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Assessment of selection and resistance risk for demethylation inhibitor fungicides in *Aspergillus fumigatus* in agriculture and medicine: a critical review

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Abstract

BACKGROUND: An increasing number of publications have claimed that demethylation inhibitor (DMI) fungicides are confronted with resistance development in the fungus *Aspergillus fumigatus* and that the origin of resistant isolates may also be outside the medical area. For resistance risk assessment and sourcing the origin of DMI resistance, the primary exposure events of *A. fumigatus* with DMI treatments have been analysed case by case, resulting in the pathogen exposure risk (PER).

RESULTS: The calculated maximum exposure concentrations (MEC) are highest during medical treatments (human and veterinary), certain fruit and seed treatments and wood preservation, and are much lower for crop protection applications. Most agricultural DMIs are intrinsically ~ 10–100 times less active than medical DMIs for *A. fumigatus* control and potential resistance selection. However, imazalil is used in agriculture and veterinary medicine (as enilconazole) expressing strong intrinsic activity against *A. fumigatus*. The majority of mutations in the target gene, *cyp51*, of DMI-resistant isolates are different in *A. fumigatus* (e.g. TR_{34}/L98H) in comparison with plant pathogens (e.g. A379G, I381V).

CONCLUSIONS: The assumed selection risk, ASR (MEC × PER) for resistance evolution to DMIs in *A. fumigatus* is estimated to be highest for human and veterinary applications. However, environmental origin of DMI-resistant spores from certain sites cannot be ruled out.

Keywords: agricultural fungicides; *Aspergillus*; azole fungicides; DMI fungicides; DMI resistance; exposure concentrations; medical fungicides; pathogen exposure risk; selection risk; veterinary fungicides

1 INTRODUCTION

Aspergillus fumigatus Fres. (Ascomycetes, Eurotiales, Trichocomaceae) is a saprophytic, thermophilic, ubiquitous fungus widespread in nature (from Antarctic to the Sahara Desert), typically found in soil (as a soil-borne fungus) and on decaying organic matter such as compost, where it plays an essential role in carbon and nitrogen recycling.[1]

When growing on dead organic matter or agar, the fungus produces millions of minute grey–green conidia (2–3 μm in diameter) on its surface, which readily become air-borne.[2] Thus, they are always present in the air both outdoors and indoors, including in homes and hospitals, reaching average concentrations of 10–200 conidia [or colony forming units (cfu)] per m^3 of air.[2–4] It is estimated that everybody inhales several hundred spores each day.

Spores can be disseminated easily, especially when contaminated material (e.g. biowaste, compost, mouldy building material) is moved. The fungus is capable of growing between at 8 and 55 °C with an optimum at 37–43 °C, conditions it regularly encounters in self-heating compost and hay heaps as well as in the human body.[1,2] However, it is not a plant pathogen, does not actively grow on plant surfaces and cannot invade living plant tissue. Thus, it is not a target for agricultural fungicides in crop protection (e.g. spray applications of field and perennial crops). However, it can cause invasive Aspergillosis in immunocompromised individuals, such as organ transplant recipients and people with AIDS or leukaemia; the fungus may become pathogenic (as an opportunistic pathogen), over-running the host's weakened defence system and causing a range of diseases generally termed (invasive) Aspergillosis.[2,5] *Aspergillus* normally enters the lungs by inhalation of air contaminated with spores, infects the alveolar system and can be disseminated through angio-invasion by the bloodstream into other parts of the body.[5,6] However, it can also infect the digestive tract (with contaminated food) and obviously survives there.[7]

The fungus is also the causal agent of several mycoses in the lungs and respiratory tract (sino-nasal Aspergillosis) of animals like cattle, sheep, horses, rodents, dogs, cats, poultry (chicken, geese), birds, reptiles, in farms, homes and zoos.[8,9]

Together with other *Aspergillus*, *Penicillium*, *Botrytis*, *Stachybotris*, *Cladosporium* and *Alternaria* species, *A. fumigatus* can cause strong allergic reactions if patients are exposed to high

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spore concentrations.[9] The fungus, especially when growing on certain building materials, can produce genotoxic and cytotoxic mycotoxins, such as gliotoxin, fumagillin, sphingofungin and Koji acid.[1,10,11]

At optimum temperatures, the fungus has a high competitive saprophytic ability and produces many enzymes such as proteases, amylases, 1,3-β-glucanases, xylanases, α- and β-glucosidases, lipases, aminotransferases, many organic acids (e.g. citric, kojic) and antibiotic metabolites (e.g. fumigacin), and can degrade polymers like starch, cellulose, pectin, chitin, materials such as tannin, peptone, fructosan, mannan, catechin, hair, wood, bark and even pesticides (e.g. atrazine, DDT) and PVC.[1]

A large number of demethylation inhibitor (DMI) fungicides have been used intensively in agriculture and medicine (human and veterinary) since the 1970s.[9,12] More than 30 molecules are registered in each application field, and they are all mechanistically cross-resistant (same mode of action in sterol biosynthesis),[9,12] but only a few (agricultural and medical DMIs) are intrinsically active against *A. fumigatus*.[13]

Resistance (shift in sensitivity) to DMIs has been known for more than 30 years in both agricultural and medical uses against a range of plant and human pathogens.[14–18] In recent years, an increasing proportion of *A. fumigatus* isolates (from patients and the environment) has been observed to be resistant due to the presence of specific single nucleotide polymorphisms (SNPs) in the *cyp51* gene.[19,20] It is possible to detect resistant isolates during treatment of patients with antifungal drugs (in hospitals),[21,22] but also in untreated patients as well as in the environment (e.g. in flower beds near hospitals and in planting substrate).[23–26] However, the origin of DMI-resistant isolates of *A. fumigatus* in the environment is unknown.

Cross-resistance exists, although at different intensities, among medical DMIs and between them and agricultural DMIs for the majority of *A. fumigatus* isolates, if *A.fumigatus*-specific SNPs are present in *cyp51*.[13,16,21,27,28] For other resistance mechanisms, e.g. ATP binding cassette (ABC) and major facilitator superfamily (MFS) transporters, cross-resistance is less pronounced.[15,28] After intensive fungicide use during medical applications, *A. fumigatus* can develop resistance against different chemical classes, especially against DMIs,[21,22,28] but occasionaly to the polyene amphotericin B and rarely to echinocandins and allylamines.[9,17,28,29] The latter three fungicide classes are not used in agriculture. Based on these cases, *A. fumigatus* is ranked as a human pathogen with a high potential risk of developing resistance.

The aim of this contribution is a critical review of the potential selection risks and likely origin of resistance to DMI fungicides in *A. fumigatus* in medicine and agriculture based on published data and theoretical considerations.

2 *A. FUMIGATUS* HABITATS IN THE ENVIRONMENT AND POTENTIAL DMI EXPOSURE EVENTS

The major habitats of *A. fumigatus*, the most important contamination routes and potential DMI exposure events are presented schematically in Fig. 1.

The fungus grows and sporulates frequently in natural soil in the landscape: litter layer, mull and mor-type humus, soil under steppe vegetation with different grasses, peat (cut and uncut), peaty habitats like open bogs, forest soils including nurseries, soil under beech, willow, poplar, hardwood, pine, larch, tropicals, coffee, flax, clover, groundnuts, onions, rice, corn, barley, oat, wheat, strawberry, beans and peas.[1] These habitats typically do not receive direct fungicide treatments, and fungicide concentrations in agricultural soil originating from spray applications of field and perennial crops are relatively low (see Section 5). The fungus can also be isolated from soil in horticulture, for example, plantations, garden soils and different planting substrates, which in some cases may be treated with specific fungicides (only very rarely with DMIs) and nematicides against soil-borne pathogens and nematodes.[25]

Probably the most important habitats for *A. fumigatus* growth and sporulation are decaying plant material of different origins, especially compost, self-heated hay and corn heaps, garden compost, and litter of, for example, ferns, cotton, barley, cabbage, potato and conifers.[1,30] There are no DMI treatments in these habitats.

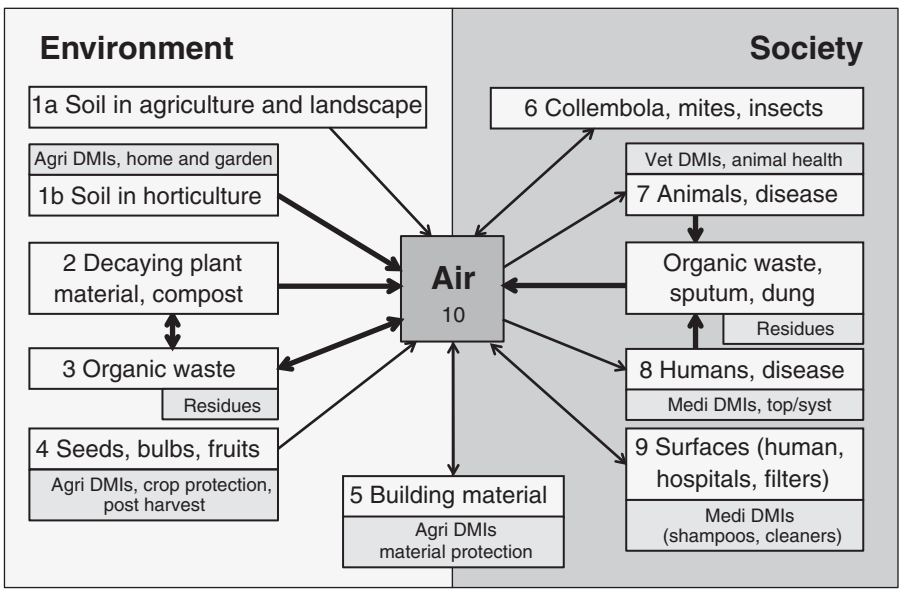

Figure 1. Habitats and potential exposure events of *A. fumigatus* with DMIs in the environment (including soil in landscape, agriculture, horticulture, compost, waste, seeds, fruits) and in society (including human and veterinary medicine, diverse material).

Growth and sporulation of *A. fumigatus* has been repeatedly reported in/on organic waste like activated sludge, slime from paper mills, garbage from homes, compost places (waste piles),[30] animal dung, birds nests, wood chips, corn silage, decaying fruits, humid woollen fabrics and hair. Some DMI residues may be expected, for example, in discarded peel from citrus fruit that has previously been treated (see Section 5).

Under favourable conditions, *A. fumigatus* may survive as spores on seeds (and bulbs, fruits), particularly of corn, but also wheat, barley, oat, rice, sorghum, grasses, cotton, groundnuts, beans, cacao beans and flower bulbs.[1] Because seed treatment, bulb dipping and post-harvest treatment of fruits with fungicides (including DMIs) are common practice, the fungus may be exposed to significant fungicide concentrations.

Like all moulds, *A. fumigatus* can sporulate on humid building materials, walls, wallpaper and surfaces of 'diverse material' (e.g. leather, plastic) although its saprophytic competitiveness is expressed only at elevated temperatures (30–50 °C).[31] Some of these materials (e.g. wood for house building) are impregnated with remarkably high concentrations of fungicide to protect against moulds and decay.

In homes, moulds including *A. fumigatus* can serve as food for collembola and mites living in/on many substrates (e.g. pillow, cat fur) and can be disseminated by these pests. Some of these substrates may be treated with fungicides including DMIs.

Aspergillus fumigatus is the most important causal agent (pathogen) of several mycoses in the lungs and respiratory tract (sino-nasal Aspergillosis) of animals including cattle, sheep, horses, rodents, dogs, cats, poultry (chicken, geese), birds, reptiles, in farms, homes and zoos.[9] DMIs are often used as preventive or curative treatments.[32,33] In addition, animal stables represent ideal conditions for *A. fumigatus* growth and survival (organic debries, dung, waste, humid and warm climate, poor sanitation).

With increasing importance, *A. fumigatus* is the causal agent (opportunistic pathogen) of systemic mycoses in the lungs and respiratory tract (invasive pulmonary Aspergillosis) of immunosuppressive (immunocompromised) human patients.[9] It normally enters the lungs by inhalation of air contaminated with spores, infects the alveolar system and can be disseminated through angio-invasion by the bloodstream to other parts of the body. However, it can also infect the digestive tract.[7] Medical fungicides, especially systemic DMIs (e.g. itraconazole) have been used successfully for many years.[9,34,35]

A major problem for any hygienist in laboratories and hospitals is the survival of mould spores on different surfaces (human skin, hair, nail, ear, nose), personal belongings, like cloths, socks and shoes, and in air-conditioning filters.[4] Antifungal (and biocidal) compounds are added to some shampoos and cleaners.

Air-borne spores of moulds including *A. fumigatus* are present everywhere, especially near composting facilities and stables, but also in private homes and hospitals. In 2012, typical concentrations of mould spores in air were reported as being 135 cfu m^{-3} (5–17 000) indoors and 145 cfu m^{-3} (15–2900) outdoors.[3] The majority of spores were produced by *A. versicolor* (60%) and other Aspergilli (25%) including 5% each of *A. fumigatus* and *A. flavus*, and several *Penicillium* species (16%) with *P. chrysogenum* being the most frequent species (9%).[36] In a recent report,[4] the medical hygienist S. Engelhart described the increased occurrence of invasive aspergilloses in immunosuppressive patients in German hospitals as being primarily related to: (1) demolition, construction and renovation work in hospitals and their surroundings; (2) the lifting of litter and dust outdoors by helicopters and indoors by ventilation systems, vacuum cleaners and air-conditioners without adequate filters; (3) work in gardens and homes with planting substrates and exposure to composting places/facilities; and (4) lack of care when handling biowaste. Compared with the rather low spore concentrations in air (2–30 m^{-3}) in a garden centre, a botanical garden and a forest in Belgium, higher values (10–70 m^{-3}) were observed in a house during renovation, a pigeon coop and a compost bin.[37] Together with other *Aspergillus*, *Penicillium*, *Botrytis*, *Stachybotris*, *Cladosporium* and *Alternaria* species, *A. fumigatus* spores can cause strong allergic reactions if sensitive persons are exposed to high spore concentrations.[10,30] According to official hygiene guidelines in Germany, threshold values for mould spores in indoor air of < 200 cfu m^{-3} are considered normal, 200–500 cfu m^{-3} should trigger long-term measures, 500–1000 cfu m^{-3} requires immediate sanitation and > 1000 cfu m^{-3} requires sanitation and short-term measures.

3 MAJOR AGRICULTURAL AND MEDICAL DMIS

DMI fungicides are very important and effective tools for disease control both in agriculture and medicine. About 30 active ingredients in each application field have been in use since the 1970s. The list of medical DMIs (in alphabetical order)[9] is: in particular as topical imidazoles, bifonazole, butoconazole, climbazole, clomidazole, clotrimazole, croconazole, econazole, enilconazole, fenticonazole, isoconazole, ketoconazole, miconazole, neticonazole, omoconazole, oxiconazole, parconazole, sertaconazole, sulconazole, tioconazole (skin treatments); as topical triazoles albaconazole, fosfluconazole, terconazole; and as systemic triazoles (with strong activity against *A. fumigatus*) fluconazole, itraconazole, posaconazole, ravaconazole, saperconazole, voriconazole and in the near future isavuconazole (oral intake). The list of agricultural DMIs comprises:[12] the piperazine triforine; the pyridines pyrifenox and pyrisoxazole; the pyrimidines fenarimol and nuarimol; the imidazoles imazalil, oxpoconazole, pefurazoate, prochloraz and triflumizole; the triazoles azaconazole, bitertanol, bromuconazole, cyproconazole, difenoconazole, diniconazole, epoxiconazole, etaconazole, fenbuconazole, fluquinconazole, flusilazole, flutriafol, hexaconazole, imibenconazole, ipconazole, metconazole, myclobutanil, penconazole, propiconazole, prothioconazole, simeconazole, tebuconazole, tetraconazole, triadimefon, triadimenol, and triticonazole. The chemical structures of agricultural DMIs can be found in the FRAC Code List 'Modes of action of fungicides' (www.frac.info).[38] The most important commercial DMIs for disease contraol in agriculture and medicine are listed in Table 1.

The most active medical DMIs are the systemic compounds itraconazole, posaconazole, ravaconazole and voriconazole used primarily in humans, but also in veterinary medicine, and enilconazole used in veterinary medicine. Similar *in vitro* activity against *A. fumigatus*, although not designed for *A. fumigatus* control, is expressed by the agricultural DMIs imazalil (identical molecule to enilconazole), metconazole and prochloraz (Table 1).[13] Moderate activity against *A. fumigatus* ($\sim$10–50 times less active than systemic medical DMIs) can be assigned to econazole, fluconazole, ketoconazole and saperconazole for medical applications (mostly topical) and to some agricultural DMIs such as bromuconazole, difenoconazole, propiconazole and tebuconazole (Table 1).[13] Epoxiconazole and prothioconazole are $\sim$20–150 times less active, all other medical and agricultural DMIs (e.g. penconazole, cyproconazole, myclobutanil) are considered

Table 1. Introduction of major DMIs to the market. Compounds with *in vitro* activity against *A. fumigatus* are highlighted: strong activity (median $MIC_{50} < 0.5\ mg\ L^{-1}$, typical for systemic medical triazoles) is in bold, moderate activity ($0.5 < MIC_{50} < 5\ mg\ L^{-1}$, i.e. 10–50 times less active than systemic medical triazoles) is underlined; others are > 100 times less active (values from Snelders *et al*)[13]

Introduction period	Agriculture Major sensitivity shifts for at-risk pathogens: 1983–2000 (for *M. graminicola*: 2000–2008)	Human and veterinary medicine Major sensitivity issues since 1994 (e.g. in *Candida*) (for *A. fumigatus*: since 1998)
1970–1979	**imazalil**, fenarimol, triadimefon, triadimenol, propiconazole	miconazole (topical), econazole (topical), clotrimazole and others
1980–1989	**prochloraz**, flutriafol, flusilazole, cyproconazole, penconazole, bitertanol, tebuconazole, myclobutanil, difenoconazole and others	ketoconazole (topical, human/vetrinary), fluconazole (systemic, human/veterinary), **enilconazole** (veterinary) and many others
1990–1999	tetraconazole, triticonazole, epoxiconazole, **metconazole**, fluquinconazole, bromuconazole	**itraconazole** (systemic, human/veterinary), saperconazole, climbazole (in shampoos, cleaners) and many others
2000–2009	prothioconazole (main introduction period for quinone outside inhibitors)	**voriconazole** (systemic), **posaconazole** (systemic), **ravaconazole** (systemic)

The identical molecule is used in agriculture under the name imazalil and in medicine under the name enilconazole.

to be not active against *A. fumigatus* (> 100 times less active).[13] Based on their strong intrinsic activity, the systemic medical triazoles, as well as some agricultural azoles (including imazalil and also prochloraz and metconazole), may select resistant mutants in *A. fumigatus*, depending on product use strategies. Other agricultural DMIs (e.g. tebuconazole, propiconazole) contribute only little (if at all) to resistance selection due to insufficient activity and low exposure concentrations in most crop protection applications (see Section 5).

Medical and agricultural DMIs share the same site of action (binding site), although with different docking affinities to the CYP51 enzyme:[13,15,27] i.e. inhibition of 14C-α-demethylase in biosynthesis of ergosterol.[9,12] Cross-resistance exists, although at different intensities, among all agricultural and medical DMIs and between the two groups for the majority of isolates in all fungal species, especially when certain SNPs (mutations) are present in the *cyp51* gene.[13,15,39] For other resistance mechanisms (e.g. overexpression of ABC and MFS transporters, see Section 4), cross-resistance is less pronounced. However, no cross-resistance exists between DMIs and molecules with other modes of action (in both agriculture and medicine). For *A. fumigatus* control in medicine, additional modes of action such as the polyenes (e.g. amphotericin B, nystatin), echinocandins (e.g. caspofungin, anidulafungin) and allylamines (e.g. terbinafine and naftifine) are available.

Whether and in what quantities a fungicide will actually reach the target site (i.e. bioavailability for pathogen) depend primarily on the physicochemical properties of the molecule, for example, solubility in water and log P (and K_{om} in soil) as well as on the application methods, fungicide retention (and interception) in the substrate and 'transport properties' of the substrate (plant, human tissue, soil) such as water potential and 'health status' of the material (e.g. living or dead tissue).

4 RESISTANCE MECHANISMS AND SELECTION

Repeated exposure of patients to antimycotics, especially to DMIs, can lead to drug resistance in *A. fumigatus*.[28] Indeed, DMI-resistant isolates have been found in patients for some years in several European countries and India,[19–22,40] and resistant spores are present in the air.[37] Fluconazole, introduced to the market in the 1980s, with limited activity against *Aspergillus* species, is frequently used as prophylaxis in leukaemia patients and bone marrow transplant recipients. Results generated by Liu *et al* suggest that pre-exposure of *A. fumigatus* to fluconazole attenuated the *in vitro* fungicidal activity of subsequent itraconazole use against it.[41] To our knowledge, extended sensitivity monitoring of *A. fumigatus* isolates has been undertaken only since the first years of this century.[19] Thus, a low level of DMI resistance in *A. fumigatus* might have been around for a long time but remained undetected. Resistance (*in vitro* and *in vivo*) of *A. fumigatus* has also been described in several isolates for the polyene fungicide amphotericin B,[17,29] a chemical class that has never been used in agriculture, but is occasionally used in veterinary medicine (dog, cat).[33] However, resistance to echinocandins and allylamines, although reported,[28] seems to be very rare. Based on these results, *A. fumigatus* may have a high potential for acquiring resistance to fungicides in medical applications.

There are basically four (five) mechanisms contributing to resistance against DMIs.

(1) Mutations in the *cyp51 (erg11)* gene. Different mutations, often in combination, have been reported to confer resistance to DMIs in pathogens with one *cyp51* paralogue. In the plant pathogen *Mycosphaerella graminicola* mainly V136A/C/G, Y137F, A379G, I381V, S524T, Y459C/D/N/P/S, G460D and Y461D/H/S have a direct impact on sensitivity to DMIs.[15,39,42,43] For *Candida albicans*, > 100 SNPs have been reported to contribute to azole resistance using *in vitro* assays, mainly G129A, Y132F/H, K143E/R, S279F, G307S, S405F/P, G448E/R/V and G450E/R/V.[17,44,45] In *A. fumigatus*, > 50 SNPs have been reported, of which mainly G54E/K/R/V/W, TR_{34}/L98H, Y121F, 138C/R, M220I/K/R/T/V/W, T289A, Y431C and G448S were commonly found in azole-resistant isolates (*cyp51A* paralogue).[21,46,47] Consequences of the presence of SNPs are decreased fungicide binding to the target enzyme. (Note: there are differences in amino acid numbering when fungi are compared: amino acid position 121 in *A. fumigatus* corresponds to position 137 in *M. graminicola* and position 132 in *C. albicans*, and 98 to 114, 220 to 239, 289 to 308 and 431 to 459 in *M. graminicola*). Only a few mutations at conserved residues encoding resistance are common between *A. fumigatus* and other fungi/yeast: Y121F

in *A. fumigatus* corresponding to Y132F in *C. albicans* and Y137F in *M. graminicola* and Y431C corresponding to Y459C in *M. graminicola*.

There are three possible explanations for the different SNP pattern in the *cyp51* gene:

i The identified mutations are species specific (e.g. L98H specific for *A. fumigatus*, A379G and I381V specific for plant pathogens) and would be selected similarly by all DMIs independent of their chemical structure and use pattern (i.e. medical and agricultural fungicides would basically select the same SNPs in a given species).

ii The identified mutations are paralogue specific because all reported amino acid changes in *A. fumigatus* are in the *cyp51A* paralogue, which is absent in most other fungi, and *cyp51A* and *cyp51B* differentially affect azole activity in *A. fumigatus* and *Fusarium graminearum* compared to other fungi.[48,49]

iii The *A. fumigatus* mutations (e.g. L98H in combination with a 34 bp tandem repeat [insert] in the *cyp51* promotor, TR_{34}/L98H) evolved through preferential selection, i.e. medical DMIs specifically select different mutations than agricultural DMIs as it is known to occur with several agricultural DMIs in field populations of *M. graminicola* and also among molecules of the SDHIs, succinate dehydrogenase inhibitors.[42]

In *A. fumigatus*, mutations at position M220 confer resistance specifically against itraconazole, whereas TR_{34}/L98H against all medical DMIs (itra-, posa-, vori-, ravu-conazole).[28] In clinical samples of resistant *A. fumigatus*, 18 SNPs were identified, which have not been found in environmental samples, except for TR_{34}/L98H and TR_{34}/Y121F/T289A which were found in both origins.[25,50] TR_{34}/L98H was found recently in clinical samples in many countries (e.g. NL, DK, E, F, B, N, India, China),[13,25,40] which may be the result of recurrent local selection rather than migration from one primary source.

(2) Increased expression of *cyp51 (erg11)* gene (promotor insertions). The consequences are an increased production of target enzyme resulting in lower activity of the fungicide (valid for both agricultural and medical pathogens). Several reports are available describing resistance to imazalil in different *Penicillium* spp. (e.g. *P. digitatum, P. italicum*) as being related to tandem repeats in the promotor (overexpression of *cyp51* gene) and nucleotide insertions in the promotor.[51,52] However, unlike in *A. fumigatus* where mutations were found in combination with promotor tandem repeats, no SNPs in the *cyp51* gene were reported in *Penicillium* species.

(3) Increased expression of multidrug efflux (transporter) genes (e.g. ABC, multidrug resistance [MDR] and MFS).[39,53] Consequences are an increased activity of specific membrane (ABC, MFS) pumps partly removing fungicides from fungal cells, resulting in lower fungicide concentrations at the target site (valid for both agricultural and medical pathogens). In *A. fumigatus*, the ABC/MDR mechanism (AfuMDR, MFS) was frequently reported as a resistance mechanism (in addition to SNPs in the *cyp51* gene).[28] In that case, cross-resistance is not always obvious including medical DMIs.

(4) Accumulation of intermediate (toxic) sterols as a result of lanosterol-C14α-demethylase inhibition in the ergosterol biosynthesis pathway.

(5) Intrinsic resistance (tolerance), probably based on the specific molecular architecture of the *cyp51* gene. Aspergillosis in patients can be caused by a range of *Aspergillus* species: of 94 isolates gained from sputa and bronchial washes from the respiratory tract of patients in UK hospitals, 71, 11, 11 and 7% were *A. fumigatus, A. flavus, A. niger* and *A. terreus*, respectively.[19] Obviously, some of the *Aspergillus* species may be intrinsically resistant to azoles *(A. lentulus)* or amphotericin B *(A. terreus)*.[25]

Selection of resistant individuals (mutants) by applying a fungicide against wild-type populations can occur only:[12,15] (1) when the fungus is in an active developmental stage (mycelial growth, spore germination, sporulation, logarithmic phase of disease development) but not, when the fungus is in a dormant stage (e.g. as spores); (2) by using effective concentrations (e.g. $> ED_{90}$ *in vitro*; recommended full n-rate for field applications in agriculture) of an effective product (intrinsically active), but not when the dose is strongly sublethal or by residue concentrations, and not, when a product is intrinsically not enough active); (3) by continuous exposure (selection) of populations over long periods (e.g. season long in agriculture, for months in patients); and (4) by broad fungicide usage (over area and time), e.g. for triazoles (in agriculture) typically after 5–10 years of intensive product use, depending on pathogen species.

In most cases, a high number of applications and long-lasting selection pressure will accelerated resistance development. Whether lower than recommended rates (e.g. half rates) may influence selection process, is debatable, in line with likely variation in selection for resistance also being dependent on the intrinsic potency of the individual compound. Thus, it is almost impossible to give a critical threshold concentration for selecting resistant induviduals. However, it can be assumed that selection may occur only at concentrations in the colonized substrate (e.g. mg dm^{-3} or mg kg^{-1} of soil, plant, human tissue) corresponding to at least 'full rates' ($> EC_{90}$, MIC_{100} values *in vitro*, full n-rates in agriculture, recommended daily dose in medicine) because resistant individuals are normally at the upper end of or even outside the log-normal sensitivity distribution of populations.[38,39] Resistance development can be delayed by using alternations of and mixtures with effective companion fungicides at effective rates (a common practice in agriculture).[38] Resistant *A. fumigatus* isolates emerged during the therapy of a patient after 125 weeks of DMI treatment.[22] After isolation, many *in vitro* resistant *A. fumigatus* isolates were less virulent in a mouse model.[22] However, resistant *A. fumigatus* isolates can obviously survive in nature[37] and may be equally fit as wild-type isolates.

5 POTENTIAL EXPOSURE OF *A. FUMIGATUS* TO DMIS IN AGRICULTURE AND MEDICINE

To assess the exposure risk, it is essential to consider the quantity, *Q*, of a fungicide per volume of substrate (e.g. soil, plant) to which a fungus is exposed over time (as done in ecology and toxicology),[54] expressed as, for example, mg dm^{-3} (or mg L^{-1} or ppm). Using this procedure, fungus exposure can be compared among different substrates. However, it is not essential for the fungus at a given site to know 'how many tons of fungicide are put into the environment'.[13,25,55] For simplicity, 1 dm^3 (10 × 10 × 10 cm) is set here as equal to a volume of 1 L or a weight of 1 kg of substrate (soil, plant, human body, assuming the substrate density is ~ 1 kg dm^{-3} = 1 g cm^{-3}). In agriculture, product

rates are often given as g ha^{-1} (1 ha = 10 000 m^2 = 1 000 000 dm^2). Assuming the total quantity of applied (sprayed) fungicide, e.g. 100 g ha^{-1}, would reach the naked soil surface, the average quantity in the top 10 cm (1 dm) of soil would be: $Q = 0.1$ mg dm^{-3}, ~0.1 mg kg^{-1}, ~0.1 mg L^{-1} = 0.1 ppm. A soil layer of 10 cm is considered significant for this purpose because > 80% of micro-organisms live in this soil layer.[54] Q would be 1 ppm if a soil layer of 1 cm is considered (theoretical soil surface).

Below, the 15 most important DMI application types in agriculture and medicine are evaluated in terms of calculated maximum exposure concentrations (MEC, mg L^{-1} or dm^{-3} or kg^{-1}; Table 2) taking into account the recommended use rates and number of applications.

5.1 Application types (1) to (4): spray applications in field crops, orchards/vineyards and vegetables/berries/ornamentals

In field crops (e.g. cereals, rape, sugar beet, potato), DMI use rates are commonly 100–200 g a.i. ha^{-1} (depending on the product, e.g. epoxiconazole, propiconazole, prothioconazole, difenoconazole) with two or three applications per year. The interception value for sprayed pesticides in cereals (amount reaching the soil surface with applications at growth stages GS 29, 39, 59) are 50, 30 and 10%, respectively. The quantity, Q, in the top 10 cm soil for the first, second and third application (each at 200 g ha^{-1}) is 0.1, 0.06 and 0.02 mg L^{-1}, respectively. The calculated total quantity in the top 10 cm soil is 0.18 mg L^{-1} soil (for a total of three applications each at 200 g ha^{-1}) and 0.08 mg L^{-1} soil (for a total of two applications each at 100 g ha^{-1}; Table 2). If a soil layer of 1 cm is considered with the same DMI use pattern (theoretical soil surface), Q values are 10 times higher (0.8–1.8 mg L^{-1}). Spray intervals in cereals are mostly ~20 d, resulting in a total exposure time for three applications of a minimum of 60 d. The degradation of triazoles in soil is rather slow (DT_{50} 30–300 d, Pesticide Manual BCPC). Thus, exposure of fungi in soil to DMIs may be ~3-4 months. However, DMIs are strongly adsorbed to soil organic matter (K_{oc} 200–3000 mL g^{-1}, depending on molecule and soil type, Pesticide Manual BCPC), and are considered more or less immobile and no longer bioavailable after a short period (also valid for resistance selection). In field experiments, the expected maximum accumulation of, for example, difenoconazole in soil (at plateau concentration) was reported to be 0.03 mg kg^{-1}, supporting the validity of the presented calculations. Based on these experimental and calculated values, DMI concentrations in soil are considered to be too low for resistance selection.

Considering the same spray regimes as described above, the amount of residues in straw can be estimated as follows. Interception values (uptake into cereal plants) are 50, 70 and 90%, resulting in fungicide concentrations of Q = 100, 140 and 180 g ha^{-1}, respectively, for three applications (total of 420 g ha^{-1}). Assuming an average grain yield (for wheat in Western Europe) of 60–90 dt ha^{-1} and an average grain–straw factor of 0.9 (for wheat 0.83, for barley 0.95, resulting in 50–80 dt straw ha^{-1}), the estimated residue concentrations (RC_{max}) are 50–85 and 30–50 mg kg^{-1} straw (for three and two applications, respectively, at last spray). Assuming that fungicide degradation in the plant reaches ~75% before harvest, the calculated maximum fungicide residue concentration in mature straw is ~8–20 mg kg^{-1} straw. Frequently measured residue concentrations for different azoles in straw harvested in controlled field trials were between 0.5 and 12 mg kg^{-1}.[56,57] In fact, the tolerances for residues (maximum residue limit [MRL] for calculating the feed burden) in wheat straw were set by the US EPA at 5 mg kg^{-1} (prothioconazole, tebuconazole) to 10 mg kg^{-1} (propiconazole).[57] These fungicide concentrations might be high enough to select resistance in fungi growing on straw. However, frequent fungal saprophytes with high competitive capacity for decomposing wheat staw in nature are *Mucor hiemalis*, *Agrocybe gibberosa*, *Chaetomium globosum*, *Sphaerobolus stellatus*, *Fusarium culmorum*, *Trichoderma viride* and basidiomycetes such as *Coprinus comatus*, *Trametes versicolor* and *Typhula* sp.,[1,54,58] but not really *A. fumigatus*. Whether straw containing high DMI residue concentrations which is contaminated with animal manure in stables allowing fungal growth, may favour selection of resistant *A. fumigatus* needs to be investigated.

In orchards and vineyards, DMI use rates are commonly 10–25 g a.i. ha^{-1} (= 1–2.5 g hL^{-1} if the spray volume is 1000 L ha^{-1}; e.g. for penconazole, difenoconazole, myclobutanil) with a maximum of six spray applications per season; interception values for the soil surface are assumed to be ~20%. Thus, the calculated total quantity in the top 10 cm soil is 0.012–0.03 mg L^{-1} soil (Table 2). Spray intervals are ~12 d, resulting in a total exposure time of a minimum of 72 d. In berries, ornamentals and vegetables, a maximum of six spray applications is carried out. The use rates are normally 1–3 g a.i. hL^{-1} (e.g. for penconazole in vegetables) to 10–20 g a.i. hL^{-1} (e.g. for difenoconazole in strawberries and roses) to 5-30 g a.i. hL^{-1} (e.g. for imazalil in ornamentals); interception values for the soil surface are assumed to be 50%. Thus, the calculated total quantity in the top 10 cm soil is 0.03–0.09 mg L^{-1} soil, for a use rate of 1–3 g hL^{-1}, and 0.3–0.9 mg L^{-1} soil, for a use rate of 10–30 g hL^{-1}. Spray intervals are ~12 d, the total exposure time is a minimum of 72 d (Table 2). The calculated exposure concentrations are considered as too low for resistance selection. One might argue that fungi on or in treated fruits may also have been exposed to DMIs. After season-long spray applications with DMIs, the MRL in edible plant material was set at 0.2 mg kg^{-1} (e.g. for penconazole). Considering a safety factor of ~10, acceptable maximal residue (R_{max}) values are ~0.02 mg kg^{-1}. Under good field practice (GFP) conditions, these values are normally not surpassed. They are much too low for resistance selection.

5.2 Application type (5): post harvest treatments of fruits (e.g. citrus)

Many fruits are treated with fungicides after harvest for protection against decay (e.g. by dipping, spray on a conveyor belt, incorporation in a wax coat). Common use rates are 2–4 g a.i. t^{-1} fruits (2–4 mg kg^{-1}; e.g. for imazalil in citrus against *Penicillium* spp.) with one application. DMI fungicides are reported to remain mainly (85–95%) in the fruit peel. Recent residue measurements of imported mandarins and clementines from Spain, Italy and Greece to Austria revealed imazalil concentrations 1–3 mg kg^{-1} fruits.[59] This is the concentration in/on decaying fruits to which a mould (*Penicillium, Aspergillus* spp., including *A. fumigatus*, e.g. in biowaste) would be exposed (Table 2). Because of ideal growth conditions for moulds in biowaste, selection for resistance cannot be excluded.

5.3 Application types (6) to (9): seed, bulb and soil treatment

Most seeds of field crops (especially cereals, corn, peas, sugar beet, cotton) are treated with insecticides and fungicides (including DMIs) before planting to protect against seed- and soil-borne pathogens and pests. Two aspects have to be considered, the

Table 2. Calculated potential MEC for DMIs applied to pathogens in agriculture and medicine.

No.	Type of application	DMI use rate	No. of applications	Interception* (%)	MEC, mg L^{-1} (kg, dm^3)	Substrate	Exposure time	Comments
Agriculture								
1	Spray: field crops	100 g ha^{-1} 200 g ha^{-1}	2 (GS 39/59) 3 (GS 29/39/59)	30/10 50/30/10	0.08 0.18	In soil (top 10 cm)	90–120 d	DT_{50} : 30–300 d; K_{oc} : 200–3000 (strong adsorption to soil)
2	Spray: orchard/vine	10–20 g ha^{-1} (1–2.5 g hL^{-1})	Max. 6	~20	0.012–0.03	In soil (top 10 cm)	Min. 72 d	Spray volume ~1000 L ha^{-1}
3	Spray: veg./berr./orna.	1–3 g hL^{-1} 10–30 g ha^{-1}	Max. 6	~50	0.03–0.09 0.3–0.9	In soil (top 10 cm)	Min. 72 d	Spray volume ~1000 L ha^{-1}
4	Residues: fruit/veg.		Season-long sprays		~0.02 (mg kg^{-1})	In/on fruit		Av. MRL: ~0.2 mg kg^{-1}
4a	Residues: straw	100 g ha^{-1} 200 g ha^{-1}	2 (GS 39/59) 3 (GS 29/39/59)	70/90 50/70/90	8–12 12–20	In straw		Av. MRL: ~0.5–12 mg kg^{-1}
5	Post harvest: citrus	2–4 mg kg^{-1} fruits	1	85–95% in peel	1–3 (mg kg^{-1})	In fruit peel	Several weeks	Dip or wax application
6	Seed treatment	5–10 (–150) g 100 kg^{-1} seeds	1		0.01–0.02 (–0.3)	In soil (top 10 cm)	Several weeks	Seeding density: 100–200 kg ha^{-1}; DMI input 10–20 g ha^{-1}
7	Seed treatmet: cereals	5–10 g 100 kg^{-1} seeds (wheat/corn)	1	100% retention to seed	65–130 (w) 100–200 (c) (mg dm^{-3})	In/on seed coat, 1 mm thick	Several weeks	TWG (wheat/corn): ~50/300 g yielding A ~380/1500 cm^2 (1 grain ~3.5/7 mm diam)
7a	Bulb dipping: flowers	0.2% a.i., 5 L 100 kg^{-1} bulbs	1	100% retention to bulb	300/3000 (mg dm^{-3})	In film (bulb) 1/0.1 mm thick		1 bulb: ~30 g, ~4 cm diam. yielding A ~50 cm^2
8	Soil drench: flowers	0.075% a.i., 1000 L ha^{-1}	Mostly only 1		0.8	In soil (top 10 cm)	Several weeks	Strong adsorption to soil; DMI use rare
9	Substrate mixture	Unknown	1		Unknown	In soil	Several weeks	Not known whether DMIs are used commercially at all
10	Wood preservation	Pressure treatment	1	Retention conc.	30–300 (mg dm^{-3})	In indoor wood	Months to years	MEC in wood for in-soil use up to 5000 mg dm^{-3}
11	Material protection	e.g. 0.2–0.6% a.i. in stain	1		Unknown	In paints, plastics	Months	DMIs not important commercially
Medicine								
12	Systemic (iv, oral): humans, *A. fumigatus*	400–800 mg d^{-1} for adults	Split in 2–3 d^{-1}	55–95	5–10 (mg kg^{-1} bw)	In human body	60–150 days (– 900 d)	50% elimination in human body: 10–30 h
13	Topical (cream): humans, *A. fumigatus*	1% (cream): 2 mg cm^{-2} cream/skin	Daily		200/2000 (mg dm^{-3})	In film/skin 1/0.1 mm thick	Several weeks	
14	Topial, systemic (iv, oral) dip, wash: aminals *A. fumigatus*		1–2 d^{-1}		5–30 (mg kg^{-1} bw)	In animal body	40–60 d	Cats, dogs, birds, chickens, rabbits, horses, cattle, sheep, reptiles, turtles, sankes
15	Surface (living and material)	3.5% (e.g. in shampoo), triazoles in cleaners	Regularly		High	On surfaces of humans/animals/materials		Shampoos for humans, dogs

Calculations based on recommended DMI use rates. *Interception (1, 2, 3, 4a) or retention (5, 7, 7a, 10, 12).

fungicide input into the soil (important for soil-borne pathogens) and the quantity of fungicide on the seed surface (against seed-borne pathogens). For most DMIs, use rates are 5–10 g a.i. 100 kg^{-1} seeds (e.g. cereals; rarely 5–60 g a.i. 100 kg^{-1} seeds [for bitertanol] and 5–150 g a.i. 100 kg^{-1} seeds [for triticonazole]), applied once before planting. Assuming a seeding density for cereals of 100–200 kg seeds ha^{-1}, the calculated maximum fungicide input into the top 10 cm soil is 10–20 (–300) g ha^{-1} or 0.01–0.02 (–0.3 [triticonazole]) mg L^{-1} soil (Table 2). These concentrations are too low for resistance selection. The 1000-grain-weight (TGW) for wheat is ~50 g; one wheat grain (average diameter 3.5 mm) has an average surface $A = 38\ mm^2$; for 1000 grains (50 g) $A = 38\,000\ mm^2$; for 2000 grains (100 g) $A = 76\,000\ mm^2$. Thus, wheat seed treatment with DMIs at 5–10 g a.i. 100 kg^{-1} seeds results in a surface concentration (C) of 6.5–13 mg 100 000 mm^{-2} (if retention of fungicide is 100%). Assuming that the fungicide penetrates 1 mm into the seed coat, the concentration to which a fungus on the seed coat is exposed to is 65–130 mg dm^{-3} (mg L^{-1} = ppm; Table 2). The same calculation can be done for corn seeds, with a TGW of ~300 g and an average seed diameter of 7 mm and a surface of 150 mm^2. For 1-mm-thick corn seed coat, $C = 100–200$ mg dm^{-3}. This is the concentration to which a seed-borne fungus sitting/growing on/in the seed coat or in the 'soil inhibition zone' (around the seed) would be exposed. These concentrations are available only at planting. The concentration around the seed (in inhibition zone) decreases exponentially over distance. Seed treatment is carried out only once (per season), mostly with fungicide mixtures to delay resistance evolution and enlarge activity spectrum; the treated seed remains covered in soil and decays shortly after germination. Thus, it is debatable whether resistance selection on treated seed surfaces represents a significant concern, although the calculated concentrations are rather high.

Occasionally, DMIs such as prothioconazole, propiconazole and prochloraz are used to treat flower bulbs (e.g. tulips) by dip or spray application. In the Netherlands, prothioconazole (480 g L^{-1}) is used at concentrations of 0.2%. One tulip bulb with an average diameter of 4 cm has a surface of ~50 cm^2 and a weight of 30 g. Assuming that 5 L of fungicide suspension per 100 kg bulbs are used, the calculated average concentration in a 1 mm (1000 μm) film around the bulb (100% fungicide retention) is: $C = 300$ mg dm^{-3}. Most likely, the film around a bulb is only 100 μm or less, then $C = 3000$ mg dm^{-3} (Table 2). These concentrations may be high enough for resistance selection, especially when infested bulbs are stored in special containments during overwintering. To consider fungicide inputs into soil with treated bulbs (10 000 kg bulbs ha^{-1}), the DMI concentration in the top 10 cm soil is ~1 mg L^{-1} soil. In garden centres or private gardens (flower beds), DMIs may also be used occasionally as soil drench. For tulips, DMIs (e.g. propiconazole) at concentrations of 0.08% (0.8 mg a.i. L^{-1}) may be applied at planting with 1000 L suspension ha^{-1}, resulting in an average concentration of 0.8 mg L^{-1} soil in the top 10 cm of soil. Because DMIs are strongly bound to soil organic matter, treatment of planting substrates (soil, peat, mixtures with perlite) is probably not feasable commercially. Frequently used pesticides for planting substrates are propamocarb, metalaxyl and some nematicides.

5.4 Application types (10) and (11): wood preservation and material protection

In some instances, wooden timber is treated with fungicides to protect from decay caused by a range of fungi such as *Coniophora puteana*, *Coriolus versicolor*, *Gloeophyllum abietinum*, *G. trabeum*, *Lentinus tigrinus*, *Poria monticola*, *P. placenta*, *Serpula lacrymans*, *Stereum sanguinolentum* and *Tyromyces palustris*. Treatments, especially against Basidiomycetes, are sometimes carried out using DMIs, although other chemical classes are commercially much more important such as butylcarbamates, different copper, zinc and ammonium formulations.[60] Typical (retention) concentrations in wood which has been pressure-treated with propiconazole or tebuconazole are 30–300 mg dm^{-3} wood for indoor and up to 1000 mg dm^{-3} wood for in-soil uses (Table 2).[61,62] The exposure time is long (months to years) and the concentrations probably high enough to express significant selection pressure for resistance. However, *A. fumigatus* is not likely to competitively attack and degrade treated wood under these conditions. In addition, treatments are made preventatively a long time before any mould attack may occur. Very little public information is available regarding the extent to which DMIs are used commercially for material protection (e.g. for wallpaper, plastics, leather, paints). Typical mould control agents are sodium hypochlorite, hydrogen peroxide, quarternary ammonium salts, dimethyl ammonium chloride, glutaraldehyde, aluminium oxide, silicic acid, fatty acids, potassium palmitate, magnesium oxide, potassium chloride, calcium hydroxide, titanium dioxide, colophonium-glycerol ester as potassium soap. Widespread moulds on wet walls are *A. versicolor* (dominant species, high spore concentrations in air, see Section 2), *A. glaucus*, *A. fumigatus*, *A. tamarii*, *Aureobasidium pullulans*, *Botrytis cinera*, *Chaetomium globosum*, *Cladosporium sphaerospermum*, *Penicillium brevicompactum*, *P. chrysogenum* and *Trichoderma longibrachiatum*.[3,31,36,] Few DMIs such as azaconazole (0.3% in stain), propiconazole (0.6%) and tebuconazole (0.2%) are mentioned in the literature in the context of material protection. However, no validated information was found about exposure concentrations in, for example, coats of paint on wood or walls. Therefore, it is very speculative to claim that selection of DMI resistance in *A. fumigatus* strains may occur in reality.

5.5 Application types (12) and (13) : systemic and topical treatment (oral or skin application) of humans against (invasive or non-invasive) *A. fumigatus*

DMIs (azoles) are the most important and widely used antifungal agents in human medicine against a range of diseases including Aspergillosis and Candidosis. The following azoles are commercially used for *A. fumigatus* control:[35] fluconazole (only weakly effective against *A. fumigatus*) at 600 mg d^{-1} (to 1800 mg d^{-1}) over 6–8 weeks; itraconazole at 400–600 mg d^{-1} over 2–5 months (up to 2.5 years); voriconazole at 400 mg d^{-1} (oral), 600–900 mg d^{-1} (intravenous) over 2–5 months; and posaconazole at 600 mg d^{-1} (preventative) to 800 mg d^{-1} (curative) over 2–5 months. To reach a constant level of antifungal drug in the body, daily application is needed, because the half-life (50% elimination) of azoles in the human body is 10–30 h. The bioavailability in the body is 55% (for itraconazole) to 96% (for voriconazole). For an adult patient (assumed average body weight, bw: 70 kg), *A. fumigatus* (in the lung) is exposed daily to calculated average azole concentrations of 5-10 mg kg^{-1} bw over several months (depending on disease severity; Table 2). Because treatments are normally made as a therapy when the pathogen has already spread in the human body, selection of resistance may develop more easily than with preventative treatments. Some azoles are specifically used for topical (skin) treatments (e.g. in creams, lotions) against *A. fumigatus* (econazole and ketoconazole are recommended as 1% cream). A standard application rate for

creams (e.g. for efficacy testing) is 2 mg cream cm^{-2} surface. Thus, the local concentration of the active ingredient is ~ 200 mg dm^{-3} skin for a 1 mm skin penetration, and 2000 mg dm^{-3} skin for 0.1 mm skin penetration (Table 2). The calculated concentrations for both topical and systemic treatments are considered to be high enough for potential resistance selection, particularly because some of the used azoles are highly active against *A. fumigatus*. Whether selection happens in reality depends on the exposure time, use pattern and disease intensity. In fact, DMI-resistant *A. fumigatus* isolates have been described in patients after 125 weeks of DMI treatment in the Netherlands and in more than 10 treated patients in UK hospitals.[21,22]. Although patient-to-patient transmission of Aspergillosis is rather unlikely (P. Verweij, pers. commun.), the number of *Aspergillus* cells in a sick patient is high enough to allow resistance selection during the rather long fungicide exposure time.

5.6 Application type (14): treatment (topical, fumigation, oral, intravenous) of animals against *A. fumigatus*

As in human medicine, *A. fumigatus* is an important pathogen for animals (not necessarily only as invasive Aspergillosis), and azoles are used quite frequently for disease control in animal health. Interestingly, enilconazole, used in agriculture under the name imazalil as seed/tuber and post-harvest treatments in a range of fruits, is an important antifungal agent in animal health[33], for example in chicken coops to protect chickens against *A. fumigatus* at a rate of 1–2 g smoke pellets resulting in 0.2-0.4 mg ai dm^{-3} air and as 0.2% solution (topical application as wash, dip, spray treatment) against ringworm disease and *A. fumigatus* in horses, cattles and dogs.[33,63] In addition, some azoles used for *A. fumigatus* control in humans are obviously recommended also for animal health treatments such as ketoconazole at rates of 10–20 mg kg^{-1} bw (in cats and dogs), 20–30 mg kg^{-1} bw (in birds) and 100 mg kg^{-1} (in crocodiles and turtles).[33] Furthermore, itraconazole is used against *A. fumigatus* and *Fusarium* spp. in cats, dogs, horses, birds, rabbits, reptiles and snakes at rates of 5–10 mg kg^{-1} bw applied daily for up to 6 weeks; and also fluconazole at rates of 2–5 mg kg^{-1} bw daily for up to 8 weeks (in dogs) to 20 mg kg^{-1} bw daily (in birds) (against *A. fumigatus*; Table 2).[33] DMI uses in animal health (probably mostly done as curative treatments) are considered as rather critical in terms of resistance selection, because the application systems are open to the environment (in contrast to hospitals in human medicine) and offer favourable living conditions for *A. fumigatus* (dung, waste, organic surfaces such as hair, skin, sputum, humid and warm climate). In addition, animals can be considered as carriers of fungal spores being collected and disseminated in and to the environment (including DMI-resistant spores).

5.7 Application type (15): surface treatment (not purposely but coincidentally for *A. fumigatus* control)

Without realizing we might be exposed in daily life continuously to 'azoles' (in a broad sense): medical DMIs, for example, ketoconazole and especially climbazole (1 – 3.5%) are ingredients of certain shampoos for dogs and humans against dandruff (*Malassezia globosa* = *Pityrosporum ovale*). Climbazole and other unrelated azoles (with unknown fungicidal activity) such as 1,2,4-triazole, tolyltriazole and benzotriazole are used in certain cleaners as Cu corrosion protectants. No information is available about exposure concentrations, interaction with CYP51 enzyme and activity of the latter compounds against *A. fumigatus*. However, a gradual but unrecognized selection of less sensitive fungal individuals including those of *A. fumigatus* persisting on surfaces of humans, animals and any materials should be taken into consideration when discussing the origin of resistance.

6 ASSESSMENT OF SELECTION AND RESISTANCE RISK

To simplify the comparison of calculated MECs in different substrates (Table 2), the following scale was chosen: MEC < 0.5 mg L^{-1} = 1; 0.5–5 mg L^{-1} = 2; 5–50 mg L^{-1} = 3; > 50 mg L^{-1} = 4 (where: 1 = low; 2 = medium; 3 = high; and 4 = very high exposure concentrations; Table 3). In addition to fungicide exposure concentrations acting on a fungal population, biological factors of the fungus (e.g. pathogenic and saprophytic competitiveness, abundance in the substrate as mycelium or sporulating body, influence of environment on fungus behaviour) must also be considered for resistance risk assessment. This was evaluated and expressed in this study as pathogen exposure risk (PER; Table 3) with a scale from 1 to 4 (low to very high exposure risk). In a human lung, *A. fumigatus* probably expresses the highest pathogenic (aggressive) behaviour; thus, PER for invasive Aspergillosis was rated as 4, followed by still high, but somewhat lower PER (= 3) for animal Aspergillosis and saprophytic activity of *A. fumigatus* on decaying fruit peel. Although MECs can be very high on seeds/bulbs, in wood and all kind of surfaces (humans, materials; factor 4), sometimes also in straw (factor 3), the saprophytic competitiveness of *A. fumigatus* is low resulting in low PER (= 1). As in other risk assessment schemes,[38] risk factors are multiplied. The product of MEC × PER is termed assumed selection risk (ASR) in this study (Table 3), that is, the likelihood that evolution of resistance may become a significant problem for pathogen (fungus, disease) control. If MEC × PER is shown graphically, we can quite easily see which ASR conditions are most critical (Fig. 2). Based on the listed prerequisites, it is postulated, taking all available factors into consideration, that medical DMI treatments (topical and systemic) in humans and animals and probably some material protection applications show the highest selection risk (ASR = 9–12), followed by topical treatments to human skin, flower bulb dipping and post-harvest fruit treatment (ASR = 6–8), followed by surface treatments, wood preservation, seed treatment and residues in straw (ASR = 3–4); all other treatments in agriculture are considered as not critical at all (ASR = 1–2; Fig. 2). However, deviations in both directions (lower or higher risk) cannot be ruled out in cases where fungal populations and DMI concentrations are lower than average (lower risk) or when fungal populations are in the logarithmic development phase and applications are not carried out according to recommendations (higher risk). In addition, the calculated MECs are order of magnitudes and may vary according to intrinsic activity and product use strategies.

7 CONCLUSIONS

The origin of DMI-resistant isolates of *A. fumigatus* is basically unknown. However, it was possible to identify certain critical situations by taking into account the calculated MER, the PER and the ASR in all major application types of agriculture, human and veterinary medicine. Exposure concentrations for DMIs (quantity per kg, L or dm^3 substrate) are highest during medical treatments (human and veterinary), certain fruit and seed treatments and

Table 3. Exposure of *A. fumigatus* to DMIs: calculated MEC, PER and ASR (ASR = MEC × PER).

	MEC (mg L^{-1})	MEC	PER	ASR
1. Spray application in field crops: in soil	0.1	1	1	1
2. Spray application in orchards and vineyards: in soil	0.03	1	1	1
3. Spray application in berries, ornamentals, vegetables: in soil	0.9	2	1	2
4. Residue concentrations in fruits/vegetables: in/on fruits	0.02	1	1	1
4a. Residue concentrations in cereal straw: in straw	8–20	3	1	3
5. Post harvest fruit treatment: in fruit peel	3	2	3	6
6. Cereal seed treatment (I): in soil	0.02	1	1	1
7. Cereal seed treatment (II): in/on seed coat	60–200	4	1	4
7a. Bulb dipping: on bulb coat	300–3000	4	2	8
8. Soil drench fungicide input: in soil	0.8	2	1	2
9. Substrate treatment: in soil (not commercially done)	Low	nd	1	?
10. Wood preservation: in wood (*A. fumigatus* not really competitive)	30–300	4	1	4
11. Material protection, paints, plastic, leather: on surface	Very high	4	3	12
12. Systemic treatment (iv, oral) of humans against *A. fumigatus*: in body	10	3	4	12
13. Topical treatment (creams, lotions) of humans against *A. fumigatus* on skin: on skin	200–2000	4	2	8
14. Treatment (top, fumig, oral, iv) of animals against *A. fumigatus*: in body	30	3	3	9
15. Surface treatment: on surface	Very high	4	1	4

Scale (exposure concentrations for MEC, exposure risk for PER): 1, low; 2, medium; 3, high; 4, very high. nd, not determined; ?, unknown (for details see text).

wood preservation, and are ~10–100 times lower for crop protection applications in agriculture than for medical treatments. Most major agricultural DMIs are intrinsically not active enough (~10–100 times less active than systemic medical DMIs) for *A. fumigatus* control and the potential selection of resistance in the environment. For agricultural uses, the potential selection of resistant *A. fumigatus* mutants by DMIs in soil is rather limited because of very low exposure concentrations and rather tight adsorption to soil organic matter resulting in limited bioavailability. Although DMI concentrations on the seed surface can be rather high after seed treatment, selection of resistant *A. fumigatus* spores is limited because seeds are treated only once (per season), mostly with fungicide mixtures delaying resistance evolution, and stay covered in soil and decay shortly after germination. Although DMI residues in straw and treated wood may be high enough for resistance selection, *A. fumigatus* expresses a rather low saprophytic competitiveness on these substrates compared with other fungi. However, *A. fumigatus* might thrive quite well on other material such as wet wallpaper and decaying plant tissue/fruits during composting in biowaste. The majority of *cyp51* SNPs associated with DMI resistance are different in *A. fumigatus* (e.g. TR_{34}/L98H) in comparison with plant pathogens (e.g. A379G, I381V).[64] They may have been selected preferentially in *A. fumigatus* by medical DMIs (including imazalil = enilconazole). Thus, the evolution of resistance to DMIs in *A. fumigatus* is likely to have emerged (and still emerges) during human and veterinary applications, especially when high concentrations of highly active systemic DMIs are used over long periods (months, years). However, an environmental origin of DMI-resistant *A. fumigatus* spores originating from fungicide-treated material at specific sites cannot be ruled out.

8 COUNTERACTIONS AND PERSPECTIVES

Because spores (including DMI-resistant spores) of *A. fumigatus* are omnipresent in air outdoors and inside buildings (including hospitals), the most effective strategies to avoid collateral resistance (between medicine and agriculture) are as follows.

(1) The implementation of strong and validated sanitation programmes (e.g. suppression of spore production and dissemination as much as possible, effective disposal of mouldy waste, use of effective air filters in hospitals and composting facilities).
(2) Analyse and review certain critical DMI application types especially in veterinary medicine, post harvest and material protection and develop strong anti-resistance strategies including rotation and mixtures of chemistries; consider the use of DMIs in agriculture with no or low potential for selecting DMI resistance in *A. fumigatus*.

This review clarifies several examples of *A. fumigatus* exposure to DMIs and the possible origin of resistant isolates from both the environment and society. It would be counterproductive to prematurely ban or reduce DMI applications in one area or the other, as discussed by certain politically driven groups without having validated the assumptions and consequences. Many uncertainties remain regarding the evolution of resistance and fungus behaviour which need to be investigated further before the risk from non-medical uses can be ranked in relation to that coming from treatments of humans and animals with DMI-antifungal drugs. Important research questions to be addressed should include induction and gene expression experiments, transformation and segregation studies, monitoring the origin of sensitive

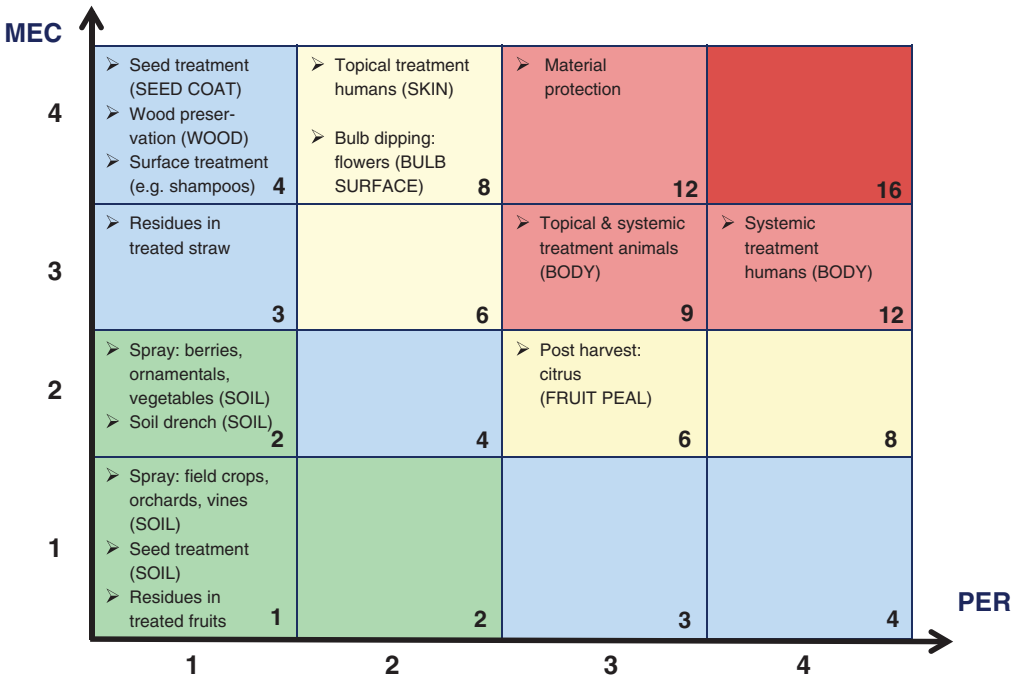

Figure 2. Assessment of selection and resistance risk during exposure of *A. fumigatus* to DMIs in the environment (including agriculture) and medicine. MEC, maximum exposure concentration ; PER, pathogen exposure risk ; ASR, assumed selection risk (ASR = MEC × PER). ASR scale: 1–2, very low risk; 3–4, low risk; 5–8, medium risk; 9–12, high risk; 13–16, very high risk.

and resistant individuals at different environmental sites, alternative treatments in all application area, although experimental work with *A. fumigatus* is rather delicate (for workers and environment) if quarantine conditions cannot be completely guaranteed.

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