Characterization of *Aspergillus fumigatus* cross-resistance between clinical and DMI azole drugs

Running Title: Cross-resistance between clinical azoles and DMIs

Rocio Garcia-Rubio[1,2*], Irene Gonzalez-Jimenez[1*], Jose Lucio[1] and Emilia Mellado[1,3#]

[1]Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III (ISCIII), Majadahonda, Madrid, 28222, Spain.

[2]Actual address: Center for Discovery and Innovation, Hackensack Meridian Health, Nutley, NJ, 07012, USA.

[3]Spanish Network for Research in Infectious Diseases (REIPI RD16/CIII/0004/0003), ISCIII, Majadahonda, Madrid, 28222, Spain.

[*] Both authors contribute equally to this work. Author order was determined alphabetically

[#] Corresponding author: emellado@isciii.es

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 work we have performed *A. fumigatus* susceptibility testing to 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* populations groups based on their azole resistance mechanism and previous WGS analysis, which suggests that the different resistance mechanisms have different origins and are specifically associated to 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 of the CDC 2019 Antimicrobial Resistance Threats Report. 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 forthcoming years.

Key Words: *Aspergillus fumigatus*, azole resistance, azole drugs, DMIs, plant pathogens

INTRODUCTION

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 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, its capacity to induce resistance in the target pathogens is weaker than other agricultural fungicides. Chemically, azoles are divided in imidazoles or triazoles (5). Several azole drugs used in crop protection have a similar molecular structure to medical triazoles (Figure 1) and cross-resistance between them has been demonstrated through lab evolution under agricultural azoles (6, 7). In the clinical setting, the introduction of azole drugs constituted 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-2000s, such as itraconazole, voriconazole, posaconazole and more recently isavuconazole (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 as 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 of the CDC 2019 Antimicrobial Resistance Threats Report (10).

Azole drugs act inhibiting the activity of Cyp51 enzymes, the azole target. Many filamentous fungi, particularly ascomycetes, harbored 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 paralogous with an unchanged enzyme activity (13).

Multiple studies in 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 (Figure 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 itraconazole (ITZ) and posaconazole (PSZ), but with unaffected minimum inhibitory concentrations (MICs) to short-tailed azoles such as voriconazole (VRZ) and isavuconazole (ISZ) (23, 24). Mutation M220 leads to ITZ resistance and variable MIC values to VRZ, PSZ and ISZ (25) while point mutation G448S shows resistance to VCZ and ISV 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 multi-azole resistant phenotype (28-30).

Given the similarity among clinical azoles and those used in crop protection, cross-resistance among DMIs and clinical azoles is common. Thus, it 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 towards the pathway involved

125 in the *A. fumigatus* environmental source of azole resistance. Different patterns of azole
126 cross-resistance were observed depending on the azole resistance mechanism.
127

128 **RESULTS AND DISCUSSION**

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

133 **The *A. fumigatus* strain collection represents a heterogeneous population**

134 Several authors have demonstrated the huge genetic diversity among *A. fumigatus*
135 strains using data from various typing techniques and whole genome sequencing (WGS)
136 (33-36). All the strains used in this work were identified as *A. fumigatus sensu stricto*.
137 Their azole resistance mechanism was analyzed by PCR amplification and sequencing
138 of the *cyp51A* gene including its promoter. Since both genetic background and
139 phenotypic features, such as antifungal resistance, may influence the susceptibility
140 testing results, the isolates included in this study were distributed in different groups
141 attending to their Cyp51A modifications, susceptibility to clinical azole drugs and WGS
142 cluster based on a previous *A. fumigatus* study performed in our group (33).
143 A description of each group, resistance mechanism and number of strains within it is
144 described in Table 2. The strains used in this work belonged to what we called cluster I
145 - azole susceptible *cyp51A*-WT strains together with azole resistant *cyp51A* single point
146 mutation strains, cluster II - azole susceptible and resistant strains with both *cyp51A*
147 single point mutations combined with TRs promoter integrations mechanisms, cluster
148 III - strains with the following five particular *cyp51A* modifications (F46Y, M172V,

149 N248T, D255E, E427K), and cluster IV - strains with three particular *cyp51A*
150 modifications (F46Y, M172V, E427K) (33).
151
152 **Antifungal susceptibility testing: clinical azole drugs**
153 Following the EUCAST guidelines the analyzed strains showed a wide range of MIC
154 values to all four clinical antifungals tested – itraconazole (ITZ), voriconazole (VRZ),
155 posaconazole (PSZ) and isavuconazole (ISZ). These differences were based on their
156 specific genetic background (WGS cluster) and azole resistance mechanism. *In vitro*
157 susceptibility testing showed ranges within one or two 2-fold MIC for each strain which
158 suggests stable and reliable results. However, MIC ranges per group may be broader
159 since several isolates are included in a group. MIC ranges for each clinical azole and
160 group of strains are shown in Table 2. There was no relevant difference in MIC values
161 among the Cyp51A-WT strains (from cluster I or II) to the clinical azoles tested. All the
162 *A. fumigatus* azole resistant strains with G54 mutation were resistant to ITZ and PSZ,
163 while the strains with M220 were resistant to ITZ, but variable to VRZ, ISZ and PSZ.
164 Strains harboring the G448S mutation were resistant to VRZ and ISZ but variable to
165 ITZ and PSZ. Finally, the isolates with the combined resistance mechanism which
166 includes a TR insertion in *cyp51A* promoter showed a multi-azole resistance profile to
167 all clinical azoles tested. No differences in susceptibility were seen to amphotericin B or
168 echinocandin drugs among all the strains tested (Table S1).
169
170 **Antifungal susceptibility testing: DMIs**
171 Susceptibility testing to eight DMI fungicides used for crop protection, three imidazole
172 drugs – imazalil (IMZ), prochloraz (PRZ), triflumizole (TFZ) – and five triazole drugs
173 – metconazole (MTZ), tebuconazole (TBZ), epoxiconazole (EPZ), bromuconazole

(BRZ), difenoconazole (DFZ) – was performed using the *A. fumigatus* strain collection. Again, *in vitro* susceptibility testing showed ranges within one or two 2-fold MIC for each strain. MIC ranges for each DMI and group of strains are shown in Table 2.

There was no remarkable differences in the MIC values to DMI drugs among the isolates that conformed the azole susceptible group (Cyp51A-WT, Cyp51A-3SNPS and Cyp-5SNPs from clusters I, II, III or IV) showing that their different genomic background is not influencing their DMI susceptibility profiles (Table 2). However, there were several relevant differences depending on the azole resistance mechanism groups (Table 2 and Figure 3). In general, most *A. fumigatus* azole resistant strains showed high MICs to all DMIs tested except for the strains with Cyp51A-G54 mutation which exhibited a hyper-susceptible phenotype to all the agricultural fungicides tested. Moreover, strains that harbored the resistance mechanisms TR_{46}/Y121F/T289A and TR_{34}/L98H/S297T/F495I were highly resistant to imidazoles, to both IMZ and PRZ or 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 to either 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.

198 Most *A. fumigatus* isolates with *cyp51A* single point mutations - G54, P216, M220,
199 G448 - were isolated from patients who had received long-term azole treatment (26,
200 37). However, mutations at positions G54, and occasionally M220 and P216, have also
201 been reported in strains from an environmental origin (38-40).
202 It is well-known that G54 mutation may emerge after long-term ITZ therapy in patients
203 with chronic aspergillosis or cystic fibrosis (41). However, the fact that it has also been
204 isolated from the environment in very different geographical locations (several
205 European countries, India, China, Tanzania and Thailand) points to for a possible
206 agricultural origin (38-40, 42). The results obtained in this work do not point towards
207 the environmental route to explain this resistance mechanism as all G54 strains tested
208 are resistant to long-tailed clinical azoles but highly susceptible to agricultural DMIs
209 and short-tailed clinical azoles, such as VRZ or ISZ (Figure 3 and Table 2). *A.*
210 *fumigatus* Cyp51A homology model studies have showed that G54R mutation can
211 prevent long-tailed azoles from entering the channel but not the more compact molecule
212 VRZ (43). In addition, the equivalent Cyp51 mutation has never been identified in plant
213 pathogens related to DMI resistance (Table 1). These strains showed even lower MIC
214 values to the new triazole DMIs tested than the *cyp51A*-WT strains (Supplementary
215 table 2). Alternatively, the possibility that G54 *A. fumigatus* azole resistant isolates may
216 develop during azole therapy within an infected or colonized patient and then spread
217 into the environment has been proposed (44). The G448S mutation has been shown to
218 confer resistance to VRZ and ISZ, together with elevated MICs to ITZ and PSZ (26).
219 Although to date the it has mainly been reported in the clinical setting, its high triazole
220 DMI resistance (Table 2) and the recent finding of *A. fumigatus* isolates with
221 environmental origin, which harbor this resistant mechanism (45, 46), would suggest

222 that this mutation could emerge under VRZ selective pressure in the clinical setting or
223 under other DMI triazoles such as MTZ in the environment (Figure 3).
224
225 Currently, the more frequent *A. fumigatus* mechanism of azole resistance involves the
226 overexpression of the *cyp51A* gene sometimes together with point mutations -
227 TR_{34}/L98H, TR_{46}/Y121F/T289A, TR_{53}- (28-30) and is associated with the
228 environmental route and the extended use of DMI fungicides in crop protection (14).
229 Moreover, strains with these resistance mechanisms have been found in azole *naïve*
230 patients but also in the environment throughout multiple worldwide locations (32, 47).
231 Since azole fungicides are used on a global scale, several resistance mechanisms have
232 been described to be common between plant pathogens and *A. fumigatus* azole
233 resistance isolates (Table 1).
234
235 In this context, the most common *cyp51* mutation in plant pathogens associated with
236 DMI resistance is the 134/136/137 tyrosine (Y) substitution to phenylalanine (F) or to
237 histidine (H) (Cyp51 amino acid position varies depending on the fungal species)
238 without known alterations in the Cyp51 promoter (Table 1). This mutation would
239 correspond to the Y121F modification commonly found in *A. fumigatus* together with
240 other modifications in the *cyp51A* gene - TR_{46}/Y121F/T289A (26, 30). Interestingly, the
241 Y121F mutation without TR integration in *A. fumigatus* has only been found in one
242 clinical isolate but the patient was never exposed to azole drugs. This strongly suggests
243 a resistance of environmental origin and it could represent the missing link between the
244 wild type gene and the TR_{46}/Y121F/T289A resistance mechanism (48). The sole Y121F
245 mutation confers resistance only to VRZ but not to ITZ or PSZ whereas the
246 TR_{46}/Y121F/T289A mutation is associated with multi-azole resistance. High-resolution

247 X-ray crystal structure analysis demonstrated that the Y140F/H mutation in
248 *Saccharomyces cerevisiae* Erg11 disrupted the binding of short-tailed triazoles but not
249 long-tailed ones (49).
250 The *A. fumigatus* strains which harbor the TR_{46}/Y121F/T289A mutation combination
251 have a pattern of resistance to all DMIs tested but particularly high to imidazole drugs.
252 Apart from *A. fumigatus*, other fungal human pathogens present the equivalent
253 Cyp51/*ERG11* mutations (*Cryptococcus neoformans, Histoplasma capsulatum,*
254 *Candida albicans, Candida auris*) (50-53) which leads to resistance to only short-tailed
255 triazoles. Similarly, the sole Y121F mutation in *A. fumigatus* leads just to VRZ
256 resistance (48). This mechanism of resistance commonly found in both plant pathogens
257 and *A. fumigatus* leads to similar activity and therefore might be developed from azole
258 selection pressure in both cases. In *Erysiphe necator*, a strong association between
259 *cyp51* gene copy number variation, which influenced expression in a gene-dose
260 dependent manner and was correlated with fungal growth in the presence of a DMI
261 fungicide has been found (54).
262 Several authors have observed elevated MIC values to the imidazole PRZ among *A.*
263 *fumigatus* isolates harboring the TR_{34}/L98H/S297T/F495I mutation (55-57). Our results
264 are in agreement with them as these strains showed a substantially stronger increase in
265 the MIC value to PRZ (range 8-32 mg/L) compared to the strains harboring the
266 TR_{34}/L98H mutation (1-8 mg/L).
267 It has been described that most of the *A. fumigatus* strains with
268 TR_{34}/L98H/S297T/F495I mutation are more genetically related than strains with
269 TR_{34}/L98H mutation, which might be due to an extremely adaptive recombinant event
270 under the selection pressure of imidazole fungicides in some countries (55-58). In one
271 of our previous works using WGS, the strains with TR_{34}/L98H/S297T/F495I mutation

grouped together in a small subcluster even when their geographical origin was non-related, such as from Spain, Denmark or the Netherlands (data not shown). Moreover, if we compare the agricultural pathogen's Cyp51s to the Cyp51A protein of *A. fumigatus*, the role of these mutations in PRZ resistance has been demonstrated even with structural *in silico* modelling (18). For instance in *P. 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 the deleterious effect of F495I on the protein function, as T289A does in TR46/Y121F/T289A, 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 their geographic origin. This common genetic background may help them to adapt to the environment or may confer them with an 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

297 contained in order to minimize the development and spread of azole resistant *A.*
298 *fumigatus* strains. Finally, it is very unlikely that the G54 mutation is being selected
299 from the most common DMIs used in crop protection and thus the fact that it has been
300 isolated from the environment should be investigated further.

301

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 (NCBI accession number AFUB_063960). 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

Clinical azoles

Antifungal susceptibility testing (AFST) was performed using a broth micro dilution 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 echinocandins caspofungin (Merk&Co., Inc., Rahway, NJ) and anidulafungin (Pfizer SA, Madrid, Spain). The final concentrations tested ranged from 0.015 to 8 mg/L for azoles, 0.03 to 16 mg/L for amphotericin B and caspofungin, and 0.008 to 4 mg/L for anidulafungin. *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains in all tests performed. Minimal inhibitory concentrations (MICs) were visually read after 24 and 48 hours of incubation at 37°C in a humid atmosphere. MICs 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≤1, R>1), VCZ (S≤1, R>1), PSZ (S≤0.125, R>0.25) or ISZ (S≤1, R>2) (64).

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 DMSO and autosterilized for 30 minutes at room temperature, as stated in the EUCAST protocol for clinical azoles. The final concentrations tested ranged from 0.064 to 32 mg/L. Clinical breakpoints for interpreting AFST results have not been established so isolates are considered susceptible or resistant based on the MIC shown by the group of clinical azole-susceptible strains. MICs were performed at least three independent times for each isolate (biological triplicates). In addition, four new DMIs that have been recently introduced in the market - bitertanol, myclobutanil, triadimenol and paclobutrazole (all purchased at Sigma-Aldrich, Química, Madrid, Spain) - were also tested against our *A. fumigatus* strain collection following the same methodology.

Author Contributions: EM conceived and designed the experiments. RGR, IGJ and JL performed the experiments. RGR, IGJ and EM, analyzed the data. RGR, IGJ and EM drafted the manuscript. All authors read and approved the final manuscript.

Funding: This research was funded by Fondo de Investigacion Sanitaria (FIS PI18CIII/00045) and also by Plan Nacional de I+D+i 2013-2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD16/CIII/0004/0003), co-financed by European

Development Regional Fund ERDF "A way to achieve Europe", Operative program Intelligent Growth 2014-2020.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the result.

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609 **Table 1.** Main Cyp51 resistance mechanisms to DMIs found in plant pathogens from 2000 to 2020.
610

Plant pathogen	DMI resistance	Cyp51 modifications	Promoter alterations	Over expression	Cyp51 gene	References
Penicillium digitatum	TFZ, FNM, BTN IMZ	Absent Absent	126 bp TR 199 bp TR	Yes Yes	Cyp51 Cyp51B	2
Blumeriella jaapii	FBZ	Absent	Truncated retrotransposon	Yes	Cyp51	2
Venturia inaequalis	MCB DFZ	Absent Absent	553 bp insertion EL3,1,2 repeated element	Yes Yes	Cyp51A 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 MCB	Y136F Y136F	ND ND	Yes Yes	Cyp51B 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 sf.sp.*hordei (Blumeria graminis* sf.sp.*hordei)*	TDM, TBZ BZZ TDM	Y136F, S509T Y136F Y136F, K147Q	ND ND ND	ND ND ND	Cyp51B Cyp51B Cyp51	2
Mycosphaerella graminicola (*Zymoseptoria tritici*)	TDM, TBZ, PRZ, TBZ, EPZ TBZ, DFZ TBZ, EPZ PTZ, EPZ	Y137F, I381V, V136A, ΔY459, ΔG460 I381V Y461S, Y137F S524T	ND ND ND ND	No ND ND ND	Cyp51 Cyp51 Cyp51 Cyp51	2 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 EPZ	Absent Absent	Absent Absent	Yes Yes	Cyp51B Cyp51	20 21
Sclerotinia homoeocarpa	PPZ	Absent	Absent	Yes	Cyp51	22

611 ND, non-determined or non-described. IMZ (imazalil), PRZ (prochloraz), TFZ (trifumizole), MTZ (metconazole), TBZ (tebuconazole), EPZ (epoxiconazole), BRZ
612 (bromuconazole), DFZ (difenoconazole), BTN (bitertanol), MCB (myclobutanil), TDM (triadimenol), PPZ (propiconazole), FNM (fenarimol), FBZ (fenbuconazole), FSZ
613 (flusilazole), PTZ (prothioconazole), BZZ (Benzimidazol), TTZ (tetraconazole), TRZ (triticonazole).

614 **Table 2.** MIC ranges to clinical and agricultural azole antifungals. *A. fumigatus* isolates are grouped based on their azole susceptibility profile and their

615 Cyp51A modifications.

616

Cyp51A modifications (# isolates)	WGS Cluster	MIC ranges to clinical azoles (mg/L)				MIC ranges to agricultural DMIs (mg/L)							
		ITZ	VRZ	PSZ	ISZ	IMZ	PRZ	TFZ	MTZ	TBZ	EPZ	BRZ	DFZ
AZL-S													
WT (20)	I-II	0.25-0.5	0.125-0.5	0.06-0.125	0.25-1	0.125-0.5	0.125-0.5	8-16	0.125-0.5	1-4	2-4	1-4	0.5-2
5SNPs* (6)	III	0.5-1	1-2	0.125-0.5	1	0.25-0.5	0.25-0.5	16- >32	0.25-1	2-8	8-16	4-16	2-8
3SNPs** (11)	IV	0.25-1	0.5 -1	0-06-0.125	1-2	0.125-0.25	0.125-0.25	8-16	0.25-1	2-4	2-4	2-8	2-4
AZL-R - point mutations													
G54 (12)	I-II	>8	0.25-0.5	1- > 8	0.25 -1	0.06-0.125	0.125-0.25	2-4	0.06-0.125	0.5-2	0.5-2	0.5-1	0.06-0.25
M220 (7)	I-II	> 8	0.25-1	0.25 -2	1-4	0.25-2	0.25-1	8-32	0.25-2	2-16	4-16	1-4	2-16
G448S (5)	I-II	1-2	> 8	0.25 -1	4-8	0.5-2	1-2	32- >32	4-8	8- >32	8- >32	4- >32	4- >32
AZL-R - TR integrations[&]													
TR_{34}/L98H (12)	II	> 8	4-8	0.5-1	8	1-8	2-8	>32	1-2	16-32	>32	8-32	16- >32
TR_{34}/L98H/ S297T/F495I (3)	II	> 8	4-8	0.5-1	>8	8	>32	>32	4-16	16-32	>32	>32	>32
TR_{46}/Y121F/T289A (4)	II	2-4	4- >8	0.5	>8	32- >32	16- >32	>32	8-16	>32	>32	>32	>32
TR_{53}(3)	II	> 8	2-4	0.5-1	8	2-8	2- 8	>32	2	16-32	>32	32	16-32

617

618 AZL-S (azole susceptible), AZL-R (azole resistant)

619 ITZ (itraconazole), VRZ (voriconazole), PSZ (posaconazole), ISZ (isavuconazole), IMZ (imazalil), PRZ (prochloraz), TFZ (trifumizole), MTZ (metconazole), TBZ (tebuconazole), EPZ

620 (epoxiconazole), BRZ (bromuconazole), DFZ (difenoconazole)

621 * 5SNPs: F46Y/M172V/N248T/D255E/E427K

622 ** 3SNPs: F46Y/M172V/E427K

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

624 **Figure legends**

625

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

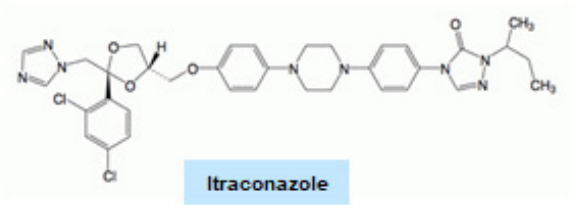
629

630 **Figure 2.** The most common azole resistance mechanisms in *A. fumigatus* and
631 susceptibility profiles to clinical azoles associated with each Cyp51A modification.

632

633 **Figure 3.** Minimum inhibitory concentration ranges to four agricultural azole
634 antifungals. *A. fumigatus* isolates are grouped based on their azole susceptibility profile
635 and their Cyp51A modifications.

CLINICAL TRIAZOLES



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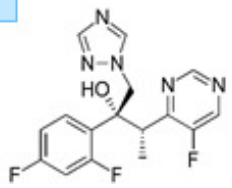

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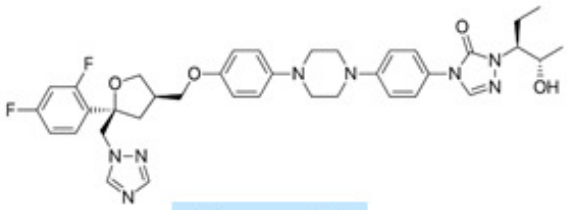
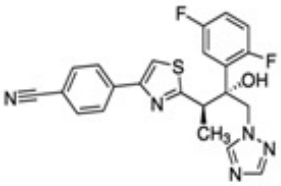
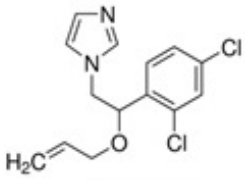


Posaconazole

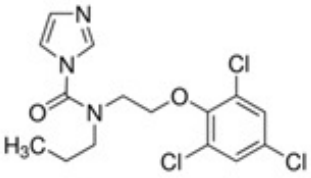


Isavuconazole

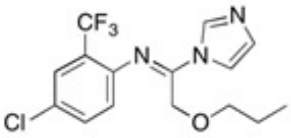
IMIDAZOLE FUNGICES



Imazalil

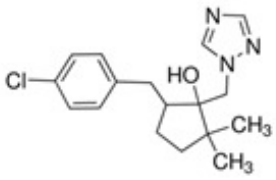


Prochloraz

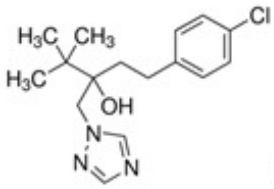


Triflumizole

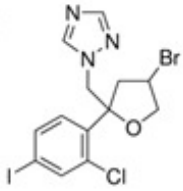
TRIAZOLE FUNGICIDES



Metconazole



Tebuconazole



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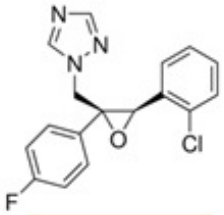
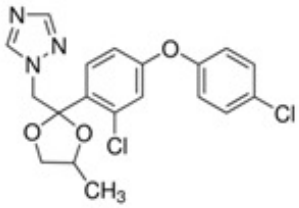


Epoxiconazole



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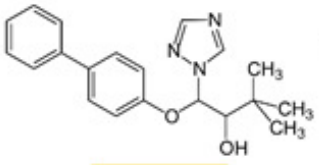
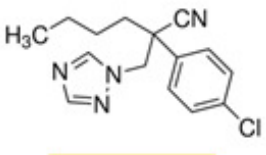
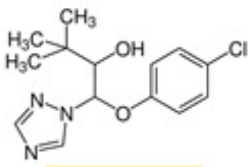


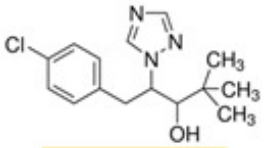
Bitertanol



Myclobutanil



Triadimenol



Paclobutrazole