25	0.125 to 0.25	8 to 16	0.25 to 1	2 to 4	2 to 4	2 to 8	2 to 4
AZL-R point mutations													
G54 (12)	I-II	>8	0.25 to 0.5	1 to >8	0.25 to 1	0.06 to 0.125	0.125 to 0.25	2 to 4	0.06 to 0.125	0.5 to 2	0.5 to 2	0.5 to 1	0.06 to 0.25
M220 (7)	I-II	>8	0.25 to 1	0.25 to 2	1 to 4	0.25 to 2	0.25 to 1	8 to 32	0.25 to 2	2 to 16	4 to 16	1 to 4	2 to 16
G448S (5)	I-II	1 to 2	>8	0.25 to 1	4 to 8	0.5 to 2	1 to 2	32 to >32	4 to 8	8 to >32	8 to >32	4 to >32	4 to >32
AZL-R TR integrations[d]													
TR_{34}/L98H (12)	II	>8	4 to 8	0.5 to 1	8	1 to 8	2 to 8	>32	1 to 2	16 to 32	>32	8 to 32	16 to >32
TR_{34}/L98H/S297T/F495I (3)	II	>8	4 to 8	0.5 to 1	>8	8	>32	>32	4 to 16	16 to 32	>32	>32	>32
TR_{46}/Y121F/T289A (4)	II	2 to 4	4 to >8	0.5	>8	32 to >32	16 to >32	>32	8 to 16	>32	>32	>32	>32
TR_{53} (3)	II	> 8	2 to 4	0.5 to 1	8	2 to 8	2 to 8	>32	2	16 to 32	>32	32	16 to 32

[a]*A. fumigatus* isolates are grouped based on their azole susceptibility profiles and their Cyp51A modifications. AZL-S, azole susceptible; AZL-R, azole resistant; ITZ, itraconazole; VRZ, voriconazole; PSZ, posaconazole; ISZ, isavuconazole; IMZ, imazalil; PRZ, prochloraz; TFZ, triflumizole; MTZ, metconazole; TBZ, tebuconazole; EPZ, epoxiconazole; BRZ, bromuconazole; DFZ, difenoconazole.

[b]5SNPs, F46Y/M172V/N248T/D255E/E427K.

[c]3SNPs, F46Y/M172V/E427K.

[d]Tandem repeat (TR) integration in the *cyp51A* promoter in combination, or not, with single point mutations.

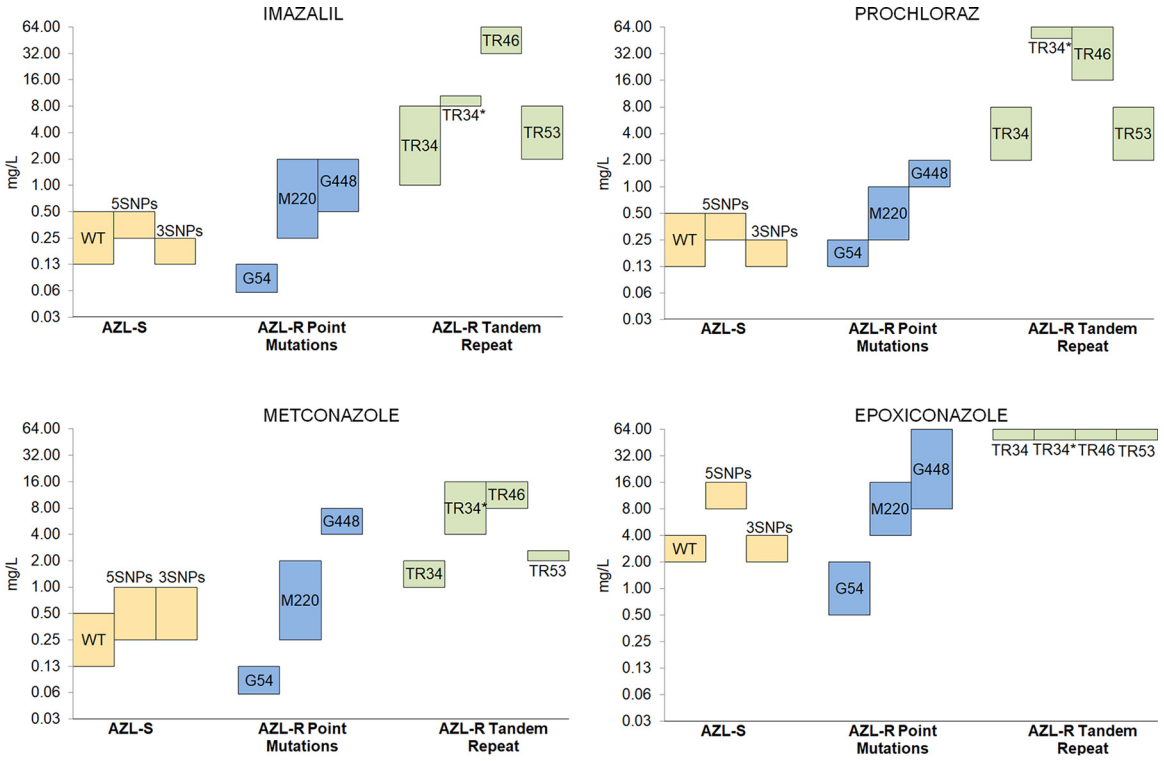

FIG 3 Ranges of MICs to four agricultural azole antifungals. *A. fumigatus* isolates are grouped based on their azole susceptibility profile and their Cyp51A modifications.

F495I were highly resistant to imidazoles, to both IMZ and PRZ and just PRZ, respectively. Strains with the G448S mutation showed a pattern of high resistance to triazole DMIs but not that much to imidazole drugs.

Role of clinical azoles and agriculture DMIs in the emergence and development of *A. fumigatus* azole resistance. It is presumed, but still currently debated, that the development of azole resistance in *A. fumigatus* may be linked either to a medical or patient-acquired route or to an environmental route (9, 14). Although azole resistance is acquired by selection pressure in both cases, it is proposed that as a result, different resistance mechanisms and susceptibility patterns are developed.

Most *A. fumigatus* isolates with *cyp51A* single point mutations—G54, P216, M220, and G448—were isolated from patients who had received long-term azole treatment (26, 37). However, mutations at position G54, and occasionally M220 and P216, have also been reported for strains from an environmental origin (38–40).

It is well known that G54 mutation may emerge after long-term ITZ therapy in patients with chronic aspergillosis or cystic fibrosis (41). However, the fact that it has also been isolated from the environment in very different geographical locations (several European countries, India, China, Tanzania, and Thailand) points to a possible agricultural origin (38–40, 42). The results obtained in this study do not point toward the environmental route to explain this resistance mechanism, as all G54 strains tested are resistant to long-tailed clinical azoles but highly susceptible to agricultural DMIs and short-tailed clinical azoles, such as VRZ and ISZ (Fig. 3 and Table 2). *A. fumigatus* Cyp51A homology model studies have showed that the G54R mutation can prevent long-tailed azoles from entering the channel but not the more compact molecule VRZ (43). In addition, the equivalent Cyp51 mutation has never been identified in plant pathogens related to DMI resistance (Table 1). These strains showed even lower MIC values to the new triazole DMIs tested than the *cyp51A*-WT strains (Table S2). Alternatively, the possibility that G54 *A. fumigatus* azole-resistant isolates may develop during azole therapy within an infected or colonized patient and then spread into the

environment has been proposed (44). The G448S mutation has been shown to confer resistance to VRZ and ISZ, together with elevated MICs to ITZ and PSZ (26). Although to date this mutation has mainly been reported in the clinical setting, the associated high triazole DMI resistance (Table 2) and the recent finding of *A. fumigatus* isolates with environmental origin, which harbor this resistance mechanism (45, 46), would suggest that this mutation could emerge under VRZ selective pressure in the clinical setting or under selective pressure from other DMI triazoles, such as MTZ, in the environment (Fig. 3).

Currently, the more frequent *A. fumigatus* mechanism of azole resistance involves the overexpression of the *cyp51A* gene, sometimes together with point mutations (TR_{34}/L98H, TR_{46}/Y121F/T289A, and TR_{53}) (28–30), and is associated with the environmental route and the extended use of DMI fungicides in crop protection (14). Moreover, strains with these resistance mechanisms have been found in azole-naive patients but also in the environment throughout multiple worldwide locations (32, 47). Since azole fungicides are used on a global scale, several resistance mechanisms have been described to be common between plant pathogens and *A. fumigatus* azole-resistant isolates (Table 1).

In this context, the most common *cyp51* mutation in plant pathogens associated with DMI resistance is the 134/136/137 tyrosine (Y) substitution to phenylalanine (F) or to histidine (H) (Cyp51 amino acid position varies depending on the fungal species) without known alterations in the Cyp51 promoter (Table 1). This mutation would correspond to the Y121F modification commonly found in *A. fumigatus* together with other modifications in the *cyp51A* gene, e.g., TR_{46}/Y121F/T289A (26, 30). Interestingly, the Y121F mutation without TR integration in *A. fumigatus* has been found only in one clinical isolate, but the patient was never exposed to azole drugs. This strongly suggests a resistance of environmental origin and could represent the missing link between the wild-type gene and the TR_{46}/Y121F/T289A resistance mechanism (48). The sole Y121F mutation confers resistance only to VRZ and not to ITZ or PSZ, whereas the TR_{46}/Y121F/T289A mutation is associated with multiazole resistance. High-resolution X-ray crystal structure analysis demonstrated that the Y140F/H mutation in *Saccharomyces cerevisiae* Erg11 disrupted the binding of short-tailed triazoles but not long-tailed ones (49).

The *A. fumigatus* strains which harbor the TR_{46}/Y121F/T289A mutation combination have a pattern of resistance to all DMIs tested but particularly high resistance to imidazole drugs. Apart from *A. fumigatus*, other fungal human pathogens present the equivalent Cyp51*/ERG11* mutations (*Cryptococcus neoformans*, *Histoplasma capsulatum*, *Candida albicans*, and *Candida auris*) (50–53), which lead to resistance to only short-tailed triazoles. Similarly, the sole Y121F mutation in *A. fumigatus* leads just to VRZ resistance (48). This mechanism of resistance commonly found in both plant pathogens and *A. fumigatus* leads to similar activity and therefore might be developed from azole selection pressure in both cases. In *Erysiphe necator*, a strong association between *cyp51* gene copy number variation, which influenced expression in a gene-dose-dependent manner and was correlated with fungal growth in the presence of a DMI fungicide, has been found (54).

Several authors have observed elevated MIC values to the imidazole PRZ among *A. fumigatus* isolates harboring the TR_{34}/L98H/S297T/F495I mutation (55–57). Our results are in agreement with them, as these strains showed a substantially stronger increase in the MIC value to PRZ (range, 8 to 32 mg/liter) than did the strains harboring the TR_{34}/L98H mutation (1 to 8 mg/liter).

It has been described that most of the *A. fumigatus* strains with the TR_{34}/L98H/S297T/F495I mutation are more genetically related than strains with the TR_{34}/L98H mutation, which might be due to an extremely adaptive recombinant event under the selection pressure of imidazole fungicides in some countries (55–58). In one of our previous studies using WGS, the strains with the TR_{34}/L98H/S297T/F495I mutation grouped together in a small subcluster even when their geographical origins were nonrelated, such as in the case of strains from Spain, Denmark, or the Netherlands (data not shown). Moreover, if we compare the agricultural pathogen Cyp51 proteins to the

Cyp51A protein of *A. fumigatus*, the role of these mutations in PRZ resistance has been demonstrated even with structural *in silico* modeling (18). For instance in *Penicillium digitatum*, the F506I mutation arose in combination with a 199-bp insertion in the *cyp51* promoter, showing even higher resemblance to the *A. fumigatus* TR resistance mechanism therefore suggesting a common and environmental evolutionary route (18, 55). Moreover, in this plant pathogen the single F495I mutation is not responsible for the whole increase in the imidazole MIC values, as L98H on its own does not lead to the same MIC values as its combination with the promoter insertion (18, 28). The possibility that the S297T mutation might be required to compensate for the deleterious effect of F495I on the protein function, as T289A does in the case of the TR_{46}/Y121F/T289A mutation, has been previously proposed (59).

In general, resistant strains with TR insertions in the *cyp51A* promoter are grouped together into one cluster based on our previous WGS phylogenetic analysis (33), which indicates genetic closeness independently of the geographic origin. This common genetic background may help them to adapt to the environment or may confer on them improved fitness that favors their selection and spread. Moreover, different TR mutations are emerging in different geographic locations (32), which suggests that the local use of DMIs may affect the development of a specific resistance mechanism (41, 58, 60).

In conclusion, this study suggests that the environmental use of imidazole fungicides might confer selection pressure for the emergence of TR_{34}/L98H/S297T/F495I and TR_{46}/Y121F/T289A *A. fumigatus* azole-resistant isolates. In any case, cross-resistance to all of them is the rule. Therefore, the use of DMIs should be further controlled and contained in order to minimize the development and spread of azole-resistant *A. fumigatus* strains. Finally, it is very unlikely that the G54 mutation is being selected from the most common DMIs used in crop protection, and thus, the fact that it has been isolated from the environment should be investigated further.

MATERIALS AND METHODS

***Aspergillus fumigatus* strain collection.** A total of 83 unrelated strains of *A. fumigatus* from different countries with clinical origin were included in this study. Fungal genomic DNA was extracted as described previously (12). All isolates were identified at the species level by PCR amplification and sequencing of ITS1-5.8S-ITS2 regions and a portion of the β-tubulin gene (61).

Characterization of azole resistance molecular mechanisms in *A. fumigatus* strains. Azole resistance mechanisms were studied by sequencing the main azole target gene *cyp51A* in the *A. fumigatus* collection. Conidia from each strain were cultured in 3 ml of GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) and grown overnight at 37°C, after which mycelium mats were harvested and DNA was extracted (62). The full coding sequence of the *cyp51A* gene, including its promoter sequence, was amplified and sequenced using the PCR conditions described before (28). Each isolate was independently analyzed twice. DNA *cyp51A* sequences were compared against the *cyp51A* sequence of the *A. fumigatus* reference strain CBS 144.89 (GenBank accession number AF338659). A total of 46 independent *A. fumigatus* strains with known azole resistance mechanisms were included in this work, as well as 37 azole-susceptible strains.

TRESPERG genotyping and whole-genome sequence analysis. All *A. fumigatus* isolates included in this study were genotyped following the previously described TRESPERG typing assay (36). Whole-genome sequencing previously performed in a collection of 101 *A. fumigatus* genomes, including azole-susceptible and azole-resistant strains, was used to divide the *A. fumigatus* collection into four different clusters (33).

Antifungal susceptibility testing. (i) Clinical azoles. Antifungal susceptibility testing (AFST) was performed using a broth microdilution method following the European Committee on Antifungal Susceptibility Testing (EUCAST) reference method 9.3.1 (63). The antifungal clinical azoles used were itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer SA, Madrid, Spain), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), and isavuconazole (Basilea Pharmaceutica, Basel, Switzerland; tested from January 2017). In addition, we performed AFST to amphotericin B (Sigma-Aldrich Química, Madrid, Spain) as well as the echinocandins caspofungin (Merck & Co., Inc., Rahway, NJ) and anidulafungin (Pfizer SA, Madrid, Spain). The final concentrations tested ranged from 0.015 to 8 mg/liter for azoles, 0.03 to 16 mg/liter for amphotericin B and caspofungin, and 0.008 to 4 mg/liter for anidulafungin. *Aspergillus flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains in all tests performed. MICs were visually read after 24 and 48 h of incubation at 37°C in a humid atmosphere. MIC determinations were performed at least three independent times for each isolate (biological triplicates). *A. fumigatus* clinical breakpoints for interpreting AFST results established by EUCAST were used to classify each isolate as susceptible (S) or resistant (R) against a specific antifungal, in this case ITZ ($S \leq 1$; $R > 1$), VCZ ($S \leq 1$; $R > 1$), PSZ ($S \leq 0.125$; $R > 0.25$), or ISZ ($S \leq 1$; $R > 2$) (64).

(ii) Agricultural azoles (DMIs). AFST was also performed against 14-α demethylation-inhibiting fungicides (DMIs) following the EUCAST methodology as described before. The antifungal DMIs tested were three imidazole drugs (prochloraz, imazalil, and triflumizole) and five triazole compounds (tebuconazole, bromuconazole, metconazole, epoxiconazole, and difenoconazole), all purchased at Sigma-Aldrich, Química (Madrid, Spain). All DMIs were dissolved in dimethyl sulfoxide (DMSO) and autosterilized for 30 min at room temperature, as stated in the EUCAST protocol for clinical azoles. The final concentrations tested ranged from 0.064 to 32 mg/liter. Clinical breakpoints for interpreting AFST results have not been established, so isolates were considered susceptible or resistant based on the MIC shown by the group of clinical azole-susceptible strains. MIC determinations were performed at least three independent times for each isolate (biological triplicates). In addition, four new DMIs that have been recently introduced into the market—bitertanol, myclobutanil, triadimenol and paclobutrazol (all purchased at Sigma-Aldrich, Química)—were also tested against our *A. fumigatus* strain collection following the same methodology.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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E.M. conceived and designed the experiments. R.G.-R., I.G.-J., and J.L. performed the experiments. R.G.-R., I.G.-J., and E.M. analyzed the data. R.G.-R., I.G.-J., and E.M. drafted the manuscript. All authors read and approved the final manuscript.

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