

Azole-Resistant Invasive Aspergillosis: Relationship to Agriculture

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Abstract Azole resistance in *Aspergillus fumigatus* has been increasingly reported particularly over the last decade. Two routes of acquisition are described: selection of resistance during long term azole therapy in the clinical setting, and primary acquisition of resistant isolates from the environment due to the considerable use of azole fungicides in agriculture and for material preservation. Three specific resistance genotypes have been found in azole naïve patients. Two of these have also been found in the environment and are characterized by a tandem repeat in the promoter region of the target gene coupled with point mutation (s) in *CYP51A* (TR₃₄/L98H and TR₄₆/Y121F/T289A). In the third a single target enzyme alteration (G432S) is found. These resistant “environmental” strains have been detected in many West-European countries as well as in the Asia-Pacific. Noticeably, these two continents account for the highest fungicide use in the global perspective (37 % and 24 %, respectively). Among the 25 azole fungicides, five have been associated with the potential to select for the TR₃₄/L98H genotype; three of these are among those most frequently used. Although the number of antifungal fungicide compounds and classes available is impressive compared to the armamentarium in human medicine, azoles will remain the most important group in agriculture due to superior field performance and significant resistance in fungal pathogens to other compounds. Hence, further spread of environmental resistant *Aspergillus* genotypes may occur

and will depend on the fitness of each resistant phenotype and the pattern of azole fungicide use.

Keywords Azole resistance · *Aspergillus* · Fungicide · Agriculture · CYP51

Introduction

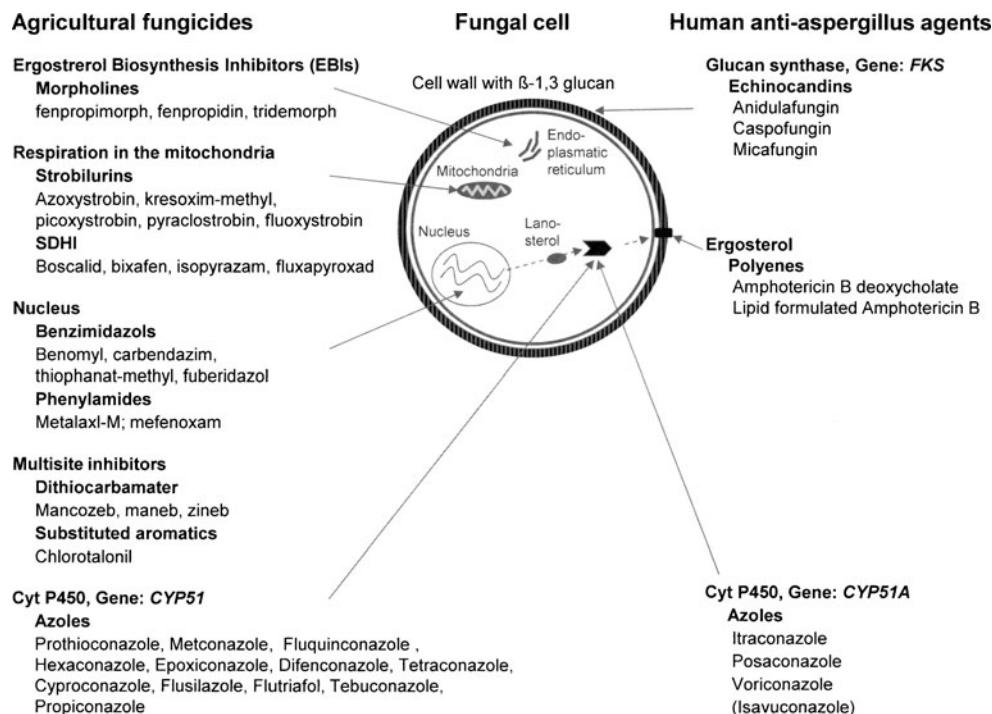
Aspergillus fumigatus is a ubiquitous saprophytic fungus associated with a variety of diseases including allergic manifestations and chronic infections in the immunocompetent population and acute invasive localized or disseminated aspergillosis in the immunocompromised host. The estimated burden of disease is 3–500,000 for the acute invasive infections, 3 million with chronic pulmonary aspergillosis and 4 million with allergic bronchopulmonary aspergillosis [1]. Azole antifungal drugs are the cornerstone in the antifungal treatment of aspergillosis due to the clinical superiority of voriconazole for invasive infections and the fact that this group is the only oral option for patients with allergic or chronic forms of aspergillosis treated outside the hospital [2–5]. Itraconazole is often the primary choice for the allergic and chronic aspergillosis, voriconazole is the first line agent for invasive infection and posaconazole is licensed for prophylaxis and salvage treatment in the immunocompromised host. Second line options are amphotericin B formulations and echinocandins (Fig. 1) [4, 5].

Azole resistance in *Aspergillus* spp. may be intrinsic or acquired. Intrinsic resistance is characteristic for some of the sibling species of the *A. fumigatus* that are not easily identified in the routine laboratory (e.g. *A. lentulus* and *A. udagawae*) and acquired resistance is seen in *A. fumigatus* isolates at rates most commonly not exceeding 5 % but with significant variation (zero to 61 %) depending on geographical location, case mix and method for resistance detection [6, 7•, 8].

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Fig. 1 Drug targets of agricultural fungicides (*left*) and antifungal agents used in human medicine for the treatment of aspergillosis (*right*)



Early and appropriate antifungal treatment is associated with lower failure rates in patients with acute invasive aspergillosis and not surprisingly azole resistance has been associated with a poorer outcome [2, 3, 9, 10, 11]. Often the diagnosis of azole resistant aspergillosis is difficult or delayed. This is in part due to cultures having low diagnostic sensitivity and hence, in many cases no fungal isolate is available for susceptibility testing. However, even if the culture is positive, susceptibility testing is unfortunately not routinely performed at many centres despite recommendations to do so and despite the fact that azole breakpoints have recently been established [6, 12, 13, 14]. Therefore, understanding of the clinical relevance of azole resistance and when it should be suspected and tested for is of utmost importance and the fundamental basis for improving management of this disease. In this review we attempt to address azole resistance in *Aspergillus* with particular focus on the link between the azole use in agriculture and the risk for acquiring azole resistant *Aspergillus* disease in humans.

Azole Resistance in *Aspergillus*

Azoles inhibit the ergosterol biosynthetic pathway by binding to its target enzyme lanosterol 14- α demethylase encoded by the *CYP51A* gene. This enzyme belongs to the cytochrome P450 family and is required for converting lanosterol to ergosterol, an essential component of the fungal cell membrane (Fig. 1). This results in the accumulation of 14- α methyl sterols and impaired cell membrane integrity [15, 16]. Multiple mechanisms of acquired azole resistance

in *A. fumigatus* have been suggested and include: 1) target gene mutations, 2) target gene up-regulation, 3) up-regulation of efflux pumps, 4) reduced membrane permeability and 5) other mechanisms (Table 1).

Azole Resistance in *Aspergillus* Isolates from Azole Exposed Patients Only

A number of *cyp51A* mutations have been detected in isolates with wild type azole susceptibility phenotype, whereas others are associated with mono- or multi-azole resistance (“hot spot mutations” [13]; Tables 1 and 2)). *cyp51A* knockout mutant data, heterologous transformation analysis, molecular dynamic simulations and studies on site-directed mutagenesis, protein folding and homology modelling have assisted in establishing a role for the gene in azole resistance [17, 18, 19, 20, 21], and identifying, confirming and predicting mutations conferring (cross-)resistance [20, 21, 22, 23]. Mutational *cyp51A* hot spot codons such as G54, G138, M220, Y431, and G448 are considered definitely involved in azole resistance and have been reported frequently from multiple centres (Table 2).

None of these mutations, however, are consistently present in clinically resistant isolates, and azole resistant isolates do not always exhibit *cyp51A* mutations [20, 24, 25, 26]. Relatively consistent azole susceptibility profiles have been described for isolates with hot spot mutations (Table 2). Most mutations confer itraconazole-resistance (Table 2), whereas pan-azole resistance typically has been reported in isolates with G138C or M220K alterations (Table 2).

Table 1 A summary of currently described azole resistance mechanisms in clinical isolates of *A. fumigatus*

Azole resistance mechanism	Description	Reference
<i>cyp51A</i> mutations	Azoles are directed against the enzyme lanosterol 14- α demethylase (<i>CYP51A</i>) required to convert lanosterol to ergosterol. Blocked access of the substrate lanosterol results in buildup of toxic sterol intermediates and fungal growth arrest. Mutations in <i>CYP51A</i> may decrease azole target affinity.	[15, 101, 102]
Target gene upregulation	Increased expression of <i>CYP51A</i> has been documented with and without tandem repeats in the promoter region. A mutation (P88L) in the CCAAT-binding transcription factor complex subunit HapE was associated with azole resistance in clinical isolates with upregulation of the target enzyme production but wildtype <i>CYP51A</i> . As Hap is a transcription factor complex, the increased resistance might be due to a gain of function mutation if the mutated Hap complex binds to a CCAAT-box in the promoter region of <i>CYP51A</i> and induces the expression of the gene.	[22, 24, 32, 42••, 103] Camps et al., (submitted)
Upregulation of efflux pumps	An <i>AfiI</i> transposon found inserted 370 base pairs upstream of the start codon of <i>CYP51A</i> may modulate <i>CYP51A</i> expression	[17•]
Upregulation of efflux pumps	Upregulation of efflux pumps decreases the intracellular concentration of antifungals. Efflux pumps consist of two superfamilies: ATP binding cassette (ABC) and the major facilitator (MF) transporters. Increased expression of genes encoding ABCs and MFs has been found in clinical azole resistant isolates and in laboratory mutants with induced resistance.	[104–108]
Reduced membrane permeability	Reduced membrane permeability results in decreased penetration of the drug.	[109]
Other	Cholesterol import as compensation for ergosterol depletion caused by triazole therapy	[110]
	<i>A. fumigatus</i> can produce an extracellular hydrophobic matrix with typical characteristics of a biofilm <i>in vitro</i> on bronchial epithelia, and <i>in vivo</i> in aspergilloma, as well as in invasive pulmonary aspergillosis. The biofilm may render the isolate resistant to antifungal agents, including azoles.	[111–113]
	Loss of SrbA, a sterol regulatory element-binding protein homologue in <i>A. fumigatus</i> , results in susceptibility to fluconazole, an azole to which <i>A. fumigatus</i> is intrinsically resistant, and the protein may indirectly mediate resistance to azoles.	[45]
	Loss of Unfolded Protein Response function detected in Δ <i>hacA</i> mutants enhances susceptibility to antifungal drugs.	[114]

Acquired multi- or pan-azole resistance, where hot spot mutations have emerged during azole therapy over a period of less than a year, has been described [11•, 27–30].

Azole-Resistant *A. Fumigatus* Found in Azole Naïve Patients

Azole resistant *A. fumigatus* has been found in azole naïve patients and has been shown to harbour one of three different resistance mechanisms: 1) a 34 bp tandem repeat (TR₃₄) in the *CYP51A* promoter region coupled with a L98H substitution in the *CYP51A* gene, 2) a 46 bp tandem repeat (TR₄₆) coupled with Y121F/T289A substitutions in the *CYP51A* gene, or 3) a G432S substitution in the *CYP51A* gene. Of particular interest is the TR₃₄/L98H genotype, which was initially detected at a Dutch centre in 12/13 itraconazole-resistant patient isolates [31••]; this mutation was subsequently found in the vast majority of itraconazole-resistant isolates from other Dutch centres [20•, 32] and associated with an up-regulation of the target enzyme level (mediated by the tandem repeat) and by decreased

affinity for azoles (mediated by the substitution) in combination leading to the pan azole resistant phenotype [21•]. Using site-directed mutagenesis, Snelders et al. [21•] showed that the multi-azole resistant phenotype could not be induced exclusively by introducing either the TR₃₄ or L98H mutation, indicating that the multi-azole resistant phenotype associated with L98H is dependent on the TR₃₄ in the promoter region.

Link to the Agricultural Use of Azoles

Dominance of a single resistance mechanism as the one observed in the Netherlands is difficult to explain by resistance development in individual azole-treated patients, since a wide array of different resistance mechanisms is normally found in patients with resistance after long term treatment [11•, 26•, 33••]. Apart from being associated with multi-azole resistance in clinical isolates from azole naïve as well as exposed patients [31••, 33••, 34•, 35•], TR₃₄/L98H has also been found in azole resistant, environmental isolates in Denmark and The Netherlands [32, 36•]. This raised the hypothesis that azole resistant patient isolates may not only

Table 2 *cyp51A* alterations found in *A. fumigatus* with and without azole resistance or reduced susceptibility

	Codon	Amino acid substitution (non-synonymous and synonymous)	Azole phenotype			Reference
			ITZ	POS	VOR	
Hotspot mutations (mutations commonly found in azole resistant isolates and confirmed by various analyses (see text for details))	G54	E	R	S/I/R	S	[11•, 20•, 25, 27, 98, 104, 115–118]
		K	R	NA	NA	[104]
		R	R	R	S	[11•, 25, 28, 104, 106, 115–119]
		V	R	I/R	S	[11•, 115, 117]
		W	R	R	S	[20•, 21•, 25, 116, 117, 119, 120]
	L98	H ^a	R	S/I/R	I/R	[7••, 10, 11•, 20•, 22, 23, 32, 33••, 34•, 35•, 36•, 98, 117, 120]
		I ^b	R	I	S	[21•]
		Q ^b	R	I	I	[21•]
		R ^b	R	I	I	[21•]
		Y ^b	R	I	S	[21•]
	G138	C	R	R	R	[11•, 17•, 20•]
		R	S	S	R	[118]
	M220	I	R	S/I/R	S	[20•, 26•, 28, 32, 33••, 98, 115, 117]
		K	R	I/R	S/I/R	[7••, 11•, 20•, 26•, 33••, 117, 121, 122]
		R	R	I/R	S/I	[7••, 20•, 26•, 32, 98]
		T	R	S/I/R	S/I/R	[11•, 115, 117, 122]
		V	R	I/R	S	[20•, 26•, 115, 117, 122]
		W	R	R	NA	[26•]
		Y431	C	I/R	S/R	S/I/R
	G448	S	R	I/R	R	[11•, 118]
Mutations present in the absence of hotspot mutations and associated with microbiological and/or clinical resistance or reduced susceptibility	N22	D	R	NA	NA	[106]
	Y121	F ^c	I	I	R	[42••]
	H147	Y	R	I	R	[11•]
	P216	L	R	I/R	S/I	[11•, 27, 120]
	F219	I	R	S/I/R	S/I/R	[27, 120]
	I266*	N	R	I	S	[25]
	A284	T ^d	Reduced susceptibility	Reduced susceptibility	Reduced susceptibility	[26•]
	T289	A ^c	I/S	I/S	R	[42••]
	F332**	K	R	I	S	[123]
	S400	I	S	S	I	[20•]
	E427	G	R	S/I	I/R	[11•]
	G432	S	R	S	S	[43••]
	G434	C	R	R	R	[11•, 17•]
T440	A	R	NA	NA	[106]	
Y491	H	R	NA	NA	[106]	
Mutations present in isolates with wt susceptibility, in combination with hotspot mutations, or present in both susceptible and resistant isolates	A9	T				[27]
	L20	L				[106]
	F46	Y ^c				[11•, 20•, 23, 26•, 119, 120, 124]
	S52	T				[20•, 23]
	Q88	H				[20•]

Table 2 (continued)

Codon	Amino acid substitution (non-synonymous and synonymous)	Azole phenotype			Reference
		ITZ	POS	VOR	
G89	G ^c				[20•, 26•, 119, 120, 124]
V101	F				[33••]
E130	D				[20•]
Q141	H				[20•, 23]
M172	V ^c				[11•, 20•, 23, 26•, 119, 120, 124]
N248	T ^c				[11•, 20•, 26•, 120, 124]
L252	L				[20•]
D255	E ^c				[11•, 20•, 26•, 119, 120, 124]
D262	Y				[124]
A284	A ^d				[20•]
S297	T				[20•, 22, 23, 32, 33••, 34•]
L339	L				[20•]
L358	C ^c , L ^c				[20•, 26•, 119, 120, 124]
S393	S				[106]
P394	L				[106]
E427	G, K ^c				[11•, 20•, 23, 26•, 119, 120, 124]
C454	C ^c				[20•, 26•, 119, 120, 124]
F495	I				[20•, 22, 23, 32, 33••, 34•]
G497	G				[119, 124]

*Annotated as I266 in the reference, however, Genbank consensus has N266

**Annotated as F332 in the reference, however, Genbank consensus has P332

^a always coupled to a 34 bp tandem repeat in the promoter region

^b laboratory induced

^c present together and coupled to a 46 bp tandem repeat in the promoter region

^d two mutations have so far been reported in A284: A (susceptible [20•]) and T (resistant/reduced susceptibility [26•])

^e reported only together with other mutations of the mutation complex made up by F46Y, G89G, M172V, N248T, D255E, L258C, L353C/L, L358L, E427K, and C454C and present in isolates with wt and resistant phenotype

result from longstanding azole therapy resulting in selection for resistant mutants, but be acquired directly from the environment [23]. Three additional observations support this notion. First, *A. fumigatus* isolates of identical microsatellite short tandem repeat (STR) genotype with and without azole resistance have been found in individual patients suggesting selection in vivo. But notably, although patients harbouring a TR₃₄/L98H as well as a susceptible isolate have been described, these isolates have never had the same STR genotype, suggesting “double infection” with unrelated *A. fumigatus* isolates and not in vivo selection of resistance [33••]. Second, five specific azole fungicides (propiconazole, bromuconazole, epoxiconazole, difenoconazole and tebuconazole) show a molecular structure very similar to the medical triazoles, adopt similar poses while docking the target enzyme, have activity against wild type *A. fumigatus*

but not against azole-resistant TR₃₄/L98H-positive isolates and have all been introduced in The Netherlands between 1990 and 1996 directly preceding the isolation of the first TR₃₄/L98H in 1998 [23, 37••]. And third, tebuconazole has been shown to be able to induce tandem repeats in the promoter region of *CYP51A* under laboratory conditions [37••].

In order to fully understand the nature and development of antifungal resistance, it is necessary to bear the following factors in mind [38]: Antifungal drug resistance (ADR) is not transmitted from person to person, 2) ADR is not conveyed by lateral gene transfer (as seen among bacteria via plasmids), 3) ADR has developed rapidly over the past few years, 4) ADR development under azole pressure (e.g. during therapy) presumably occurs during asexual reproduction, which is much more likely to occur in patients with

chronic aspergillosis and aspergillomas than in patients with acute invasive aspergillosis, and 5), insertion of a tandem repeat acting as a transcriptional enhancer under azole pressure might be introduced more often during sexual reproduction requiring the presence of two opposite mating types which occurs mainly in the environment in *A. fumigatus* (teleomorph: *Neosartorya fumigata*) [39]. These factors have major implications for the interpretation on reports on *cyp51A* mutations, and may also to some extent predict the pattern of mutations seen in particular cohorts of patients in particular geographic areas. Hence, the finding of TR₃₄/L98H mutations in azole resistant clinical isolates in patients with invasive aspergillosis in a country where this combination of mutations occurs in the environment may not be surprising. On the other hand, azole resistant isolates from patients with chronic aspergillosis and azole therapy may harbour either different, sporadic *cyp51A* mutations or the TR₃₄/L98H genotype, depending on exposure and relative fitness of competing mutants. Conspicuously, TR₃₄/L98H was recently found in 55.1 % of culture-negative, PCR positive sputum samples from patients with allergic bronchopulmonary/chronic pulmonary aspergillosis [7••]. If TR₃₄/L98H mutants are less fit than wild type isolates and non-TR₃₄/L98H mutants and thus more difficult to culture, this could explain why they were detectable only by PCR [7••]. This raises concern, since susceptibility testing is dependent on cultured isolates. On the other hand, the TR₃₄/L98H genotype appears to be at least as fit in the environment as suggested by its clonal expansion across the Netherlands [40] and virulence studies in the animal model have failed to detect loss of virulence [41].

Recent reports of additional *cyp51A* mutants, namely TR₄₆/Y121F/T289A, found in both clinical and environmental isolates in the Netherlands [42••], and a G432S mutant in an azole-naïve patient in France [43••] add support to the hypothesis that azole resistance acquired in the environment is often but not exclusively associated with upregulation of *CYP51A* induced by the presence of a tandem repeat in the promoter region. As described below this is also the case in fungal plant pathogens. Interestingly, both the Y121 and the G432 codons in these two resistance genotypes are also recognised as hot spot codons involved in azole fungicide resistance in the fungal plant pathogen *Mycosphaerella graminicola* (corresponding to the G460 and Y137 codons in this organism) [44].

Azole-Resistance in Other *Aspergillus* Spp.

All *Aspergillus* spp. are intrinsically resistant to fluconazole [45]. Whereas wild-type *A. fumigatus* sensu stricto is susceptible to other azoles, intrinsic multi/pan-azole resistance may operate in some morphologically similar species in the

section *Fumigati* [46]. Thus, azole resistance has been reported in *A. lentulus*, which is characterised by primary *CYP51A* dependent resistance, and in *A. fumigatiaffinis*, *Neosartorya pseudofischeri*, and *A. viridinutans* [36•, 46–48]. However, recognition of these cryptic species happened only recently, and their respective roles in clinical aspergillosis and potential contribution to resistance problems remain to be further clarified.

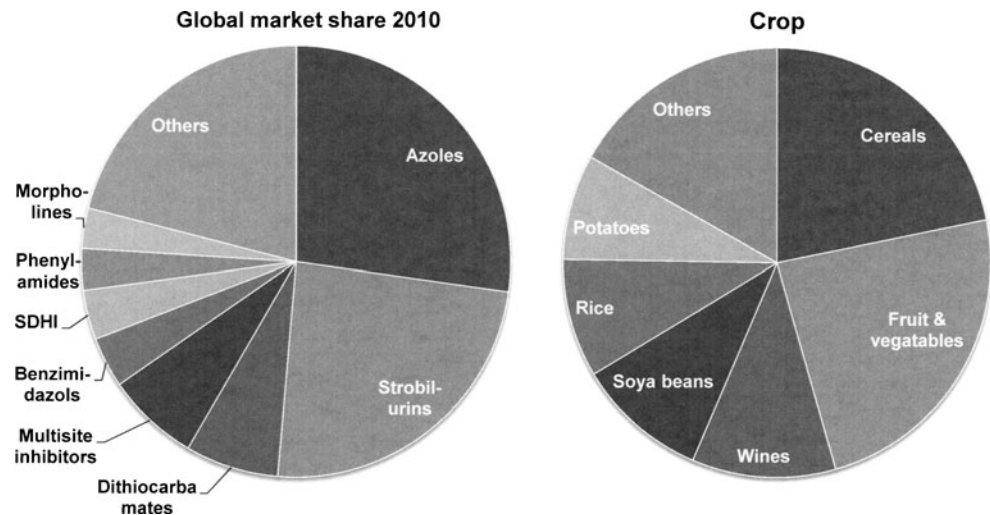
Acquired resistance in other *Aspergillus* spp. has only been sporadically investigated and reported. However, since some of these species (e.g. *A. terreus*, *A. flavus* and *A. nidulans*) exhibit reduced susceptibility to amphotericin B [49–56], the importance of azole susceptibility surveillance of such species should not be underestimated. We recently demonstrated elevated azole MICs for two *A. terreus* isolates [57, 58•], one of which had a *cyp51A* M217I mutation (equivalent to M220I in *A. fumigatus*) [58•]. An S240A alteration in *CYP51C* was recently associated with clinical voriconazole-resistance in *A. flavus* [59•]. Itraconazole resistance in species belonging to the *A. niger* complex is not unusual [60, 61] but has not so far been linked to any particular *cyp51A* gene mutation [61]. The *A. ustus* complex includes *A. calidoustus*, which has been detected in transplant patients and appears to be intrinsically pan-azole resistant [49]. Resistance profiles of other *Aspergillus* species rarely reported as causes of clinical aspergillosis were recently reviewed by van der Linden et al. [62]. Noticeably, resistance conferred by *cyp51A* mutations coupled to tandem repeats or in azole naïve patients have so far only been demonstrated for *A. fumigatus* isolates, indicating that this type of resistance acquired in the environment may not yet be a significant issue except in *A. fumigatus*.

Control Practice in Agriculture

Fungal plant pathogens cause disease in many agricultural and horticultural crops compromising yield and quality [63]. Yield losses in the range of 10 % to 30 % are not uncommon. The approach for infection control varies significantly between countries and over the seasons as many plant pathogens are crop and climate associated, often with the most severe attacks in wet seasons. Effective fungicides have been available for more than 30 years and fungicides are today commonly used for many crops. Depending on the crop and local risks for attack the number of treatments may vary between 5 and 15 times/year in orchard crops and potatoes to 0–4 in cereal crops (Fig. 2). Fungicide use in European cereal crops and in wheat in particular is the largest market for fungicides worldwide [64] (Figs. 2 and 3).

Several classes of fungicides are available for plant protection including triazoles, strobilurins, morpholines, SDHIs and chloronitriles (Figs. 1 and 2). Fungicide resistance has

Fig. 2 Global market-shares of the various agricultural fungicides in 2010 (a), and proportional use of individual crops (b). Compounds and classes are indicated as relative proportions of the total market value (11,475 mill \$) (personal communication Phillips McDougall, 2010)



been reported for the majority of fungicides although less commonly for the multisite inhibitors. Concerns related to human health specifically for the azoles have included the risk of endocrine side effects following exposure of farmers and green house workers from preparing spray mixtures or handling azole treated plants. Recently documented selection for azole resistance in human pathogenic fungi adds to this concern.

Triazoles in Agriculture and for Material Preservation

Azole fungicides constitute the most widely used class of antifungal agents for the control of fungal plant diseases (Fig. 2) [64] (personal communication Phillips McDougall,

2010). In agriculture, the first azoles (triadimefon and imazalil) were introduced in 1973 [65], and triazoles have been widely used since the beginning of 1980. In comparison with several other groups of fungicides the field performances of azoles have been relatively stable, suggesting that emergence of acquired resistance in fungal plant pathogens has been limited (www.FRAC.info; [66]). Triazoles are also commonly used for material preservation, but no official statistical information is available to verify to what extent. Examples are tebuconazole and propiconazole which are both used to protect the surface of materials or objects such as paints, plastics, sealants, wall adhesives, binders, papers, art works, wood, and for the preservation of fibrous or polymerised materials, such as leather, rubber, paper and

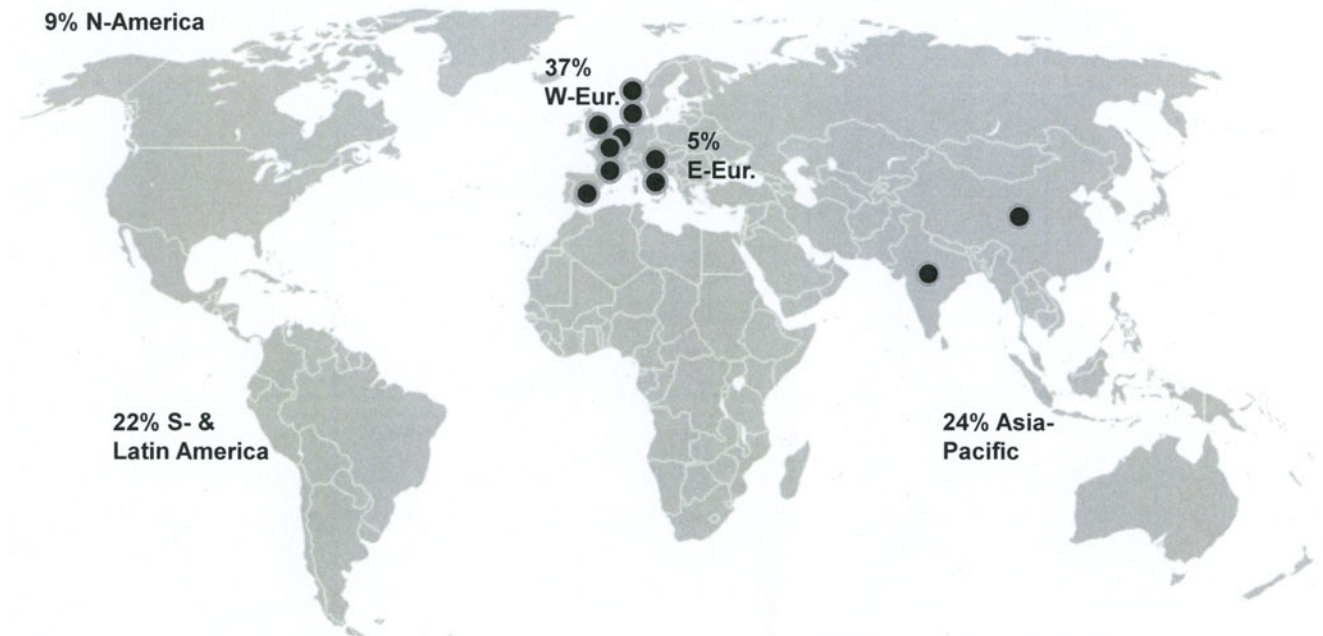


Fig. 3 Countries where *A. fumigatus* with the TR₃₄/L98H have been reported (dots) and percentage of agricultural fungicide use by continent (3 % used outside the regions shown) [7•, 8, 11•, 22, 32, 33•, 34•, 35•, 64, 97, 98, 99•]

textile products. Additionally tebuconazole is used for preservation and remedial treatment of masonry or other construction materials (EU regulation).

A total of 25 different triazoles/imidazoles have been developed for agricultural crops [65]. The products are applied either as a seed treatment or as foliar applications (sprayed on growing plants), the latter potentially applying a greater selection pressure on other fungal pathogens. In addition to protecting against fungal plant pathogens, triazole compounds may offer plant growth-regulating properties [67] and the ability to protect plants against various environmental stresses [68, 69]. Each new triazole often offered some new advantages in basic activity, spectrum, persistency or mobility in the crop. Initially triadimenol followed by propiconazole and prochloraz were most commonly used, whereas today these have largely been replaced by more potent triazoles including tebuconazole, metconazole, epoxiconazole and prothioconazole. Three of these are among the five azole fungicides which have been associated with a high potential for selecting the TR₃₄/L98H *A. fumigatus* genotype as described above [37••].

Even though other groups of chemicals e.g. strobilurins and SDHI fungicides (Fig. 1) [70], have been made available, problems related to resistance have been so significant that they are no longer appropriate for control of major diseases in many crops [71–73]. Hence, azoles alone or combinations of several agents (typically including at least one azole) are used in order to limit further selection of resistance [71, 74, 75].

Azole Resistance Mechanism in Fungal Plant Pathogens

Triazole resistance has over the years appeared in several plant pathogenic fungi. Field resistance was first reported for the cucumber pathogen *Sphaerotheca fuliginea* [76], and subsequently in several other pathogens like *Penicillium digitatum* [77], *Blumeria graminis f.sp. hordei* [78], *Venturia inaequalis* [79], *Rhynchosporium secalis* [80], and *Mycosphaerella graminicola* [71].

In several European countries a 10–100 times loss of susceptibility in vitro of *M. graminicola* populations has been reported over the last 20 years [71, 73, 81, 82]. Four azole resistance mechanisms have been found, most of which are identical to those described for *A. fumigatus* above: 1) point mutations in *CYP51*, 2) upregulation of target gene production, 3) efflux pumps, and 4) altered sterol biosynthesis; the latter has been found only in laboratory selected mutants and thus will not be dealt with any further here.

Point Mutations in Target Gene *CYP51*

A variety of different point mutations has been found in the *CYP51* gene in plant pathogens but at a relatively low

prevalence. Initially, *Blumeria graminis f.sp. hordei* and *Uncinula necator* with an Y136F alteration were associated with triazole resistance [83]. Since then, a total of 22 different alterations have been verified in *M. graminicola* [75]. Amino acid sequence alignment of CYP51 from *M. graminicola* and *Candida albicans* showed eight identical alterations in azole resistant isolates of the two organisms, while other eight unique alterations were found specifically for *M. graminicola* [75]. Moreover, alterations often accumulate in a single isolate leading to a stepwise shift in resistance which is also typical for *C. albicans* but apparently not for *A. fumigatus* [71, 84]. For example the alterations Y459D/C, G460D, and Y461H have each been linked to low level resistance, whereas I381V in combination with one of these resulted in a significantly higher level of resistance to all azoles [85]. Sequence alignment for *M. graminicola* and *A. fumigatus* identifies three codons found in azole resistant isolates of both species, namely Y137, Y459 and G460 in *M. graminicola* and Y121, Y431 and G432 in *A. fumigatus*, respectively [42••, 43••, 44]. Notably, two of these have been involved in azole resistance in azole naïve patients as described above [42••, 43••], whereas the third (Y431) was found in a patient with chronic aspergillosis and bilateral aspergilloma, and was shown to have been selected for in vivo [11•] (Fig. 4). The latter observation suggests that some alterations can be induced by fungicide as well as human azole use.

The European population of *M. graminicola* is currently dominated by two molecular types, one being tebuconazole susceptible (V136A) and another being tebuconazole resistant (A379G, I381V and Δ Y459/G460) [71, 85–87]. This has had major impact on the field performances of tebuconazole but less impact on other azoles such as epoxiconazole and prothioconazole [75].

Over Expression of the *CYP51* Gene

Over expression linked to insertions or duplications in the promoter region of *CYP51* resulting in elevated intracellular levels of the target enzyme has been detected in different plant pathogens with reduced azole susceptibility [88, 89]. Specifically for *M. graminicola* an insertion in the promoter of *CYP51* was found coupled with a *cyp51* alteration I381V in several isolates, but the increase in *cyp51* expression remains to be demonstrated experimentally [44].

Role of Efflux Pumps

The simultaneous resistance to a variety of structurally unrelated toxic compounds is most commonly caused by upregulation of efflux pumps and was initially described in

<i>A. fumigatus</i>	MVPMLWLTAY	MAVAVLTAIL	N22 LNVVYQLFFR	LWNRTEPPMV	FHWVPFLGST	50
<i>M. graminicola</i>GLGF	LAFSTL.AIL	LNVLSQLLFR	.GKSSDPPLV	FHWVPPFIGST	
<i>A. fumigatus</i>	S52 ISY G54 GIDPYKF	FFACREKYGD	IFTFILLGQK	Q88 TTVYLVGV Q88 EN	EFILNGK L98* LKD	100
<i>M. graminicola</i>	ITYGIDPYKF	FFSCREKYGD	VFTFILLGKK	TTVCLG T TKGN	DFILNGK L LKD	
<i>A. fumigatus</i>	V101 VNAAEVYSPL	TTPVFGSDVV	Y121* YDCPN S KLME	G138 QKKFIK Y GLT	Q141 QSALE S HVPL	150
<i>M. graminicola</i>	VNAEEIYSPL	TTPVFG KD WV	YDCPN S KLME	QKKF V KYGLT	TSALQ S YVTL	
<i>A. fumigatus</i>	IEKEVLDYL	RDSPN..FQG	SSGRMDISAA	MAEITIFTAA	RALQGQEVRS	197
<i>M. graminicola</i>	IAAETRQFFD	RNNPHKKFAS	T STIDLPPA	LAELTIYTAS	RSLQGKEVRE	
<i>A. fumigatus</i>	KLTAEFADLY	HDLDKGFT P I	P216 M220 F219 NFMLPWAPLP	HNKKRDAAHA	RMRSIYVDII	247
<i>M. graminicola</i>	GFDSSFADLY	HYLDMGFT P I	NFMLPWAPLP	QNRRRDYAQK	KMSETYMSII	
<i>A. fumigatus</i>	NQRRLDGDKD	SQKSDMIWNL	N266 MNCTY K NGQQ	VPDKEI A HMM	T289* ITLLMAG Q HS	297
<i>M. graminicola</i>	QKRR.ESKTG	EHEEDMIHNL	MQCKYKDGNA	IPDKEI A HMM	IALLMAG Q HS	
<i>A. fumigatus</i>	SSSISAWIML	RLASQPKVLE	ELYQEQLANL	P332 GPAC E DGSLP	PLQYKDLDKL	347
<i>M. graminicola</i>	SSATESWITL	RLASRPDIQD	ELLQE Q KDML	G.VNADG S IK	ELTYANLSKL	
<i>A. fumigatus</i>	PFHQHVIRET	LRIHSS I HSI	MRKVK S PLPV	PGT P YMIPPG	P394 RVLLAS Q GVT	397
<i>M. graminicola</i>	TLLNQVVKET	LRIH A HSI	LRKVK S PMPI	EGTAYVIPTT	HTLLA A PGTT	
<i>A. fumigatus</i>	S400 ALS S DEHFPNA	GCWDPHRWEN	QATKEQEN..D	E427 G432 Y431 G434 EVVD Y GGAV	436
<i>M. graminicola</i>	SRMDEHFPDC	LHWEPHRWDE	SPSEKYK H LS	P T TALG S IAE	EKEI Y GH L LV	
<i>A. fumigatus</i>	T440 SK T SSPYLP	G448 FGAGR H RCIG	EKFAYV N LGV	ILATIVRHLR	LFNVDG K KGV	486
<i>M. graminicola</i>	SKGAASPYLP	FGAGR H RCIG	EQFAY V QLQT	ITATMVRDFK	FYNVDG S DNV	
<i>A. fumigatus</i>	Y491 PET V SS I FS	F495 GPMKPS I IGW	EKR S KNTSK.	515		
<i>M. graminicola</i>	VGTDYSS L FS	RPLSPAV V KW	ERREERE E KN			

Fig. 4 Alignment of amino acid (AA) sequences of Cyp51 from *A. fumigatus* (Genbank accession no. AAF32372) and *M. graminicola* (Genbank accession no. ACI29117) where alterations have been associated with azole resistance. References: Leroux and Walker, Pest Management Sci (2011) and as in Table 2. **O** = Codons associated with azole resistance in azole exposed patients. **O** = Codons associated with azole resistance in azole naïve patients or in *M. graminicola*; * associated with a tandem repeat in the promoter region of *A. fumigatus*

plant pathogens by De Ward et al. [90]. This has been studied for azoles in *Botrytis cinerea* [91], *Pyrenophora tritici repentis* [92] and *M. graminicola* [84]. Pump inhibitors like e.g. promazine slightly increase the susceptibility to azoles [87] and the biological potential for efflux resistance exists in the population of *M. graminicola*, but the genes identified have not so far been identified in field isolates [75].

In France, isolates of *M. graminicola* cross resistant to azoles, thiolcarbamates and SDHI's have been reported, suggesting a combination of mutations in *CYP51* and over-expression of drug efflux transporters to be involved (Figs. 1 and 2) [44]. Azole resistant *A. fumigatus* without *CYP51A* mutations have been found in up to 40 % of the resistant isolates in the clinical setting [26•]. To what extent efflux pumps may operate in these isolates and if so to what extent such may be induced by human azole medication or environmental use remains to be understood.

Future Prospects for the Control of Fungal Plant Pathogens

Although the number of fungicide classes from the perspectives of human medicine appears impressive, many are not true options for use as single agents either due to resistance having already emerged or because the risk when used as single agent is too high. Hence, azoles will remain the most commonly used class in cereal crops for the foreseeable future in agriculture. Various initiatives have been undertaken by European authorities as well as by the industrial community (Fungicide Resistance Action Committee, FRAC) to promote practises that reduce risk of selection of resistance although consensus as to how has not been established [82, 93–95]. How this will proceed and to which extent it may influence selection of resistance in human pathogens is yet to be seen.

Conclusions

Substantial data today support that azole resistance in *A. fumigatus* has been induced during long term azole treatment in individual patients but also occurs in naïve patients due to the selection for resistant mutants in the environment. The first azole resistant environmental strain (the TR₃₄/L98H genotype) has spread throughout The Netherlands since 1998 and now accounts for between 6 % and 12.8 % of clinical *A. fumigatus* in this country illustrating the fitness and competitiveness of this genotype [96]. Subsequently, TR₃₄/L98H has also been detected in many other West-European countries including clinical isolates from Denmark [33•], Norway [32], the UK [7•, 11•], Belgium [97], France [97, 98, 99•], and Spain [22], and in the Asia-Pacific region

including India [35•] and China [34•]. Noticeably, West-Europe and Asia-Pacific represent the regions with the highest and second-highest fungicide use in a global perspective, respectively (Fig. 3) [8, 33•, 35•, 36•]. Additionally, two more resistant genotypes have recently been reported in azole naïve patients in France and The Netherlands, and thus again in West-Europe accounting for 37 % of the global fungicide market [42•, 43•]. Importantly, susceptibility testing is not routinely performed in many centres and the resistance rates reported may therefore very well represent the tip of the iceberg. We have yet to see if resistance emerges in S-America where the fungicide use has increased over the recent years and to which extent the resistance rates increase further in the parts of the world where it is already present. Important players are the fitness of the resistant phenotypes and the pattern of azole fungicide use where not only amounts but also choice of individual compounds plays an important role.

Obviously, these changes have clinical implications. Whereas acquired resistance in clinical practise may be expected after long term treatment, it is important to realise that azole susceptibility is not obligate in the azole naïve patients with aspergillosis. This suggests susceptibility testing should be performed in all patients with *Aspergillus* infection requiring antifungal therapy and highlights the need for better diagnostics improving the culture positivity rate and establishing alternative options for culture negative cases like direct detection of prevalent environmental mutants by PCR [100•]. Moreover, initial combination therapy may be considered in areas with higher prevalence of environmental azole resistant isolates for patients with severe infection. And finally, surveillance studies in both the clinical setting and the environment should be conducted in order to provide updated local data on susceptibility rates.

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