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Review

The one health problem of azole resistance in *Aspergillus fumigatus*: current insights and future research agenda

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ABSTRACT

Azole resistance is a concern for the management of diseases caused by *Aspergillus fumigatus* in humans. Azole fungicide use in the environment has been identified as a possible cause for development of resistance, which increases the complexity and number of stakeholders involved in this emerging problem. A workshop was held in Amsterdam early 2019 in which stakeholders, including medical and agricultural researchers, representatives from the government, public health, fungicide producers and end-users, reviewed the current evidence supporting environmental selection for resistance and to discuss which research and measures are needed to retain the effectiveness of the azole class for environmental and medical applications. This paper provides an overview of the latest insights and understanding of azole resistance development in the clinical setting and the wider environment. A One Health problem approach was undertaken to list and prioritize which research will be needed to provide missing evidence and to enable preventive interventions.

1. Introduction

Azoles represent an important class of chemicals for the management of fungal diseases in plants, animals and humans, and for material preservation. In all these areas azoles have brought major benefits in terms of food security, animal health, quality of life, and survival of patients with life-threatening fungal diseases through prevention and effective treatment. However, the broad use of the same antifungal class in different biotopes comes with a risk, as the selection of antifungal resistance through usage in one area of application might affect the efficacy of similar molecules in other areas of application (Fisher *et al.*, 2018). Antifungal drug resistance can be defined as a heritable change in the sensitivity of the fungus to a drug/fungicide that reduces its efficacy in controlling the fungus. *In vitro* assays of azole susceptibility are internationally standardised and yield reproducible results that are predictive of therapeutic response in experimental models and patients with aspergillosis. Evidence of such an adverse event is accumulating for *Aspergillus fumigatus*, a saprobic fungus that thrives on decaying plant material. Although the fungus does not represent a threat to living plants, it is a common cause of fungal diseases in humans ranging from allergic conditions and chronic lung infection to acute invasive aspergillosis (IA) in immunocompromised patients. Resistance to medical azoles with activity against *A. fumigatus*, i.e. itraconazole, voriconazole, posaconazole and isavuconazole, may emerge during long-term azole therapy, especially in patients with cavitary lung disease (Howard *et al.*, 2009). In addition, an environmental route of resistance development has been postulated for *A. fumigatus*, as a result of exposure to sterol 14α-demethylation inhibitor (DMI) fungicides in the environment, affecting fungal sterol biosynthesis, and selecting for azole resistance against medical azoles (Verweij *et al.*, 2009a). As agronomically used DMI fungicides are not directly targeted against *A. fumigatus*, resistance selection is an unintended side effect.

The environmental route of resistance selection is thus a one-health challenge that involves many stakeholders, including medical doctors, fungal and agricultural researchers, fungicide producers, a wide variety of fungicide users, policy makers, regulatory bodies and the composting industry. The complexity of the azole resistance problem, the diversity of the stakeholders involved, and varying interests may compromise or delay progress on necessary interventions to safeguard the future use of this important antifungal class for human health and agronomic use. We therefore convened an expert meeting on azole resistance selection in *Aspergillus* spp., primarily *A. fumigatus*, involving a group of experts representing the diversity of interested parties. In early 2019, a two-day workshop was held in Amsterdam (the Netherlands), in which the problem of azole resistance was discussed. The main aim was to present and discuss current insights into azole resistance selection, to review the underlying evidence and to list and prioritize necessary research areas to provide missing evidence and enable preventive interventions.

2. Participants and methods

The panel comprised 21 experts from six European countries, representing clinical mycology, molecular mycology, fungal genomics, plant biology, phytopathology, and evolutionary biology. The following organisations were represented: Mycology reference and research centres (National Aspergillosis Centre, Manchester University, UK, National Reference Center for Mycosis at University Hospitals Leuven, Belgium;

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Mycology Unit of Statens Serum Institut, Copenhagen, Denmark; Centre of Expertise in Mycology Radboudumc/CWZ, Nijmegen, the Netherlands; University of Nottingham, UK; and Imperial College, London, UK), the Fungicide Resistance Action Committee (FRAC) represented by agrochemical companies, agricultural research organisations (Rothamsted Research, Harpenden, UK and Wageningen University and Research, the Netherlands), food security unit of the Netherlands Institute for Public Health and the Environment (RIVM), the Royal General Bulb Growers' Association (KAVB), the Dutch Board for the Authorisation of Plant Protection and Biocides (CTGB), and the Dutch Association of Biowaste Processors (BVOR). The meeting was prepared by PEV, BF, JL and BJZ. The panel discussions were chaired by KL, PSD, AR and JL; their role was to guide the expert panel, propose working arrangements, record findings, encourage contributions, and to facilitate debates. Three topics related to the problem of azole resistance in *A. fumigatus* were discussed, namely the medical implications, resistance selection in the environment, and stakeholder views and regulations. Each topic was reviewed and presented at the meeting: medical implications by DWD, MAC, PEV, WJGM, and PB; resistance selection in the environment by UG, BF, PSD, SES, and KS; and stakeholder views and regulations by EK, AH, AJFB and PLG. Discussions on the quality of evidence and knowledge gaps were held and a final discussion session aimed to identify and prioritize the topics for future research. Where consensus was not achieved, different opinions were recorded and are stated in this report. Many of the results presented remain as yet unpublished but are described below to provide current insights.

Funding

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3. Results

Medical implications

The spectrum of *Aspergillus* diseases is wide and can be divided into (1) Allergic manifestations including allergy in the nose and sinuses (allergic *Aspergillus* rhinosinusitis) through to the lungs (allergic bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitization (SAFS)). (2) Slowly progressive pulmonary or sinus infections with destruction in apparently normal individuals (and other mammals and birds; chronic pulmonary aspergillosis (CPA) and granulomatous *Aspergillus* rhinosinusitis) and (3) immediately life-threatening invasive infection in immunocompromised patients (IA) (Kosmidis and Denning, 2015). Globally, there are estimated to be 4.8 million cases with ABPA complicating asthma in adults, 6–15 million with SAFS, 1.2 million patients with CPA following pulmonary tuberculosis (30–80% of the total CPA caseload, estimated at 3 million) and >400,000 annually with IA (Bongomin *et al.*, 2017). Co-infections with *Aspergilllus* have also been reported in severe influenza and COVID-19 cases (Arastehfar *et al.*, 2020). Azoles represent the main class for antifungal therapy of most aspergillus diseases, including itraconazole and voriconazole for CPA and other chronic aspergillus diseases, voriconazole and isavuconazole for acute invasive disease, and posaconazole for prophylaxis and salvage therapy (Patterson *et al.*, 2016; Denning *et al.*, 2016; Ullmann *et al.*, 2018). The azoles are the only class that can be administered both intravenously and orally. Alternative treatment options are limited and include liposomal polyene amphotericin B and the echinocandins. The latter drug class is not used for primary therapy of IA due to limited efficacy in neutropenic patients (Viscoli *et al.*, 2009; Herbrecht *et al.*, 2010). In areas with resistance rates exceeding 10%, experts and current guidelines recommend moving away from azole monotherapy, instead recommending voriconazole or isavuconazole in combination with an echinocandin or liposomal amphotericin B (Verweij *et al.*, 2015; Ullmann *et al.*, 2018).

Azole resistance in *A. fumigatus* is found throughout the world in the environment and in clinical isolates (Lestrade *et al.*, 2019b; Beer *et al.*, 2018). Resistance rates are particularly high in the environment in northern Europe (6–20%) (van der Linden *et al.*, 2015) and in patients with chronic and allergic aspergillosis (15–20%) (Howard *et al.*, 2009; Bongomin *et al.*, 2018). The incidence of resistance started to rise in 2003 (Bueid *et al.*, 2010; Buil *et al.*, 2019a), while resistance in *A. fumigatus* prior to 2000 is rarely reported (Moore *et al.*, 2000; Verweij *et al.*, 2002; Snelders et al., 2008; Verweij et al., 2009b). The resistance frequency has increased over the last 15 years presumably through DMI fungicide applications on plants, animals, and materials leading to the emergence of distinctive mutations in *A. fumigatus* found in the environment (the 'environmental route') and in patients with CPA and 'fungal asthma' occurring during therapy (the 'patient route') (Verweij *et al.*, 2009a; Verweij *et al.*, 2016). Azole insensitive environmental strains have two specific signature mechanisms of resistance involving a tandem repeat (TR) of 34 or 46 base pairs in the promoter region of the *cyp51A* target gene, which upregulates the expression of *cyp51A*, combined with point mutations in the target gene, which decreases the affinity of the azoles for the target protein (mainly TR_{34}/L98H and TR_{46}/Y121F/T289A genotypes) (Chowdhary *et al.*, 2017). A much-more diverse panel of resistance mechanisms have been found in resistant clinical isolates, which have developed as a result of selection during medical azole therapy, including many point mutations in the *cyp51A* azole target, altered *cyp51A* expression, altered efflux pump activity and increased *cyp51A* copy number (Bromley *et al.*, 2016; Camps *et al.*, 2012a, Chowdhary *et al.*, 2017; Fraczek *et al.*, 2013; Hagiwara *et al.*, 2018; Rybak *et al.*, 2019). In-host resistance selection has been reported in patients with a pulmonary cavity, such as an aspergilloma, which allows the fungus to reproduce through sporulation (Bongomin *et al.*, 2018; Verweij *et al.*, 2016; Vergidis *et al.*, 2020). Spontaneous mutations are found in the progeny, which may confer azole resistance and may outcompete other (wildtype) genotypes during azole therapy. Recently azole resistance was shown to develop through mitotic recombination in patients with

cystic fibrosis (CF) (Engel *et al.*, 2020). Despite that the fungus is confined to a biofilm in the epithelial mucus in the CF-lung, diploid formation was observed in compatible hyphae, which underscores the versatility of the fungus to adapt to various environments. This is further illustrated by the recent in-host selection of TR-variants including an isolate harboring TR_{120} and one with three copies of TR_{34} (TR_{34}^{3}) cultured from a CF-patient (Hare *et al.*, 2019; Risum *et al.*, 2020).

Establishing the diagnosis of *Aspergillus* infection is often difficult, or never achieved. Culture of airway specimens is limited by low sensitivity of <50%, although new high-volume culture methods have been proposed for increased sensitivity (Vergidis *et al.*, 2020). Once a culture of *A. fumigatus* has been isolated, azole susceptibility testing of *A. fumigatus* is well established and highly reproducible, involving resistance screening and minimal inhibitory concentration (MIC) determination. The use of azole agar screening (EUCAST E. Def 10.1) received a strong recommendation as a screening method and is suitable for routine testing (Guinea *et al.*, 2019; Ullmann *et al.*, 2018). The EUCAST reference microdilution MIC testing method (E. Def 9.3.1) (Arendrup *et al.*, 2012; Hope *et al.*, 2013) also received a strong recommendation for use due to being reproducible and associated with clinical breakpoints (Ullmann *et al.*, 2018). Most clinical microbiology laboratories do not perform susceptibility testing of *A. fumigatus* but refer isolates to regional or national mycology reference laboratories if testing is deemed necessary. Hence, azole resistance is in general diagnosed late with a negative impact on the outcome for the individual patient and, consequently, knowledge on the epidemiology of azole resistance in *A. fumigatus* is incomplete.

Environmental resistance causes many challenges for the management of patients with IA. There are no clinical risk factors for azole-resistant IA as in some cases up to two-thirds of patients with azole-resistant IA have not previously been treated with medical azoles (van der Linden *et al.*, 2011). Furthermore, mixed pulmonary infection does occur (at an unknown frequency), i.e. some lung lesions are caused by azole-susceptible conidia, while others may originate from a resistant spore (Kolwijck, *et al.*, 2016). Recent retrospective cohort studies detected a 20% lower survival in patients with voriconazole-resistant IA compared with voriconazole-susceptible infection (Lestrade *et al.*, 2019a; Resendiz-Sharpe *et al.*, 2019), indicating a significant clinical impact of resistance. Despite intensive resistance screening, the median time to change to appropriate therapy was 10 days, which indicates that appropriate primary therapy is critical for patient survival (Lestrade *et al.*, 2019a).

To overcome the low yield of fungal cultures and slow detection of resistance, it is possible to use molecular tools directly on clinical specimens to rapidly detect molecular markers of azole resistance (Denning *et al.*, 2011; Buil *et al.*, 2019b; Singh *et al.*, 2020). Molecular tools to detect resistance markers in the *cyp*51A-gene are less sensitive compared to detection of *Aspergillus* DNA for diagnosis as the *cyp*51A-gene is a single copy gene whereas the usual target for *Aspergillus* diagnosis is a multi-copy gene (White *et al.*, 2017). Ideally, a diagnostic PCR should be able to detect the full range of resistance mutations, preferably in a single-assay to exclude or confirm the presence of an azole-resistant *A. fumigatus* infection, but with >25 different mutations in >15 locations, this is unlikely to be possible with most standard PCR systems. Interpretation of results from patients with a mix of both susceptible and resistant isolates may also be difficult (Schauwvlieghe *et al.*, 2017). Consequently, molecular tools can detect resistance but not susceptibility.

Resistance selection in the environment

Azole antifungals are widely used in medicine (e.g. itraconazole, voriconazole, posaconazole, and isavuconazole), cosmetics (e.g. ketoconazole, miconazole and clotrimazole), veterinary treatment (e.g. itraconazole, voriconazole and enilconazole (imazalil)), material preservation (e.g. propiconazole, tebuconazole and cyproconazole), and agriculture (e.g. prothioconazole, tebuconazole and difenoconazole). In particular, DMI fungicides (triazoles and imidazoles) have been widely used for control of foliar and seed-borne diseases in cereals in the UK since their introduction in the mid-1970s. Due to differences in chemical properties and disease spectrum, different DMI molecules have been introduced and/or phased out from the market over time. Due to their efficacy and initially slow rates of resistance development with a shifting type of resistance, azoles have maintained their position as the most important fungicide group for disease control (Price *et al.*, 2015).

Several studies (Fraaije *et al.*, 2020) have recently been undertaken to address the question of whether the use of azoles in cereal crops might lead to the emergence of azole resistance of *A. fumigatus* in the environment. Of particular value has been the ability to analyse samples from long-term experiments (LTEs) in agricultural fields at Rothamsted, UK. Soil samples from LTEs have recently been analysed for the presence of *A. fumigatus* from the 'Park Grass' pasture (no fungicide applications) and 'Broadbalk' winter wheat (sampling of plots receiving no fungicides and plots receiving fungicide applications (including DMIs) since late 1970s), and two further LTEs involving straw incorporation with fungicide inputs. In addition, 15 commercial wheat fields from either the UK (5), France (5) and Germany (5) were investigated (Fraaije *et al.*, 2020). In all instances, no or only very low numbers of azole-resistant *A. fumigatus* strains (TR_{34}/L98H or TR_{46}/Y121F/T289A) were detected. Indeed, the frequencies of highly resistant strains in cereal soils were generally lower than or similar to those detected by air sampling in the Netherlands and the UK, where TR_{34}/L98H and/or TR_{46}/Y121F/T289A were detected at frequencies of 1–2%. Therefore, the use of azole fungicides in cereal production appears to present a low risk for selection of azole resistance in *A. fumigatus* (Barber *et al.*, 2020).

These observations are in line with a risk assessment model for selection of resistance to DMIs in *A. fumigatus* that was developed for all known DMI applications in crop and material protection and for treatment of fungal disease (Gisi, 2014). This resistance risk matrix was revisited and adapted based on recent experimental data and used to identify so called "hotspots" defined as an environment in which: (1) the physical, biotic and abiotic conditions facilitate the growth of the fungus and from which the fungus can spread; (2) this growth can take place for prolonged periods and the fungus can complete all the stages of its growth cycle; and (3) azoles

are present, in different concentrations sufficient to select in populations, and combinations (Schoustra *et al.*, 2019) (Fig. 1). Studies that sampled environments for the presence of azole-resistant *A. fumigatus* and DMI fungicide residues, indicated that resistance was found during stockpiling of plant waste material (for example peelings from bulbs or from household organic waste), underscoring the necessity to sample the full production cycle to identify particular stages that represent potential hot spots for the evolution and selection of azole resistance in *A. fumigatus*.

Development of resistance is an evolutionary process driven by natural selection acting on genetic variation. This genetic variation can already be present in the population, but ultimately, genetic variation is generated by novel mutations. Key factors that determine the outcome of evolution, and thus resistance development, are natural selection acting on genetic variation, a process that is further influenced by recombination, gene flow and genetic drift. In the context of azole resistance, there is some limited evidence that TR-based environmental resistance might have arisen very rarely, and that genetic diversity has then been introduced

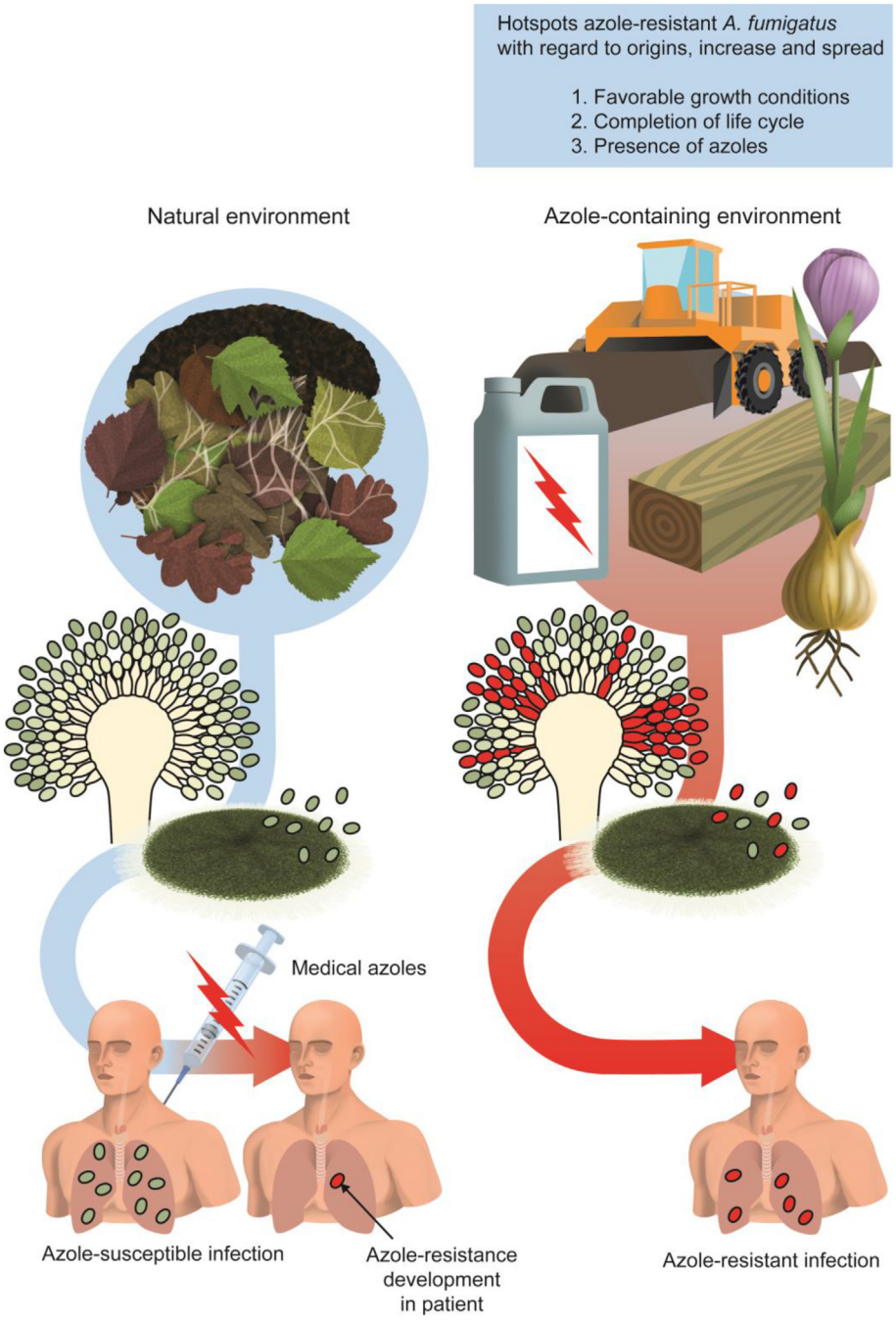

Fig. 1 – Routes of azole resistance selection through medical treatments and in specific conditions in the azole-containing environment.

as a result of sexual recombination (Camps *et al.*, 2012b). Indeed, the sexual cycle of *A. fumigatus* may be important since this can generate the specific changes (TR variation in the promotor region of the *Cyp51A*-gene) that are commonly observed in azole-resistant strains (O'Gorman *et al.*, 2009; Zhang *et al.*, 2017a). In addition to field studies, experimental evolution experiments under laboratory conditions are important to understand the dynamics of azole resistance selection in *A. fumigatus* and the role of individual variables (Zhang *et al.*, 2017b, 2019).

Although resistance selection may occur locally within a hotspot, TR-mediated resistance has now been reported globally (Lestrade *et al.*, 2019b; Beer *et al.*, 2018). Microsatellite-based STRAf genotyping of a collection of 2,784 isolates from around the globe showed that isolates broadly grouped into two well-defined clusters. Azole resistance was found on both clusters but mostly in one, associated with the TR_{34}/L98H and TR_{46}/Y121F/T289A genotypes (Sewell *et al.*, 2019). Reduced genetic diversity was evident in the TR_{34}/L98H and TR_{46}/Y121F/T289A populations compared with susceptible isolates, consistent with a recent selective sweep of these alleles. Identical TR_{34}/L98H and TR_{46}/Y121F/T289A STRAf clones were found worldwide from both environmental and clinical sources, strengthening the evidence-base for patient acquisition of azole-resistant *A. fumigatus* airborne conidia. A similar clustering has also recently been observed using a comparative genomics approach which showed the presence of two main subgroups of *A. fumigatus*, termed Clades A and B. Azole resistance linked to the TR_{34}/L98H and TR_{46}/Y121F/T289A genotypes was found specifically in Clade A, with azole resistance very rare in Clade B. Further research is ongoing to identify the mechanism and impact of gene flow amongst globally emerging azole resistance in *A. fumigatus*.

Stakeholder perspectives

The Fungicide Resistance Action committee (FRAC) was established in 1982 as a joint industry forum to provide resistance management advice and guidelines to sustain the general effectiveness of fungicides in agriculture. It represents a centre of knowledge and expertise, including scientists from all major researching agrochemical companies, and comprises a series of Working Groups, Expert Fora and Regional Groups covering different fungicide modes of action (MoA), crops and countries. Technical advice is provided on the main anti-resistance strategies to ensure long-term effectiveness of all fungal MoA's in agriculture markets. The sterol biosynthesis inhibitors (SBIs), DMIs in particular, are an important group of fungicides representing almost one third of global fungicide use on a wide variety of crops and diseases. DMI fungicides have several unique properties, including a broad spectrum of efficacy, systemic behaviour (uptake and translocation within plant tissues) and curative activity, making them an indispensable group of fungicides in conventional agriculture. FRAC has been involved in long-term monitoring of the efficacy of the azole group of DMIs to detect shifts in sensitivity in pathogens of major crops such as cereals in Europe and soybean in Brazil. While less-sensitive strains of some plant pathogens have emerged over time, the azoles have not suffered the rapid high-level resistance development seen with other single-site of action fungicides (e.g. quinone outside inhibitors (QoIs) and methyl benzimidazole carbamates (MBCs)) (Lucas *et al.*, 2015). Due to concerns over the possible environmental selection of azole resistance in *A. fumigatus*, FRAC commissioned an expert review of the literature (Gisi, 2014) and provided funding for an independent research project carried out at Rothamsted Research, UK. This is contributing new knowledge on potential hotspots for selection of azole-resistant *A. fumigatus* isolates in crop protection and might lead to improved resistance risk evaluation and identification of measures for mitigation, if required.

Another fungicide industry initiative is product stewardship aimed to manage risks and improve product performance regarding safety, health and the environment during its entire life cycle. The current focus of the (fungicide) Product Stewardship projects in the Dutch flower bulb sector is the protection of surface water. Furthermore, voluntary stewardship advice on the labels of all fungicides has been implemented over the past year. According to these restrictions, fungicides can only be used on farms that control the run-off and drainage of water very well; spillage to surface water is not permitted.

The Royal General Bulb Growers' Association (KAVB) is the association for breeders, growers and traders in the flower bulb industry in the Netherlands. There are about 25,000 ha of flower bulbs in the Netherlands, which make up around two-thirds of the world's production. The flower bulb industry uses fungicides for two applications: (1) dipping before planting, to control *Fusarium*, *Penicillium* and *Rhizoctonia*; and (2) spraying on the field to protect against *Botrytis*. Two DMIs[1] are used in flower bulbs, according to Statistics Netherlands (2016): prothioconazole (1.1% of total fungicide use) and tebuconazole (1.5% of total fungicide use). Although the volume of use is relatively low, these DMIs are important for growers for both broad spectrum disease control and fungicide resistance management. Growers are working towards using less pesticides in general and are aiming to replace single-site fungicides, acting on a specific target and therefore at high risk for resistance development, with low-risk multi-site (acting on multiple targets) fungicides. This approach has led, in the period from 2012 to 2016, to a decrease of 16.9% in total fungicide use, including a decrease of 29.7% and 24.2% of prothioconazole and tebuconazole, respectively.

Although stockpiling and handling of plant waste are emerging as important steps in azole resistance selection in *A. fumigatus*, active composting was shown to reduce the level of (azole-resistant) *A. fumigatus* (Schoustra *et al.*, 2019). Composting is a natural biological process, carried out under controlled aerobic conditions (Fig. 2), where aeration can be obtained through natural convection or forced aeration. The temperature is an important parameter as high temperature

[1] After the workshop it became clear that also imidazole fungicides may play a role in the development of cross-resistance. A third DMI, prochloraz, is used in flower bulb production. According to Statistics Netherlands (2016) prochloraz comprises 3.6% of total fungicide use. In the period 2012–2016 the approach of the Dutch flower bulb growers to reduce pesticide use has led to a decrease of 29.8% of prochloraz.

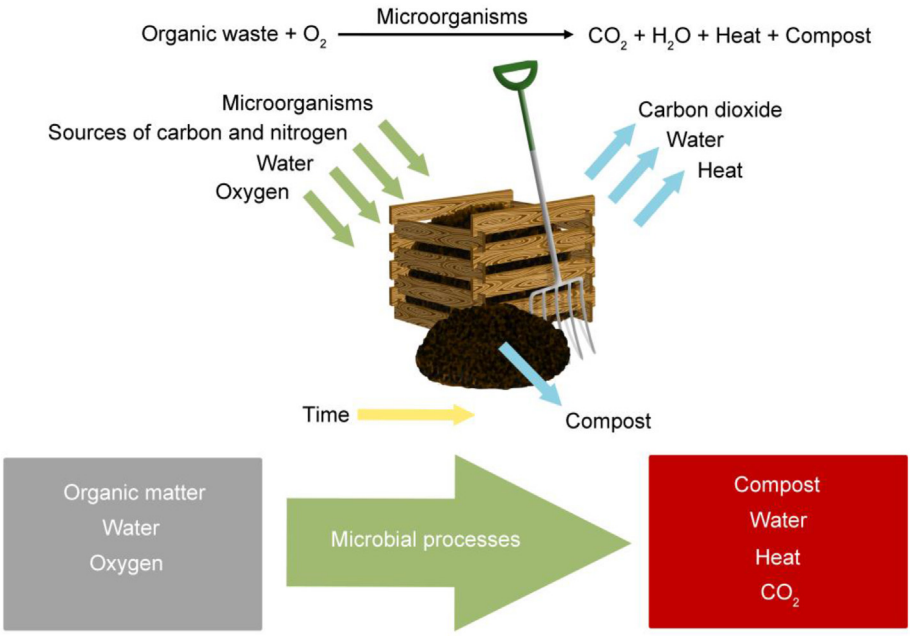

Fig. 2 – Principles of aerobic composting.

favors the destruction of weed seeds, larvae and microbial pathogens (Fig. 3).

There are various types of composting facilities in the Netherlands, including 20 large-scale enclosed facilities; 80 large-scale open facilities; and several hundred smaller scale composting facilities. Large-scale enclosed facilities have a capacity of >10,000 tonnes per annum and process primarily household biowaste and industrial organic waste, whereas the large-scale open facilities primarily compost green waste, with an annual capacity of >5,000 tonnes. Smaller scale composting facilities have a capacity of <1,000 tonnes and process primarily farm residues, including waste from the bulb sector. Feedstocks for composting include household biowaste, industrial organic waste, green waste, residues from greenhouses and farm crop residues. Approximately 90% of the large-scale composting facilities operate according to a certified procedure involving a quality assurance scheme with registration of operational performance, product quality requirements and process requirements (Keurcompost). Feedstocks at risk for high levels of chemical residues such as flower bulb waste are excluded from processing in these certified large-scale composting facilities.

Fungicides undergo a rigorous evaluation process before they can be used. In the Netherlands, the Dutch Board for the Authorisation of Plant Protection Products and Biocides (CTGB) acts as an independent authority which is commissioned to assess and authorise plant protection products and biocides, which can then be sold and used in the Netherlands. The assessment of submitted products follows EU legislation and includes physical and chemical characteristics, safety for humans, animals and the environment, and efficacy according to the user instructions. The efficacy assessment includes resistance development but is restricted to the target microorganisms. In addition, the producer is obliged to report any new unexpected events such as resistance development, which may result in advice and instruction on resistance risk management for the users or label restrictions. Several DMIs, triazoles as well as imidazoles, are authorized for use in the Netherlands, including five azole compounds (bromuconazole, difenoconazole, epoxiconazole, propiconazole and tebuconazole), initially implicated to select for cross-resistance to medical azoles in *A. fumigatus* based on both azole susceptibility testing and cyp51A docking studies (Snelders *et al.*, 2012). High levels of cross-resistance between other azoles were measured using TR_{34}/L98H and TR_{46}/Y121F/T289A strains (e.g. Chowdhary *et al.*, 2012; Sharma *et al.*, 2015; Ren *et al.*, 2016). The availability of azoles as plant protection products, new actives and renewal of approvals, is regulated by EU legislation (Regulation (EC) No 1107/2009). After evaluation for renewal under this regulation, propiconazole is no longer approved and has recently been withdrawn from the market. Several other azoles are so-called candidates for substitution (e.g. tebuconazole) and are currently under evaluation for renewal. Table 1 shows the current available azoles on the market in France as an example; biocides are not included.

Challenges related to azole resistance selection in *A. fumigatus* include the fact that *A. fumigatus* is a non-target microorganism and that the main risk of resistance selection is likely associated with waste rather than DMI application itself. Interventions that prevent resistance selection in *A. fumigatus* such as label restrictions can only be enforced if the user is responsible for the waste, which is not the case for wood chippings and green waste. Avoiding the use of fungicides belonging to similar MoA's

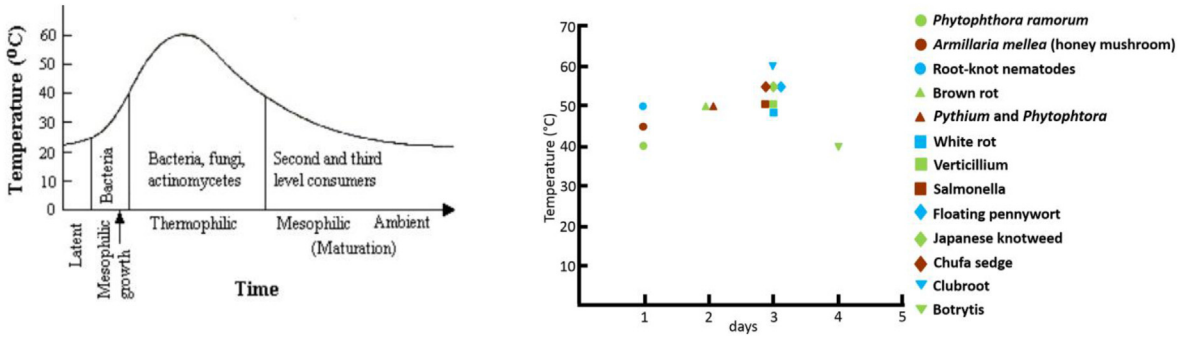

Fig. 3 – Relation between temperature and destruction of weed seeds, larvae and microbial pathogens over time during composting (source BVOR).

Table 1 – Active phytopharmaceutical substances belonging to the DMI group of fungicides and authorised for use in France in 2020.

Active substances[a]	Chemical group	Number of products	Usage[b]	Approval expiration date[c]
Bromuconazole	Triazole	1	F	31/01/2024
Cyproconazole	Triazole	9	F	31/05/2021
Difenoconazole	Triazole	67	F	31/12/2020
Fenbuconazole	Triazole	5	F	30/04/2021
Fluquinconazole	Triazole	0	F	31/12/2021
Flutriafol	Triazole	1	F	31/05/2024
Ipconazole	Triazole	6	F	31/08/2024
Imazalil	Imidazole	6	F	31/12/2024
Mefentrifluconazole	Triazole	5	F	20/03/2029
Metconazole	Triazole	38	F, G	30/04/2021
Myclobutanil	Triazole	5	F	31/05/2021
Paclobutrazol	Triazole	5	G	31/05/2023
Penconazole	Triazole	7	F	31/12/2021
Prochloraz	Imidazole	22	F	31/12/2023
Prothioconazole	Triazolinthione	105	F	31/07/2021
Tebuconazole	Triazole	94	F, G	31/08/2021
Tetraconazole	Triazole	9	F	31/12/2021
Triticonazole	Triazole	4	F	30/04/2021

a Data obtained from https://ephy.anses.fr/, accessed on 12 October 2020.
b F (fungicide), G (growth regulator);[3]Data from EU Pesticides database.
c Data from EU Pesticides database.

in plant protection, biocides and medical applications would be an effective intervention but current legislation would need to be changed to enable this. If evidence supports the development of cross resistance to specific use or specific compounds, proportional label restrictions can be implemented.

4. Discussion

Current evidence and research agenda

Overall there was a broad awareness among the experts of the problem of azole resistance in *A. fumigatus*; with recognition that selection of resistant genotypes in the environment is an unintended side effect of DMI use with consequent medical implications which demands immediate attention. Given the prominent role of the azole class for food security, material preservation and treatment of fungal diseases, the main aim of research and interventions should be to preserve this class of antifungals for continued use in all areas, and especially for medical treatments.

There are, however, still many knowledge gaps including the population dynamics of resistance selection in the environment, the role of the various reproduction modes of *A. fumigatus*, and the implications of resistance for fitness and virulence. The number of identified hotspots remains limited and their contribution to human resistant disease remains poorly understood, including transmission of *Aspergillus* conidia and respiratory tract colonization. Furthermore, the role of the chemical structure and concentration of DMIs in resistance selection as well as bioavailability and half-life in the environment under different conditions are not well characterized.

Based on the literature and expert opinion it is clear that not all DMI uses are equal with respect to selection of azole

resistance in A. *fumigatus*. Certain DMI applications appear to have a relatively low risk of selection for resistance, although it was noted that some risk is still present. By contrast, there was consensus that hotspots exist in the environment which appear to represent the main risk for selection and amplification of azole-resistant A. *fumigatus*. Key characteristics of a hotspot includes decaying plant waste that supports the growth and reproduction of A. *fumigatus* for relatively long periods of time in the presence of DMI residues. These DMIs must have activity against A. *fumigatus* in order to enable selection of resistant strains. However, many factors related to the process of resistance selection remain unclear including the concentration of fungicides that might be encountered in the environment and their bioavailability. For instance, a high DMI concentration would maintain a high selection pressure leading to a highly resistant population, but the number of fungal cells that can grow in those conditions would be low. Conversely, at lower concentrations the selection pressure would be low, but the population of fungal cells that could survive in that environment would be larger, thus increasing the probability of mutant populations with reduced susceptibility to emerge (van den Bosch *et al.*, 2011). Studies that have measured DMI concentrations in hotspots showed a broad variation in concentrations, often with very low concentrations that could not be accurately quantified (Schoustra *et al.*, 2019). Indeed, DMI concentrations would be expected to be highly variable within stockpiles of decaying waste as new material is added regularly. In addition, the bioavailability of the DMIs is an important and not well-investigated factor in resistance selection. The DMI half-life in specific environments under different conditions also requires investigation.

Other areas of research include the mutation frequency of A. *fumigatus* within the hotspot environment and its relation to the specific azole molecule the fungus is exposed to. As resistance usually comes with a fitness cost, the question remains if and how fitness and virulence of A. *fumigatus* are affected in resistant strains. It was recently shown that selection of resistance in A. *fumigatus* comes with a fitness cost when grown in the absence of azoles, but that this cost can be overcome by compensatory mutations (Verweij *et al.*, 2016). Although the spread and accumulation of TR_{34} and TR_{46} in the environment indicates that they can survive in competition with wild-type isolates, further research is needed to determine the implications of resistance in terms of fitness and virulence and how these factors can be reliably measured.

Predominantly two resistant *cyp51A* genotypes are found globally that are associated with environmental resistance selection, TR_{34}/L98H and TR_{46}/Y121F/T289A. Population genetics studies suggest that *de novo* development of these mutations is an extremely rare event (Snelders *et al.* 2012), indicating that dispersal (gene flow) is an important means for spread. More research is needed to determine how gene flow across the globe takes place. On a smaller scale, the dynamics of release of A. *fumigatus* from a hotspot and transmission to patients also remain largely unknown. While new resistance mutations have been found in hotspots and patient samples, the resistance mechanisms mainly involve novel TR variants characterized by variations in the number of repeat duplications (e.g. triplication of TR_{34} and TR_{46}) (Snelders *et al.*, 2012; Zhang *et al.*, 2017a; Risum *et al.*, 2020), as well as the length of the repeat (e.g. TR_{120}) (Hare *et al.*, 2019), or additional nonsynonymous single-nucleotide mutations in the *cyp51A* gene (Zhang *et al.*, 2017b). However, non-*cyp51A*-mediated resistance mechanisms may also play a role in resistance in A. *fumigatus* (e.g. Fraczek *et al.*, 2013; Zhang *et al.*, 2017b; Rybak *et al.*, 2019; Bowyer *et al.*, 2020), many of which are not yet characterized. Also, single-nucleotide resistance mutations resulting in Cyp51A alterations (e.g. M220I and G54E), which are commonly associated with patient-derived resistance are found in the environment indicating that the distinction between patient-derived and environmental-derived routes may be less well defined (Buil *et al.*, 2019b).

Three hotspots have been identified in a study recently performed in the Netherlands, including flower bulb waste, green waste processing and wood chippings (Schoustra *et al.*, 2019). However, not all potential hotspot sites were investigated. One important question is to determine whether the characteristic features of these three hotspots can be extrapolated to other sites and practices. This would help to risk assess potential sites and select those that need sampling. Indeed, azole-resistant A. *fumigatus* was not detected in commercial compost from various European countries despite the abundant presence of wild-type A. *fumigatus* (Santoro *et al.*, 2017). Clearly, further studies would be required to confirm that hotspots identified in the Netherlands would also constitute hotspots in other countries, and to assess the risk posed in other settings.

Interventions aimed to reduce development of resistance

Although the relation between DMI usage and azole-resistant A. *fumigatus* infection has not been proven, and as indicated above many research questions remain unanswered, the experts felt there is enough evidence to justify the implementation of appropriate mitigative interventive measures. As the DMI environment is considered the main driver for resistance in A. *fumigatus*, interventions should primarily be aimed at reducing the burden of resistance in the environment. The main aim would be to alter environmental conditions in such a manner that potential hotspots are turned into cold-spots. This can be achieved by applying measures that alter the conditions that support the growth and reproduction of A. *fumigatus* and/or by interventions that reduce DMI selection pressure. Creating conditions unfavourable for A. *fumigatus* would diminish or halt the supply of resistant genotypes emerging via spontaneous mutations or genetic recombination and lower the airborne conidial inoculum levels. As at present variation in small-scale composting already exists, therefore a practical approach would be to compare current composting practices for the presence of (azole-resistant) A. *fumigatus*. Composting practices that prevent growth (and subsequent resistance selection) in A. *fumigatus* could then be advocated as best practice and broadly implemented. As the flower bulb growers in the Netherlands have already agreed to take responsibility, a pilot study has commenced in the Dutch bulb sector and, if successful, could be applied to other sectors and a wider geographical area. This pilot study involves sampling the different phases of waste

collection and composting for *A. fumigatus*, to characterize the azole phenotypes and genotypes, and measuring the presence of DMI residues. It is also important to study the full cycle of waste management including collection, storage and composting as these phases may not all take place on-site at farms. Waste from other sectors containing DMI residues may be collected for commercial composting and stored at a composting facility, thus providing a potential source of azole-resistant *A. fumigatus* at a location other than where the waste is created. Therefore, even if the bulb sector is willing to take the initiative, it is critical to involve all relevant stakeholders that are part of the composting procedure.

Alteration of the storage conditions or duration of (treated) organic waste is an important target for interventions. A previous study showed that *A. fumigatus* was not recovered from specific sampling sites, and resistance was thus not selected for despite the presence of DMI residues (Schoustra *et al.*, 2019). As *A. fumigatus* has specific requirements regarding temperature, humidity, and water content, these can be altered in a way that prevents the growth and spread of *A. fumigatus* without affecting the quality of the compost.

With respect to DMI exposure, several fungicide product stewardship programs already exist initiated by both fungicide producers and users. These programs are aimed at preserving the performance of the fungicides, while also maintaining safety and safeguarding a clean environment. These initiatives are not targeted at reducing the burden of azole resistance in *A. fumigatus* but might contribute to reducing the DMI selection pressure. However, it remains unclear whether reducing the DMI-concentration in a hotspot would reduce the long-term burden of azole-resistance as azole-resistant *A. fumigatus* strains were recovered from sites with very low levels of DMIs (Schoustra *et al.*, 2019). Alternatively, substitution of DMIs by other compounds from the same class, but with a lower risk of resistance selection in *A. fumigatus*, or by fungicides with other MoAs that are not shared with medical antifungal drugs might be a potential approach to be investigated. However, alternative compounds would need to be authorized for the required application and it remains to be determined which DMIs do not select for cyp51A-mediated resistance in *A. fumigatus*. Another option could be to include resistance development against non-target microorganisms as part of the documentation that is to be provided to authorisation boards for plant protection products and biocides that share MoAs with drugs that are critical for treatment of human infections. Some experts suggested restricting the use of DMIs in hotspots only, as this would reduce the extent of use while not compromising global food production.

Regulating the use of compounds with potential antifungal activity overlap between medicine, veterinary, crop protection and material preservation is a necessary measure to protect patients from the enhanced risk deriving from the environmental route of resistance selection, and to safeguard the use of antifungal molecules in both medicine and crop protection. This is highly relevant as new antifungal targets are being discovered in both areas and which are undergoing clinical evaluation for treatment of fungal diseases in humans and are in development for the control of plant diseases. This requires close consultation between fungicide producers and pharmaceutical companies. If a new mode of action reaches an advanced stage of development as a potential agent against life-threatening fungal diseases of humans and is also under development for environmental use, the crop protection industry should perform a risk assessment based on the level of activity of their candidate molecules against the same human fungal pathogens. If such activity is confirmed, then the crop protection industry should devise stewardship approaches to minimize the risk of resistance selection in environmental populations of these fungi and should focus on use in only low-risk crop segments. Some experts at the meeting expressed the view that the use of molecules with the same mode of action in medicine and crop protection should be avoided.

The clinical implications of azole resistance are significant and present a plethora of challenges. Dissimilar to bacterial resistance, there are no international resistance surveillance programs for resistance in aspergilli. Only two countries, the Netherlands and Denmark, have a national surveillance program for resistance in *A. fumigatus*. These programs are critical to determine the frequency of azole resistance in *Aspergillus* disease, and to support decisions regarding primary antifungal therapy of patients as medical azoles are the current first choice treatment option. International guidelines recommend considering moving away from azole monotherapy if the resistance frequency exceeds 10%. This is the case in the Netherlands and in 2017 the national guideline was revised and recommends combination therapy in patients with IA. A problem for resistance surveillance and patient management is that many clinical microbiology laboratories do not routinely perform resistance testing in mould fungi and cultures are frequently negative anyway. As any delay in the initiation of appropriate antifungal therapy may reduce patient survival, new rapid diagnostic tests need to be developed. As indicated the current PCR-based assays are suboptimal in terms of sensitivity and the number of mutations that are detected, and new approaches are urgently awaited. As the number of treatment options in *Aspergillus* disease are currently very limited, new drug targets are urgently needed and although there are several new drug targets under investigation, the road to a clinical licensed drug is long and uncertain.

5. Conclusions

The problem of azole resistance in *A. fumigatus* clearly poses a significant threat. In contrast to bacterial infection, fungal diseases have until recently not been considered a health threat to the public, but with millions affected by fungal asthma, several 100,000s with aspergillosis complicating COPD and TB, and life-threatening aspergillosis after severe influenza and COVID-19, the realisation of its importance is rising. Indeed, azole-resistance in *A. fumigatus* was recently listed on the 2019 watch list of the Centres for Disease Control and Resistance (CDC) threat list to the USA (https://www.cdc.gov/drugresistance/biggest-threats.html#azole). Nevertheless, few antimicrobial resistance (AMR) efforts and AMR global action plans include fungi. The increasing incidence of resistance in fungal pathogens, such as multi-drug

resistant *Candida auris* might increase awareness of the scale of the global threat now being posed by antifungal drug-resistant fungal diseases. From an agricultural perspective little research has been performed into resistance monitoring in *A. fumigatus* up to now, as the fungus is not a targeted plant pathogen. Initiatives aimed to move further towards sustainable and environmentally sound agriculture should include associated health threats by non-target microorganisms as possible unintended side-effects. This should also include initiatives associated with "circular agriculture" that are signified by the aggregation of often large volumes of organic waste and the accompanying accumulation of (chemical) residues.

Our current understanding of azole resistance in *A. fumigatus* indicates that the problem is global and leads to excess mortality in patients with IA. Participants of the expert meeting acknowledge the need for further studies and are willing to investigate how current practices can be altered to reduce the burden of resistance and to retain the azole class for medical and non-medical applications. Multidisciplinary collaboration between researchers and relevant stakeholders is critical, and we call on policy makers to prioritize and facilitate the initiatives that are necessary to achieve these goals.

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Declaration of competing interest

None declared.

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