Aspergillus fumigatus

Subjects: Agronomy

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Aspergillus fumigatus is a ubiquitous saprophytic fungus. Inhalation of A. fumigatus spores can lead to Invasive Aspergillosis (IA) in people with weakened immune systems. The use of triazole antifungals with the demethylation inhibitor (DMI) mode of action to treat IA is being hampered by the spread of DMI-resistant “ARAf” (azole-resistant Aspergillus fumigatus) genotypes.

Aspergillus fumigatus DMI fungicides azoles resistance

1. Introduction

1.1. Aspergillus fumigatus

Aspergillus fumigatus is a ubiquitous saprophytic ascomycete fungus typically found in nature growing on decaying vegetation on and in soil [1][2][3]. It is thermotolerant, with a broad temperature range for growth (12–65 °C, optimum growth temperature of ~35 °C), and it benefits from high humidity (85–100%), characteristics that allow it to thrive in plant waste heaps [4][5][6].

A. fumigatus is a widespread constituent of the air microflora. Prolific release of conidia [1][7] from natural sources or others associated with human activity (e.g., composting, building work) can lead to high spore counts in aerosols, reaching up to 10^6 colony-forming units (CFUs) per m^3 [8], depending on the location, context, and season. People can inhale *A. fumigatus* spores from the air in both the indoor and outdoor environments [2][4].

A. fumigatus is also the most important of several *Aspergillus* species capable of opportunistic infection of humans and many species of domesticated animal [4][9][10]: its clinical effects range from allergic reactions to chronic pulmonary Aspergillosis (CPA) and acute invasive Aspergillosis (IA) in immunocompromised patients [11][12][13].

1.2. Demethylation Inhibitor (DMI) Antifungals in the Clinic and Fungicides in the Environment

The most important DMI antifungals used in medicine and DMI fungicides used in the outside environment (often referred to generically as “azoles” in the scientific literature) belong to the triazole and imidazole groups, which share the ability to inhibit sterol 14-alpha-demethylase, a member of the P450 family encoded by the *cyp51* gene. Sterol demethylation is intrinsic to the biosynthesis of ergosterol, an essential component of fungal membranes, the lack of which causes a loss of functionality, followed by fungistasis [13][14][15].

Since their first introduction in the 1970s, DMI fungicides have been used for various purposes in the outside environment: as crop protection agents in agriculture and horticulture; as material protectants (e.g., in timber treatment, paints), and for veterinary treatments [4][12][16]. In agriculture, DMI fungicides continue to be indispensable crop protectants for foliar application and seed treatment [17][18], a position they have maintained over several decades because their high-level, broad-spectrum efficacy has been largely resilient towards loss of efficacy against target pathogens: they show partial “shifting” resistance only, in contrast to the full resistance that has developed quite rapidly to other modes of action [12][19].

Many triazoles and imidazoles are active against *A. fumigatus* [20][21], hence the introduction of medical triazole therapies to combat CPA and IA in the late 1980s [6][10]. Triazoles are currently the first-line choice of medical treatment for patients suffering from these conditions. Therapeutic alternatives to triazoles exist (echinocandins and polyenes), but unlike triazoles, they cannot be applied orally (resulting in higher cost) and are less favourable in terms of efficacy and side effects. Thus, they are generally administered in combination with triazoles or are positioned as salvage treatments if triazole therapy fails [9][10][12][13][15].

1.3. Resistance to DMI Fungicides in A. fumigatus

Resistance to DMIs in *A. fumigatus* was first observed in the clinical setting in the late 1990s, followed by a rise in incidence over the last two decades [15][22]. The initial findings were followed by the recovery of resistant isolates from patients in many countries [9][23]. Resistant isolates hinder triazole therapy, and the infection with DMI-resistant *A. fumigatus* (ARAf) is associated with significantly higher patient mortality [9][10][12][13][15][24][25][26][27].

Triazole resistance can be acquired during therapy, particularly in patients undergoing long-term DMI treatment [10][15][28]. ARAf isolates recovered from patients typically carry resistance mechanisms based on point mutations at various positions in the *cyp51A* gene (which decrease the affinity of DMIs for the target protein), altered efflux pump activity, or increased *cyp51A* copy number [11][15] as well as other mechanisms [9][12][13][14]. However, a recent finding indicates that a mutation in the *cyp51B* gene can also confer DMI resistance [29].

Strong evidence for the emergence of resistance within patients during DMI therapy has been provided by applying microsatellite typing to isolates recovered serially from infected individuals [10][11][30]. In-patient emergence of triazole resistance is probably associated with pulmonary cavities, within which the fungus can sporulate asexually from an aspergilloma, providing an opportunity for resistant variants to emerge that are then capable of being selected by the medical DMIs [6][11][12][23].

The recovery of resistant isolates from triazole-naïve IA patients in the late 1990s indicated that the external environment presents an additional route for ARAf infection [11][15][20][25][31][32]. “Environmental” ARAf strains typically combine the insertion of tandem repeats (TRs) in the promoter region of the *cyp51A* target gene (which up-regulate the expression of *cyp51A*) together with single or multiple point mutations in the coding sequence of the gene [9][12][15][33]. The most ubiquitous common mutants recovered from patients that are generally held to be of environmental origin are TR_{34}/L98H, TR_{46}/Y121F/T289A, and TR_{34}/L98H/S297T/F495I [9][10][34][35][36]. TR_{34}/L98H,

which combines a 34 base-pair tandem repeat with a leucine-to-histidine substitution at position 98 in the amino acid coding sequence, was first recovered from a Dutch patient in 1999 [37][38]. It was later found in a sample taken from an Italian patient in 1998 [39], and it has since been detected in clinical settings (e.g., hospital corridors, intensive care units) and recovered from patients and environmental samples from around the globe [15][25][33][40]. A second widespread "environmental" mutant, TR_{46}/Y121F/T289A, was first recovered from a Dutch patient in 2009 [9][10][41][42] and later found in a sample from a US patient taken in 2008 [43]. It too has been detected widely in the environment [44], albeit less frequently than TR_{34}/L98H [45]. Further TR-associated mutants, such as a TR_{53} mutant without substitutions in the *cyp51A* gene, have also been detected [9][46].

While there is some evidence for in-patient emergence of TRs [11][47][48] and nosocomial transfer (i.e., within the clinic) of ARAf isolates [49], the environment is likely to be a relevant source of IA patient contamination with resistant isolates [6]. Several studies have shown correspondence between specific ARAf genotypes isolated from patients and the general or their local environments [15][50][51][52]. ARAf carrying the TR_{34} mutation has also been detected in hospital air and dust samples [53][54][55], as well as hospital gardens, suggesting that the immediate hospital surroundings can contribute to the clinical spore load [53].

A number of DMI fungicides used in the outside environment are intrinsically active against *A. fumigatus* [20][21][56][57]. Cross-resistance in ARAf isolates to these DMIs and the different triazoles used in medicine is common, but not always the case, depending on the genetic configuration of the ARAf genotype [20][52][57]. Unlike other Aspergillus species such as *Aspergillus flavus* and *Aspergillus niger*, *A. fumigatus* is not able to infect living plant tissues, so it is not a crop pathogen and therefore not a target of fungicide applications in agriculture [4]. However, "collateral" exposure of the fungus is possible in environmental settings. For example, DMI fungicides applied to crops start to dissipate after treatment; however, their presence in the treated field can also extend beyond the cropping cycle in the form of steadily declining residues in the soil [58] and on non-harvested parts of the crop, including vegetative substrates capable of colonization by *A. fumigatus*. If DMI residues are present in these substrates at concentrations sufficient to inhibit the growth of the wild-type portion of the *A. fumigatus* population, but not that of resistant isolates, then this convergence may lead to the selection of ARAf. Snelders et al. [20] identified a group of five agricultural DMI fungicides with structural similarity (and cross-resistance) to medical triazoles and pointed out that the timing of market introduction of these compounds immediately preceded the estimated date of origin of TR_{34}/L98H. Toda et al. [59] also related the first recovery of a TR_{34} mutant from a patient in the United States in 2016 to the preceding decade of intensification of national agricultural DMI use. A further indication of the potential participation of agronomic settings in selection for resistance derives from the characterization of ARAf isolates—deriving from clinical, environmental and plant material sources—that are also resistant to other modes of action which are used in agriculture but not in medicine, such as the quinone outside inhibitor (QoI), methyl benzimidazole carbamate (MBC), and succinate dehydrogenase inhibitor (SDHI) fungicide classes [60][61][62].

1.4. Emergence of Resistance in A. fumigatus during Exposure to Agricultural DMIs

Environmental saprophytic fungi such as *A. fumigatus* are often phenotypically and genotypically plastic, allowing them to respond to stressful stimuli (environmental factors, nutrient availability, competitive stress, toxins) via stable genetic or epigenetic changes that are then capable of selection [10].

Laboratory studies have demonstrated the emergence of *cyp51A* gene mutations during exposure to agricultural DMIs in culture media or soil, with mutants showing increased MICs (Minimum Inhibitory Concentrations) and either pan-DMI resistance or resistance to specific medical DMIs [20][63][64][65][66][67][68][69]. For example, Snelders et al. [20] showed the emergence, during in vitro exposure to agricultural DMIs, of *cyp51A* substitutions that are also known to emerge within patients during therapy. They did not detect the emergence of TR_{34}/L98H, although a tripling of the 34 base-pair tandem repeat occurred in an isolate already possessing the TR_{34}/L98H mutation. In contrast, Ren et al. [64] reported the recovery of the resistant genotype TR_{46}/Y121F/T289A after repeated subculturing of an initially wild-type strain on medium supplemented with agricultural DMIs, and Cao et al. [67] recovered TR_{34}/L98H and TR_{34}/L98H/S297T/F495I mutants from tomato leaves and soils treated with the agricultural DMI tebuconazole.

The emergence of mutants in these studies (and in the environment) is assumed to be the result of the spontaneous generation of genetic changes followed by selection under DMI pressure. DMIs have not been shown to be mutagenic: mass production of conidia from fungal colonies provides ample opportunity for spontaneous mutations to occur during the myriad mitotic events leading to the formation of spore masses [6][70]. Evidence for the role of asexual sporulation in the emergence of resistant mutants during exposure to agricultural DMIs in vitro was provided by Zhang et al. [71], who later showed the emergence of a TR_{34}^{3}/L98H isolate following asexual reproduction of a TR_{34}/L98H ancestor in the presence of voriconazole [72].

Alterations in the promotor region (of which tandem repeats are an example) and *cyp51A* single nucleotide polymorphisms typical of environmental ARAf isolates have generally been found to emerge separately in DMI-resistant isolates of plant pathogens directly targeted with agricultural DMIs [9]. However, a combination of these resistance mechanisms has recently been found in the environment in isolates of the plant pathogens *Pyrenopeziza brassicae* and *Pseudocercospora fijiensis* showing decreased sensitivity to DMIs [73][74].

The mutations leading to DMI resistance in ARAf do not generally impose a significant fitness cost, allowing them to survive and spread in the absence of DMIs [11][23][51]. Verweij et al. [70] raise the possibility that tandem repeats in the promoter region may provide a compensatory mechanism for metabolically costly point mutations in the *cyp51A* gene.

1.5. Population Dynamics of Azole-Resistant A. fumigatus in the Environment

DMI resistance is present in environmental populations of *A. fumigatus* around the globe [13][45]. There is evidence that TR-associated resistance has arisen rarely in the environment but has subsequently spread widely, acquiring various point mutations in the meanwhile [12][75]. For example, genomic analysis of both clinical and environmental TR_{34}/L98H and TR_{46}/Y121F/T289A isolates from different geographical locations [35], across India [76] and from

across the UK and Ireland [51] showed that the TR-associated DMI-resistant genotypes show relatively low genetic diversity, evidence that frequent emergence of these mutations is unlikely. The less common TR_{53} mutation first reported from the clinical setting in the Netherlands in 2009 has since been detected in the environment in Europe as well as in Colombia [15][77].

A. fumigatus conidia can be dispersed over long distances in the air, and it is conceivable that the global distribution of TR_{34}/L98H and TR_{46}/Y121F/T289A is the result of conidial spread, as shown by the detection of identical clones in different European countries [60] and by the isolation of identical TR_{34}/L98H and TR_{46}/Y121F/T289A clones from the environment and clinical settings worldwide [35]. However, the transfer of ARAf isolates on plant material such as flower bulbs has been demonstrated [53], and the long-distance spread of resistant isolates by birds is also considered a possibility [78].

2. Characteristics of Agronomic Hotspots for the Amplification and Spread of ARAf

An agronomic hotspot is an agricultural or horticultural setting that fulfils the following conditions: it provides a substrate capable of supporting the growth, reproduction and dispersal of a population of *A. fumigatus*: for selection of resistant genotypes to occur, the prevailing environmental conditions must also allow *A. fumigatus* to grow and complete its life cycle to the point of spore production, and the substrate must contain DMI residues at concentrations capable of selecting ARAf isolates from within a mixed population of susceptible and resistant genotypes [5]. The greater the population size, the greater the probability that mutations conferring DMI resistance emerge that are then capable of selection [11].

Within a hotspot, the selective action of DMI residues results in the resistant proportion of the population being "amplified," i.e., its ability to develop and release propagules into the environment is favoured at the expense of the DMI-susceptible portion [12][56]. This can increase the proportion of ARAf spores in the air spora over "background" levels (**Table 1**). Mass release of conidia is considered essential to the definition of an ARAf hotspot because airborne conidia are the predominant route of infection of patients by *A. fumigatus* deriving from the environment [3][4][15].

Table 1. List of requirements for classifying an agronomic setting as a hotspot for the amplification of DMI fungicide resistance in *Aspergillus fumigatus*.

Requirement	Comments
Favourable conditions for growth and multiplication of *A. fumigatus*	Availability of a suitable organic substrate capable of supporting a sizable population of *A. fumigatus*; prevailing conditions of temperature and humidity provide optimal growth conditions, leading to a competitive advantage for *A. fumigatus* within the fungal/microbial community
Exposure of *A. fumigatus* to residual concentrations of DMI	Residue levels of a specific DMI fungicide exceed its Minimum Inhibitory Concentration (MIC) for wild-type (susceptible) *A. fumigatus*, leading to the selection of resistant *A. fumigatus* genotypes

Requirement	Comments
fungicides that are selective for resistant genotypes	
Mass release of airborne spores of *A. fumigatus* into the environment	Selection of resistant genotypes leads to preferential reproduction and release into the air spora, resulting in an “amplification” of resistance over background levels

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