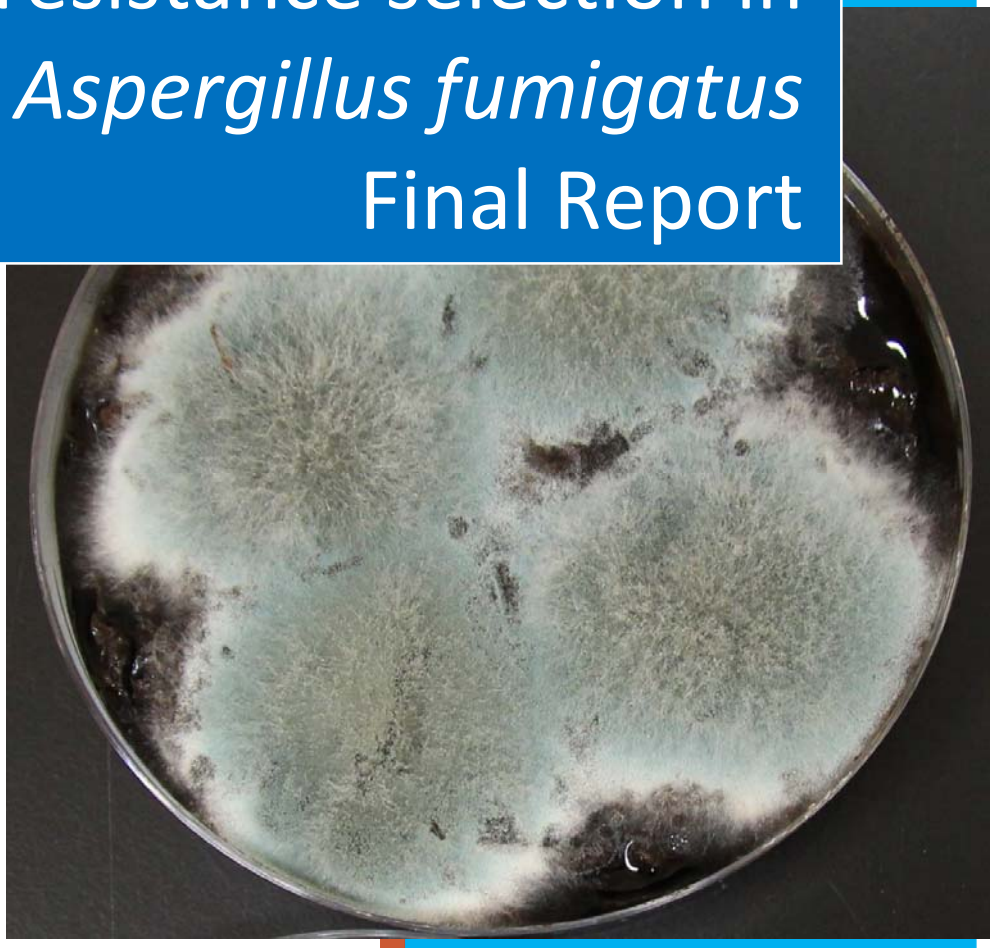
2015-2017

Azole-resistance selection in *Aspergillus fumigatus* Final Report



CLM; WU; Radboudumc

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Contents

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Front cover: Growth of *Aspergillus fumigatus* on compost agar.

Summary

Background. *Aspergillus fumigatus* is a saprophytic mold that grows on dead plant material (compost). The fungus is thermotolerant and produces billions of airborne spores, which are inhaled by humans. In healthy individuals the spores are rapidly cleared from the airways, but the fungus causes a range of diseases in specific patient groups including allergic syndromes, chronic pulmonary infections in patients with lung disease, and acute invasive infections in immunocompromised patients. Many aspergillus diseases are treated with triazoles, but the use of medical triazoles is threatened by the emergence of triazole resistance.

Resistance may develop during triazole therapy (patient route), but may also develop in the environment (environmental route). There is evidence that *A. fumigatus* acquires resistance mutations when exposed to azole fungicides. The presence of environmental resistance mutations reduces the efficacy of medical triazoles thus causing patients to fail therapy. Environmental resistance mutations are found in 80% to 90% of resistant patient *A. fumigatus* isolates, and are an important cause of triazole-resistant aspergillus disease.

Hypothesis. Specific conditions and sites are thought to exist in the environment that facilitate the emergence, amplification and spread of triazole-resistance mutations in *A. fumigatus*.

Aim. To identify and characterize conditions and sites that facilitate triazole resistance selection in *A. fumigatus* and to investigate the spread of triazole-resistant aspergoillosis cases.

Results. Through consultations of experts from various backgrounds two main conditions were identified: the ability of *A. fumigatus* to grow and complete its life-cycle (in order to achieve genetic diversity) and the presence of azole residues with activity against *A. fumigatus* (selection pressure). Sites with these conditions are considered hotspots for resistance selection. Through expert consultations ten sites were listed that might fulfill the hotspot-criteria. These sites were sampled for the presence of triazole-susceptible and triazole-resistant *A. fumigatus* and of azole fungicide residues. Three hotspots were identified: compost from flower bulb waste, green compost and wood chippings.

Flower bulb waste was further investigated as case study to understand the dynamics of resistance selection. The phase preceding the active composting, i.e. the storage of organic waste from the field, was identified as a phase that allows active growth of *A. fumigatus* in the presence of azole residues. Azole residues selected for triazole-resistant *A. fumigatus* and a new triazole resistance mechanism was identified, which was also found in clinical isolates. *A. fumigatus* was found to be able to complete both the sexual and asexual life cycle in compost. Geographic variation in resistance frequency was found in clinical isolates.

Conclusion. Hotspots for triazole resistance in *A. fumigatus* were identified and characterized. This research provides first leads to develop strategies to reduce the resistance selection pressure in hotspots.

Background

Aspergillus fumigatus is a saprophytic mold that is known to grow well in compost, where it contributes to degrading of dead plant material. The fungus can tolerate high temperatures (up to 50°C) and releases abundant amounts of spores into the air. Each day humans inhale hundreds of *A. fumigatus* spores but healthy individuals do not develop aspergillus disease. Patients with hyperactivity of the immune system may develop allergic aspergillus diseases, while patients with chronic lung disease may develop chronic colonization of the lung or chronic pulmonary aspergillosis. Immunocompromised patients, such as patients with leukemia, patients receiving corticosteroids and patients with lung damage due to influenza, may develop acute invasive aspergillosis. Invasive aspergillosis is a life threatening infection with a mortality rate of approximately 30%.[1]

Patients with aspergillus diseases are treated with antifungal compounds, of which the triazoles are the most important. Clinically licensed agents with activity against *Aspergillus* species include itraconazole, voriconazole, posaconazole and isavuconazole. The agents are recommended for the prevention of invasive aspergillosis in high risk patients (posaconazole), the treatment of chronic pulmonary aspergillosis (itraconazole) and the treatment of invasive aspergillosis (voriconazole and isavuconazole). Alternative treatment options are limited to one alternative agent, amphotericin B, which is more toxic than the triazoles.

Since 1998 triazole-resistant *A. fumigatus* isolates have been found in the Netherlands. *A. fumigatus* can acquire resistance through exposure to azole compounds. Azoles are widely used for the management of aspergillus disease in animals and humans, but also for crop and plant protection (fungicide) and for preservation of materials (biocide). It is generally accepted that resistance can develop through patient treatment (patient route) and through exposure of *A. fumigatus* to azole fungicides in the environment (environmental route)(Figure 1).[1] Resistance development during treatment of patients may occur when the patient has a cavity. *A. fumigatus* can then produce spores in the cavity (asexual sporulation), which is probably an important condition that facilitates resistance selection. Patients are infected with a triazole-susceptible *A. fumigatus* isolates which then acquires resistance mutations during triazole therapy.[2]

The alternative route of resistance development is through exposure of *A. fumigatus* to azole compounds in the environment. The following evidence supports the environmental route of resistance development:

- Specific triazole-resistance mutations have been found in patient isolates as well as in environmental *A. fumigatus* isolates. These mutations comprise of a tandem repeat (TR) in the

promoter region of the Cyp51A-gene in combination with single or multiple point mutations (TR_{34}/L98H; TR_{53}; TR_{46}/Y121F/T289A).[3-5] Given the complexity of these mutations development during azole therapy is highly unlikely. As these mutations are found both in patient and in environmental isolates we refer to these mutations as environmental mutations.

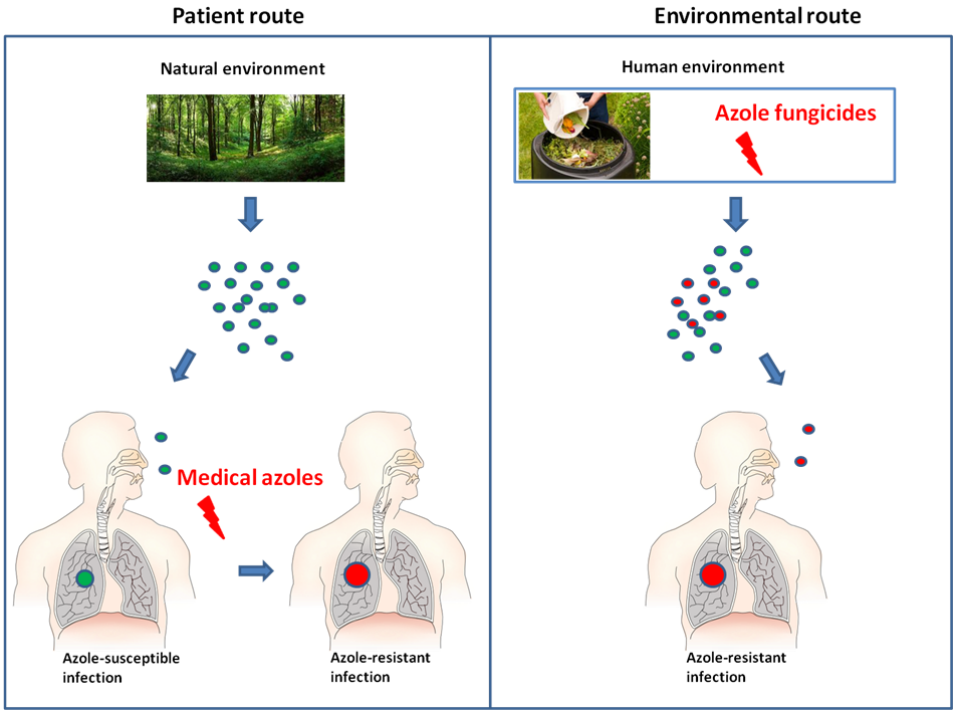

Figure 1. The two routes of azole resistance development in *A. fumigatus*. Airborne *A. fumigatus* spores inhaled by susceptible hosts and may cause aspergillus infection. Green represents triazole-susceptible and red triazole-resistant *A. fumigatus*.

- Resistance mutations found in the environment differ from those found to emerge through patient therapy. The latter consist of point-mutations in the Cyp51A-gene, such as G54W or M220I, without a tandem repeat.[2] As these point-mutations are not found in the environment in the Netherlands, we refer to these mutations as patient-derived mutations.
- Patient-derived mutations are only found in patients with chronic lung disease and a pulmonary cavity during triazole therapy. In some patients the conversion from a triazole-susceptible phenotype to a triazole-resistant phenotype has been documented.[2] Environmental resistance mutations can be found in all types of aspergillus diseases, most notably in patients with invasive aspergillosis.
- Two-thirds of patients with triazole-resistant invasive aspergillosis have no history of previous treatment with medical triazoles, thereby excluding the possibility of resistance selection

during therapy in these patients.[4,5] Resistance mutations in the isolates from these patients are from environmental origin.[3-5]

- Genotyping of environmental and clinical isolates that harbor the environmental resistance mutations shows clustering, indicating that these are genetically similar.[6]
- Despite the fact that *A. fumigatus* is not a phytopathogen, many azole fungicides used against phytopathogens show in vitro activity against *A. fumigatus*.[7] Five azole fungicides from the triazole class were highly active against wild-type *A. fumigatus*, but showed no activity against resistant isolates with TR_{34}/L98H. These five azole fungicides showed similarities with the molecular structure of medical triazoles and therefore the medical triazoles have also lost activity.[7]

Patients with triazole-resistant aspergillus disease have a high probability of treatment failure. Several case series reported treatment failure in patients with resistant chronic pulmonary aspergillosis,[8] and in patients with acute invasive aspergillosis. The mortality rate in patients with triazole-resistant invasive aspergillosis was 88%,[4] which is between two and three times higher than in triazole susceptible disease, although direct comparisons of triazole-susceptible and triazole-resistant infection are lacking.

Research assignment by the Dutch Ministry of Health

As resistance selection in the environment is an important cause of triazole-resistant aspergillus disease, the Ministry of Health provided a grant to investigate sources of triazole resistance in *A. fumigatus* in the environment.

Hypothesis:

Specific conditions and sites are thought to exist in the environment that facilitate the emergence, amplification and spread of triazole-resistance mutations in *A. fumigatus*.

Approach:

1. **Selection of sources of triazole-resistant *A. fumigatus*.** Through consultation of experts the characteristics of a source for triazole resistance selection were explored and a selection of sites was made that had a high probability of triazole resistance.
2. **Sampling of selected sites.** Samples were taken from the sites which were considered at high risk for resistance selection. Both the presence of *A. fumigatus* (resistant and not resistant) as well as the presence of azole fungicides was measured.
3. **Understanding the dynamics of *A. fumigatus* resistance.** Phenotypic and genotypic analysis of *A. fumigatus* populations recovered from resistance sources was performed to understand the process of resistance selection.
4. **Exploration of the geographical spread of triazole resistance aspergillosis cases.** The clinical implications of triazole resistance, the proportion of cases through environmental mutations and the spread of cases across the Netherlands was determined.

Aim. To identify and characterize conditions and sites that facilitate triazole resistance selection in *A. fumigatus* and to investigate the spread of triazole-resistant aspergillosis cases.

In addition, a project funded by the Ministry of Economic Affairs (RIVM triazolen-resistentie (3184200029)) will use an “experimental compost model” to investigate the dynamics of azole-resistance selection and the contribution and importance of various factors under controlled laboratory conditions. This research project is currently ongoing and the results will be reported separately.

A consortium of institutes conducted the research projects: the Centre for Agriculture and Environment (CLM), the Laboratory of Genetics of Wageningen University (WU) and the Department of Medical Microbiology of Radboud University Medical Center (Radboudumc). The RIVM acted as delegated mandator.

Results

1. Selection of sources of triazole-resistant *A. fumigatus*.

Approach

Three expert meetings were organized involving experts from different fields [attachment 1] to define the characteristics of a source of triazole-resistant *A. fumigatus*, and to select sites for sampling. We have called such sites **hotspots** for resistance development. Hotspots were characterized by combining current knowledge on resistance selection, the molecular mechanisms, and the ecology and life cycle of the fungus.

In addition, an Academy Colloquium was organized in collaboration with the Netherlands Royal Academy of Sciences (KNAW) in March 2015 entitled "Fungicides and azole-resistance selection in *Aspergillus fumigatus*". This meeting hosted international experts from various backgrounds and helped to further define the problem of azole resistance from a one-health perspective.

Biology of azole-resistance development

From the extensive discussions and from feedback of the various stakeholders we were able to define a hotspot for azole resistance of *A. fumigatus* as a location where the fungus can thrive while exposed to azoles. In principle resistance development to azoles is a stress response of the fungus that enables the fungus to survive and thrive in the presence of the stress factor (adaptation). The fungus can adapt to an environment with azoles through creating genetic diversity. Genetic diversity can be created through asexual sporulation, sexual reproduction (meiosis resulting in ascospores) or parasexual reproduction (fusion of fungal hyphae and nuclei). Asexual reproduction causes diversity through the production of new mutations, while the other two reproduction modes enable diversity through exchange of genetic information (recombination). Through chance the offspring could include a genotype, for instance a resistance mutation, that is better adapted to the azole containing environment. Through selection this genotype will emerge as the main survivor.

Hotspot characteristics

Thus a hotspot provides physical, biotic, and abiotic conditions with two main characteristics.

1. Facilitation of abundant and prolonged growth of *A. fumigatus*, so that the fungus can fulfil its full life cycle to exploit its full capacity for genetic variation and dispersal;

+ Conditions that support the growth of *A. fumigatus*
    - plant biomass
    - high temperatures (12°C to 65°C; 35°C is considered optimal growth temperature)

- high relative humidity (85%-100% is optimal)
- acidity (pH 3.7 – 7.6)
- specific nutrients

+ The fungus has to be able to escape (disperse) from the hotspot

2. Presence of azoles; azole characteristics that influence resistance development are:

+ Activity against *A. fumigatus*
+ Concentration
+ Combinations of (azole) fungicides
+ Relevant metabolites

The expert opinion underscored the importance of analyzing the full cycle of use of azole fungicides in the production of materials or crops and to review the specific applications within the production process.

Selection of potential hotspots

Based on these criteria a selection of potential hot spots was made that would be sampled for the presence of azole-susceptible and azole-resistant *A. fumigatus*, and for the presence of azole compounds. The selected hotspots included: peals from flower bulbs; residential organic waste; green composting; wood chippings compost; exotic fruit waste; fruit waste; commercial composting; wheat; horse manure; poultry manure; cattle manure; and maize silage. A number of materials and crops where azole fungicides are used (e.g. onions, horticultural crops) were not selected by the expert group in this stage, since these crops or the remains are not used in physical, biotic, or abiotic conditions that facilitate fungal growth of *A. fumigatus*.

2. Sampling and initial analysis of hotspots.

Approach

Sites that form potential hotspots were identified and sampled on three occasions. If possible, contrasting sites were selected and compared, i.e. those with known application of azole fungicides and sites where no azole fungicides were used.

(i) The samples were cultured for the presence of *A. fumigatus*, to determine if the conditions favored the growth of the fungus.

(ii) Furthermore, the resistance phenotypes were determined by culturing the samples on standard agar plates and plates supplemented with a triazole. On the standard agar both azole-susceptible and azole-resistant *A. fumigatus* colonies will grow, while on the azole-containing agar plates only azole-

resistant colonies will be able to grow. The proportion between azole-susceptible and azole-resistant colonies was recorded.

(iii) The presence of fungicides was measured in the samples. In all samples the following pesticides were analyzed: eleven triazoles (bromuconazole, difenoconazole, epoxiconazole, flusilazole, flutriafole, metconazole, penconazole, propiconazole, prothiconazole (and the metabolite prothioconazole-destio), tebuconazole and thiabendazole), four diazoles (cyazofamid, fenamidone, iprodione, triazoxide) and two imidazoles (imazalil en prochloraz).

Results from sample locations

Sample locations with no of low counts of A. fumigatus

Cereals are sprayed with azole fungicides in conventional farming. The grain is collected and stored in warehouses. The straw is partly used in animal stables and turns into manure. Different animal manure was collected. Both organic and conventional grain and straw were analyzed.

Horse manure

Wheat grain storage

No *A. fumigatus* was found in grain (either dry or wet) nor in manure from different locations and animals, while azole fungicides were present in some of the samples (Table 1). It was concluded that grain and animal manure were not hotspots for *A. fumigatus.*

Maize is sometimes sprayed with azole fungicides in conventional farming from 2014 onwards. The maize is stored in silage after harvesting. In this study two types of silage were collected, sprayed (conventional) and unsprayed with azole fungicides. Both sprayed and unsprayed silage was analyzed.

Maize silage

No *A. fumigatus* was found in maize silage (either with or without azole fungicides; Table 1). Normal maize silage undergoes anoxic fermentation creating unfavorable conditions for *A. fumigatus* to grow in. Still, in poorly constructed silage *A. fumigatus* is sometimes found. Preliminary conclusion is that maize silage is not a hotspot for *A. fumigatus*. Subsequent aim is to sample a poorly constructed silage for the presence of *A. fumigatus* and azole fungicides to confirm this conclusion.

Fruit (both regional and exotic fruit) is sprayed with azole fungicides in conventional farming. A small part of this fruit may start to rot during transportation or storage, and is then separated from healthy fruit in waste heaps. In this study both organic and conventional fruit waste (regional as well as exotic) was analyzed.

Regional fruit waste

Exotic fruit waste

No *A. fumigatus* was found in any of the fruit (either with or without azole fungicides; Table 1). Conclusion is that fruit waste is not a hotspot for *A. fumigatus*.

House hold green waste (consisting of vegetable, fruit and garden waste) might contain azole fungicides. This household green waste is collected using household containers and is processed in hydrolysis steps. In this study the household green waste was sampled at the central collection station simultaneously at three positions in the hydrolysis process: just before the start of the hydrolysis process, during the process (1 week in process) and at the end of the process (3 weeks).

Table 1. Sampled sites with no *A. fumigatus* or with low counts.

Origin	Growth of *A. fumigatus*?	Azole-resistant *A. fumigatus*?	Azole residues
Wheat grain	No	No	Yes
Manure	No	No	Yes
Maize silage	No	No	Yes
Fruit waste	No	No	No
Exotic Fruit waste	No	No	Yes
House hold waste hydrolysis start	Yes	Yes	Yes
House hold waste hydrolysis end	Yes	No	Yes
House hold waste private	Yes	Yes	No

This waste contained counts of *A. fumigatus* at the start (with 9% of isolates exhibiting an azole resistant phenotype) but no *A. fumigatus* at the end of the hydrolysis process (3 weeks) (Table 1), while in part of the household green waste samples traces of azoles were detected [Attachment 3]. Conclusion is that household green waste undergoing hydrolysis is not a hotspot for *A. fumigatus*. One sample was collected in a private household green waste container filled with green waste during 3 weeks. No *A. fumigatus* nor azoles were detected in this sample [Attachment 3].

Private garden owners sometimes use household green waste (consisting of vegetable, fruit and garden waste) in compost heaps. These heaps may contain residues of azole fungicides. Two of these heaps were sampled. In one of the two heaps *A. fumigatus* (2.3% resistant and 7% resistant a month later) was found, while no azole fungicide residues were measured (Table 1). It is expected that the presence of resistant *A. fumigatus* in the absence of azole residues is due to the presence of resistant *A. fumigatus* in the materials that were added to the heap, rather than an indication that this sample type is a source of resistance.

Private compost heap with household green waste

Sample locations with high counts of A. fumigatus and presence of azole residues

Flower bulbs are sprayed with or dipped in azole fungicides in conventional farming. Bulb waste (peals, and bulb and leaf waste) are collected and stored for composting. Different bulb waste heaps were sampled, both from an organic and a conventional bulb grower.

Flower bulb compost heap

A. fumigatus and azole fungicides were found in the flower bulb compost heaps in high quantities, with high percentages of resistant *A. fumigatus* varying between 6.2% and 24.5% (Table 2, Attachment 3). The heap from the organic flower bulb grower also contained high levels of azole fungicides. This grower indicated that he had just started the conversion of his farm to organic flower bulb production and therefore the bulb waste still contained the fungicides (from previous applications). A second sampling and analysis was done in the heaps one year later and the initial findings were confirmed. Thus the waste of bulb production met the criteria of a hotspot.

Green compost is professionally produced in large compost heaps at several locations in the Netherlands. These compost heaps contained traces of azole fungicides and *A. fumigatus* was found in high quantities, with high percentages varying between 8.5% and 30% of resistant *A. fumigatus* (Table 2, Attachment 3). However, during the composting process the counts of *A. fumigatus* decreased, indicating that professional composting may lead to a reduction of the burden of *A. fumigatus* (1.3% - 2.7% in the final compost) The presence of azole fungicides can be explained by the use of these fungicides in tree culture. Also in green compost a second sampling and analysis was done in the heaps one year later at the same location, and also at another location. Again the initial findings were confirmed. The green compost heaps met the criteria of a hotspot.

Waste of **processed wood** is collected and stored at some of the professional green compost companies. This wood is a mixture of several kinds of wood varying from railway sleepers, wooden boxes to wooden fences. The processed wood contained traces of azole fungicides and *A. fumigatus* was found in the wood heaps in high quantities, with relatively high percentages (5.8% - 20%) of resistant *A. fumigatus* (Table 2, Attachment 3). Presence of azoles can be explained by the use of these fungicides in some wood preservation culture. Also in the stored wood a second sampling and analysis was done one year later at the same location, and at another location. Visual observation indicated that the temperature in the stored wood was lower than in the compost heaps. Waste of wood is considered a hotspot for azole resistance selection.

Professional storage of processed wood

Table 2. Sampled sites with high *A. fumigatus* counts and the presence of azole residues.

Origin	**Growth of *A. fumigatus*?**	**Azole-resistant *A. fumigatus*?**	**Azole residues**
Bulb compost	Yes	Yes	Yes
Green compost	Yes	Yes	Yes
Wood chopping storage	Yes	Yes	Yes

Identification of hotspots

Overall **three hotspots** for azole-resistance selection were identified: compost from flower bulb waste, green compost and wood chippings. At these sites there was growth of *A. fumigatus*, azole-resistant *A. fumigatus* was recovered and residues of azole fungicides were found. Repeated sampling confirmed the presence of the three parameters. This was in line with the results of a previous study of Radboudumc and CLM[9]: in this pilot study azole-resistant *A. fumigatus* was also found in both

compost from flower bulb waste and green compost. Results from the other studied sites indicate that processes like hydrolysis (of regional and exotic fruit, and of household green waste), fermentation (silage and Bokashi) and manure storage (cattle, horses, goats) do not show growth of *A. fumigatus* irrespective of the presence of azole residues.

3. **Understanding the dynamics of *A. fumigatus* resistance selection.**

To obtain further insights in resistance selection the full process of application of fungicides and waste handling was investigated. For this purpose one application of azole fungicides was investigated in more detail. The flower bulb cultivation was selected as our research indicated abundant growth of *A. fumigatus* in flower bulb waste, high azole selection pressure and a high proportion of azole-resistant *A. fumigatus*.

How well does *A. fumigatus* thrive in the different steps of azole fungicide application and organic waste handling?

The application of azole fungicides, processing of flower bulbs and the composting procedures were sampled and investigated in detail. The main fungal threats for flower bulbs include *Botrytis* and *Fusarium*, which are the target pathogens for the application of mixtures of fungicides including azoles. Flowers are cut to enhance growth of the bulb, and the flower cuttings are left in the field. Organic waste, including flower bulbs, is collected during the year at designated sites in the field. Over a period of approximately 10 months (May to February) the flower bulb waste is collected, with fresh waste being added over time (pre-compost phase). From March onwards active composting takes place. The resulting compost is then used to fertilize the soil. Longitudinal sampling of these composting phases shows persistence of resistance over time. High counts of *A. fumigatus* and azole-resistant *A. fumigatus* were found. Furthermore, repeated sampling of flower bulb waste showed persistence of fungicide residues over time (Figure 2).

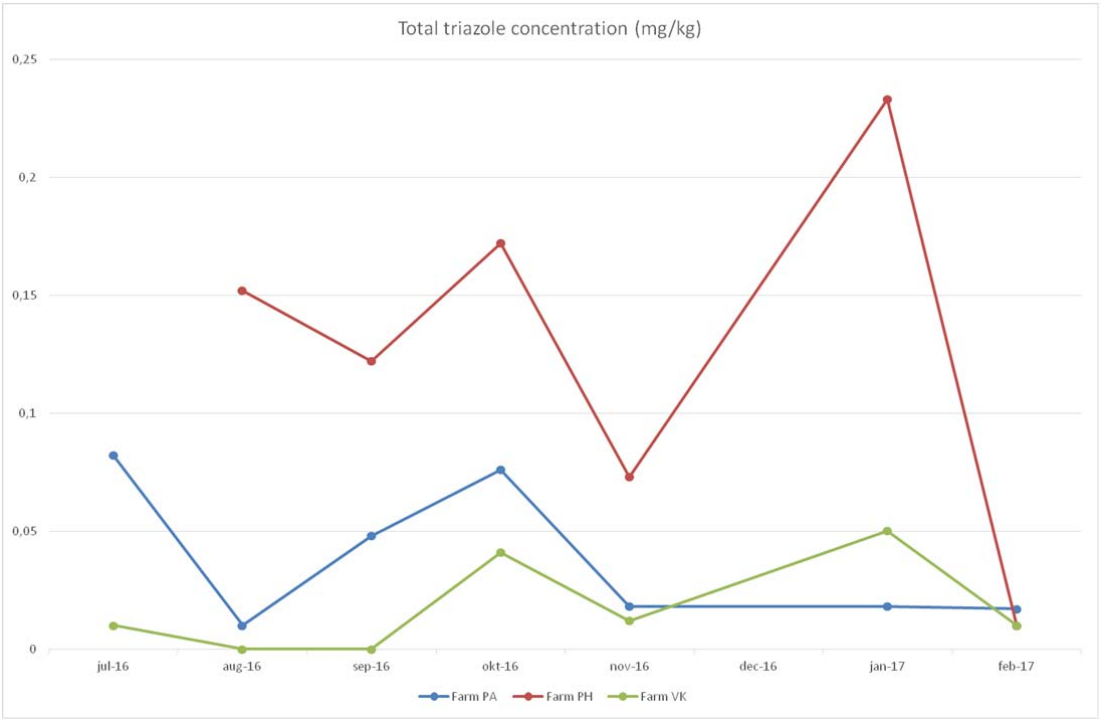

Figure 2. Triazole fungicide residues measured in flower bulb waste (pre-compost phase) between July 2016 and February 2017 at three different producers.

To determine the dynamics of *A. fumigatus* during active composting, samples were obtained from a commercial compost manufacturer and analyzed for the presence of azole-susceptible and azole-resistant *A. fumigatus*. Material used for the composting, such as grass and twigs and wood chippings contained high numbers of azole-resistant *A. fumigatus*, but sampling of the subsequent stages of composting showed a decrease of *A. fumigatus* in time. The high temperatures, i.e. between 50°C and 60°C, may be too high for the asexual spores (so-called conidia) to survive. The end product thus contained very low counts of *A. fumigatus* conidia.

The commercial compost manufacturer follows a strict scheme of different steps over a period of at least six weeks. In this period the material is mixed regularly to obtain an optimal composting process including the high temperatures mentioned. Non-professional composting by flower bulb growers or private garden owners may be less strict leading to an incomplete process with lower temperatures. Compost is a very favorable substrate for *A. fumigatus:* In the laboratory *A. fumigatus* produces up to $5x10^9$ spores per gram flower bulb-peal compost within 5 days.

What is the effect of azole exposure on *A. fumigatus* phenotypes and genotypes?

Azole-containing flower bulb compost and an azole-free vegetable and fruit compost heap were sampled. Predominantly azole-susceptible *A. fumigatus* (98%) was retrieved from the azole-free

compost heap (2% azole-resistant), while 91% of the *A. fumigatus* recovered from the azole-containing compost was resistant. The resistance mutations included TR_{46}/Y121F/T289A (80%), TR_{46}/Y121F/M172I/T289A/G448S (2%) and 9% contained a new mutation harboring three 46 base pare repeats: TR_{46}^{3}/Y121F/M172I/T289A/G448S. Microsatellite typing showed the presence of 26 unique genotypes in the azole-containing heap and 39 from the azole-free heap. Genetic studies showed that the new mutation could arise through sexual crosses of two isolates harboring TR_{46}/Y121F/M172I/T289A/G448S, although genotyping of the isolates recovered from the compost heap indicated that the new mutation might have evolved through asexual reproduction.[10] This result underscores that environments that facilitate all aspects of the fungal life cycle to be fulfilled are potentially in particular prone to producing new/emerging resistant genotypes.

The clinical *A. fumigatus* surveillance database was investigated for the presence of the new TR_{46}^{3} mutation in clinical isolates. Three patients were identified who harbored this resistance mutation: one from Zwolle (2012), one from Leiden (2013) and one from Amsterdam (2105). Thus the resistance mutations were found both in the environment and in clinical isolates.[10]

How does *A. fumigatus* become resistant - Is there evidence for sexual recombination as a mode for resistance selection?

Azoles are not mutagenic and *A. fumigatus* can respond to azole stress through creating genetic diversity, as individuals of the progeny might be better suited to survive and reproduce in the azole-containing environment. The presence of the azole then selects for the progeny best adapted. Genetic diversity can be achieved through asexual sporulation, as new spontaneous mutations will occur. Alternatively, sexual reproduction might occur, where two different mating types will undergo meiosis and diversity is achieved through recombination of the genetic background of the parent isolates. Sexual reproduction is known to be a very efficient way for fungi to create genetic diversity, but in *A. fumigatus* this reproduction mode has been observed only under laboratory conditions and never in nature.

Given the complex azole-resistance mutations found in environmental resistant *A. fumigatus*, we hypothesize that sexual recombination might be a strategy for *A. fumigatus* to escape from the stress caused by the azole-containing environment. A so-called heat-shock procedure has been described to select for the sexual spores of *A. fumigatus*, as the ascospores (sexual spores) survive such high temperatures and asexual spores do not. Although the *A. fumigatus* ascospores have never been found in nature, heat shock was applied to the compost samples collected for the abovementioned hot spot analyses. The compost was cultured following heat shock and if *A. fumigatus* colonies are recovered, it is likely that they originated from (sexual) ascospores, as the asexual spores do not survive such high temperatures. Indeed *A. fumigatus* colonies were recovered following heat shock of compost samples,

supporting (but not proving) a possible role for sexual reproduction in resistance development. However, further studies are needed to obtain more supporting evidence.

Until now sexual reproduction was achieved only on specific nutrient agar (oatmeal agar) under laboratory conditions.[11] In order to determine if sexual reproduction could take place in compost, a sexual crossing was performed in the laboratory on compost samples that were collected from a hotspot. Under laboratory conditions *A. fumigatus* was able to complete the sexual cycle and produce ascospores on compost agar. This observation further supports the possibility of sexual reproduction in compost.

Is there a cost of resistance and can we make use of this for control of the resistance problem?

Point mutations in the *Cyp51A*-gene are associated with ligand access channels and it is believed that due to these mutations the azole can no longer pass through this channel and thus in unable to reach and bind to its target. The environmental resistance mutations, however, poses a tandem repeat in the promoter region of the gene, and previous studies have indicated that causes overexpression of the Cyp51A-gene as resistance mechanism. As overexpression of a gene might not be a favorable condition for the fungus, we investigated if the fungus could switch of this mechanism when grown in the absence of azole exposure.

Using two *A. fumigatus* isolates with tandem repeat resistance mechanisms, TR_{46}/Y121F/T289A and $TR_{46}{}^{3}$/Y121F/T289A, the expression levels were determined when the isolates were cultured in agar with and without an azole. These studies indicated that the expression of the *Cyp51A*-gene was similar under both conditions. This indicates that the fungus has lost control over the regulation of the *Cyp51A*-gene, and that the overexpression cannot be switched off when it is not needed, i.e. in an azole-free environment.

Continued overexpression of the *Cyp51A*-gene in the absence of azole pressure is an unfavorable condition for *A. fumigatus* and might make the fungus prone to losing the tandem repeat, with subsequent reversal of the triazole-resistant phenotype, to a triazole-susceptible phenotype. Therefore two isolates were cultured in the laboratory for 20 generations in the absence of azoles and alternating between standard Sabouraud agar and Takasio agar (aimed to stimulate asexual sporulation). After 20 generations the phenotype and genotypes were assessed. However, no change was observed, indicating that the mutations associated with resistance remained present. Further laboratory studies are needed to understand how loss of resistance mutations is achieved by *A. fumigatus*.

The role of the azole fungicide. Do other azole fungicides that are not structurally related to the medical triazoles have the ability to select for azole resistance in *A. fumigatus*? And if so do the resistance mutations exhibit cross resistance to the medical triazoles?

A. fumigatus is not the target microorganism for azole fungicides, and the first question was to investigate the activity of azole fungicides against *A. fumigatus* isolates.[7] The in vitro activity of fungicides was investigated against wild type (azole susceptible) *A. fumigatus* and isolates harboring the TR_{34}/L98H resistance mutation. The distribution of minimum inhibitory concentrations (MICs) of azole fungicides against both groups of isolates was compared using the correlation effect size. If the correlation effect size is zero, the MIC distributions of the fungicide against the two groups overlapped, indicating no difference in activity against wild type and TR_{34}/L98H *A. fumigatus*. However, if the effect size was approaching 1, the drug was active against the wild type isolates, but not against the azole-resistant isolates. The results for 31 fungicides is shown in Figure 3. Eight fungicides showed correlation effect sizes close to 1: itraconazole (medical triazole), and seven fungicides: epoxiconazole, difenoconazole, propiconazole, bromuconazole, metconazole, imazalil, and tebuconazole (Figure 3). Five of these were found to be of the triazole class and have a similar molecule structure to the medical triazoles: epoxiconazole, difenoconazole, propiconazole, bromuconazole and tebuconazole, which are indicated in blue in the figure. However, other azole fungicides, such as imazalil showed good activity against wild type *A. fumigatus* but not against the resistant isolates.

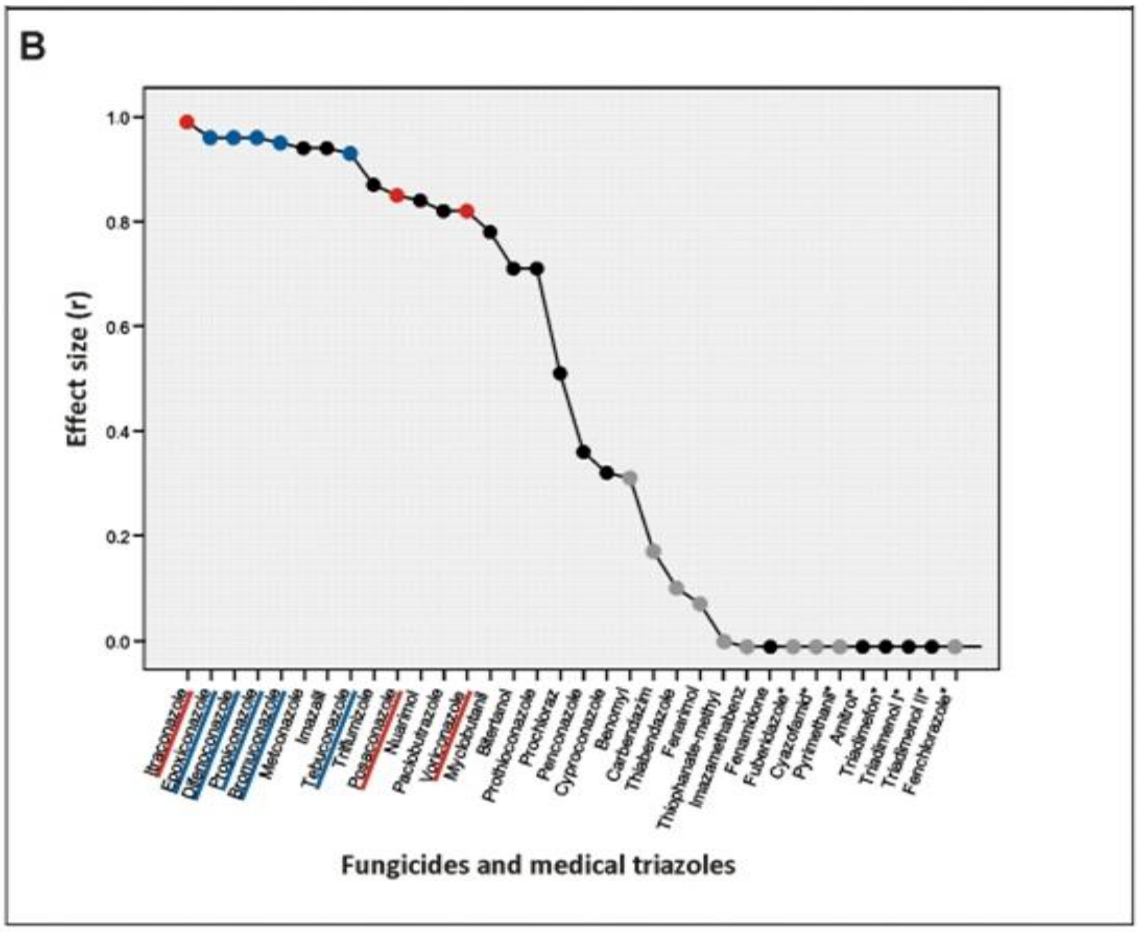

Figure 3. Correlation effect size of 31 fungicides against wildtype *A. fumigatus* and isolates harboring the TR34/L98H mutation.[7] Three medical triazoles, itraconazole, posaconazole and voriconazole are indicated in red. Five triazole fungicides, epoxiconazole, difenoconazole, propiconazole, bromuconazole and tebuconazole are indicated in blue. These compounds have a molecule structure that is similar to that of the medical triazoles. The remaining fungicides are indicated in black.

When wild type *A. fumigatus* was cultured in the presence of the five fungicides structurally related to the medical triazoles (epoxiconazole, difenoconazole, propiconazole, bromuconazole and tebuconazole), the isolates became resistant to the five fungicides and also against the medical triazoles (cross-resistance). Our hypothesis is that cross-resistance occurs because of the molecule similarity between azole fungicide and medical triazole. The hypothesis implies that if the molecule is not similar, cross-resistance might not occur or to a lesser extent. This question was investigated under laboratory conditions.

An *A. fumigatus* strain was evolved in the presence of either difenoconazole, metconazole or imazalil at MIC_{50} concentrations over seven weeks. MICs were measured weekly to monitor the development of resistance against the compound used for evolution itself as well as development of cross-resistance

against the other fungicide compounds and the three medical triazoles (itraconazole, voriconazole, and posaconazole).
Preliminary results show that two non-medical triazoles (difenoconazole and metconazole) with a high effect size and similar molecule structure were able to induce cross resistance against the medical triazoles. However, despite the high effect size, imazalil did not show the potency to induce resistance or cross-resistance against the medical triazoles. This compound is not a triazole but an imidazole.

To further explore the relation between molecule structure and cross-resistance additional confirmative studies are needed that include various older azole fungicides as well as those with lower correlation effect size against *A. fumigatus*, such as for instance prochloraz. In addition the pyrimidine fenarimol could also be tested as the compound has a low effect size and is not a triazole. Of course the activity of these alternative fungicides for use against the target pathogen should also be carefully assessed.

4. Exploration of the geographical spread of triazole resistance aspergillosis cases.

Triazole-resistance frequencies in patients are monitored through a national surveillance network, coordinated by Radboudumc, where five Dutch University Medical Centers (ErasmusMC, Rotterdam; LUMC, Leiden; VuMC, Amsterdam; UMCG, Groningen; Radboudumc, Nijmegen) systematically screen all clinical *A. fumigatus* isolates for azole resistance. This basic surveillance gives information on differences between centers and on trends in distribution of resistance mutations. As a positive culture is not always indicative of an aspergillus disease, these results cannot be linked to specific aspergillus diseases or risk groups. Resistant isolates are sent to Radboudumc for MIC-testing and genetic analysis. The findings are published each year in the Nethmap report.[12] The overall resistance frequency in 2016 was 12.9%, and resistance frequencies vary between the medical centers (Table 3). Some centers such as LUMC consistently show high resistance frequency, while other such as Radboudumc show lower resistance frequencies. It is likely that multiple factors contribute to the resistance frequency such as frequency of fungal culture, variations in underlying disease of the patients, and possibly geographic variation in exposure.

Table 3. Triazole resistance frequency in clinical *A. fumigatus* isolates in five Dutch University Medical Centers, 2013 to 2016.

	2013		2014		2015		2016	
	#pat. screened	#pat. with azoleR (%)	#pat. screened	#pat. with azoleR (%)	#pat. screened	#pat. with azoleR (%)	#pat. screened	#pat. with azoleR (%)
EMC[&]	231	10 (4.3)	265	10 (3.8)	22	7 (31.8)[*]	186	24 (12.9)
LUMC	99	19 (19.2)	113	15 (13.3)	141	23 (16.3)	88	18 (20.5)
RUMC	123	6 (4.9)	143	7 (4.9)	145	12 (8.3)	210	20 (9.5)
UMCG	194	16 (8.2)	191	18 (9.4)	225	15 (6.7)	215	26 (12.1)
VUmc	113	8 (7.1)	104	9 (8.7)	89	14 (15.7)	85	13 (15.3)
Total	760	58 **(7.6)**	814	59 **(7.2)**	600	64 **(10.7)**[¥]	784	101 **(12.9)**

[&]University Medical Centers: EMC, Erasmus Medical Center; LUMC, Leiden University Medical Center; RUMC, Radboud University Medical Center; UMCG, University Medical Center Groningen; VUmc, VU University Medical Center.

[*]Included only high risk patients.

[¥]Triazole resistance frequency calculated for four centers.

Studies investigating resistance frequency in specific risk groups generally show higher triazole-resistance rates when compared to the general surveillance. In one study performed in the intensive care unit of LUMC a resistance frequency of 26% was found in culture-positive patients with invasive aspergillosis.[13] ErasmusMC reported high resistance rates in ICU patients and hematology patients: 31.8%.[12] A recent nation-wide study of invasive aspergillosis in association with influenza, which involved the ICUs of all eight Dutch UMCs, reported an azole-resistance frequency of 29%.[14] Several of these studies indicated the presence of mixed infection, i.e. infection by both azole-susceptible and azole-resistant *A. fumigatus*.[14,15] Mixed cultures are recovered from approximately 25% of patients, and increases the risk of resistance being missed at diagnosis.[14,15]

Analysis of the underlying resistance mutations of clinical *A. fumigatus* collected in the National general surveillance program indicated that these were dominated by mutations that are associated with environmental resistance selection, i.e. TR_{34}/L98H and TR_{46}/Y121F/T289A.[12] Between 2009 and 2016, these two mutations comprised between 83% and 94.6% of clinical triazole resistant *A. fumigatus* isolates.

Interestingly the distribution of environmental resistance mutations varies over time. TR_{46}/Y121F/T289A was first found in the Netherlands in 2009 and then comprised 1.4% of environmental mutations. The proportion of the TR_{46}/Y121F/T289A mutations increased to 44.6% in 2014, but then decreased with only 10.8% of environmental resistance due to TR_{46}/Y121F/T289A in 2016 (Figure 4).

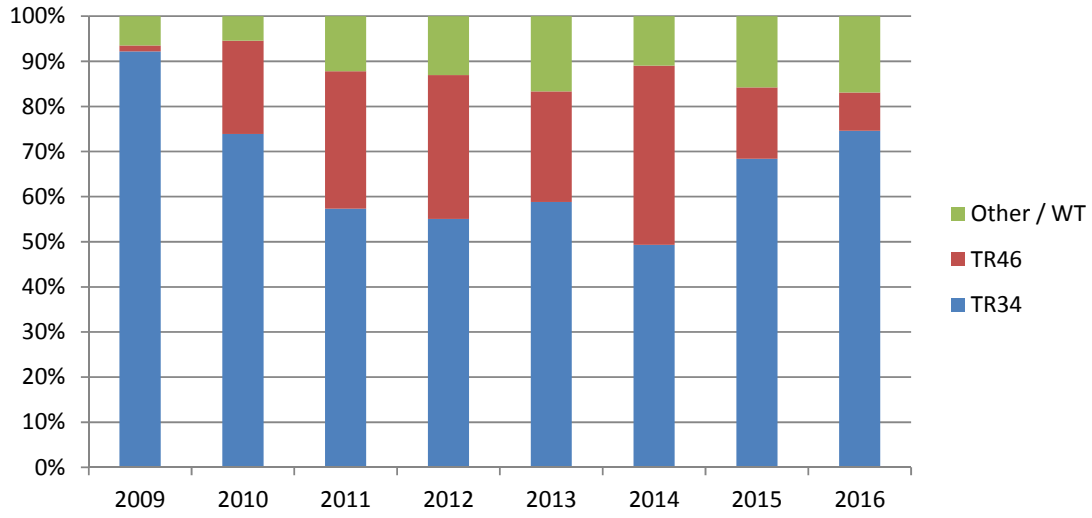

Figure 4. Distribution of resistance mutations found in clinical *A. fumigatus* isolates over an eight year period (2009 - 2016). TR_{34}/L98H is represented in blue; TR_{46}/Y121F/T289A in red. Other Cyp51A mutations or absence of mutations in this gene (wild type, WT) is represented in green.

General discussion

The aim of our project was to investigate potential sources of triazole resistance in *A. fumigatus* in the environment. We identified three hotspots for resistance development and defined the critical factors that facilitate resistance selection: *A. fumigatus* should be able to grow and complete its life cycle, and the presence of azoles. The sources that we sampled confirmed these principles as sites fulfilling the definition were found to contain high resistance counts, while resistance was not found at sites that did not fulfill the definition. We consider this a general principle for resistance selection which can be used to identify other hotspots for resistance selection.

In parallel to our research project a similar project is ongoing in the United Kingdom supported by the Fungicide Resistance Action Committee (FRAC), an organization installed by the producers of fungicides and aimed at providing management guidelines to prolong the effectiveness of fungicides.[16] This project is conducted by Rothamsted Research (Harpenden, United Kingdom), and information from both projects is exchanged. The Rothamsted Research project has identified a number of other products that contain azole-resistant *A. fumigatus* including tea, pepper and onions. The tea and pepper were bought at supermarkets and online and originated from various areas in the world including China, other Asian countries and South America. The onions originated from the United Kingdom and The Netherlands. Genotyping of the azole-resistant isolates from different products showed various profiles, which suggests that resistant genotypes might arise independently. These results indicate that other sources of triazole-resistant *A. fumigatus* may be present. In the Rothamsted Research Project air samples were collected to identify *A. fumigatus* in the air at three locations (United Kingdom, The Netherlands and Poland).

The presence of azole fungicides favors the growth of azole-resistant *A. fumigatus:* high resistance frequencies were found in compost with azole residues while wild type *A. fumigatus* was predominantly present in azole free compost. How *A. fumigatus* acquires azole resistance mutations requires more research although we did provide evidence that resistance mutations can develop through both sexual and asexual reproduction and that *A. fumigatus* can complete both reproduction cycles in compost. Our study also showed that new resistance mutations continue to emerge in the environment, which underscores the non-sustainability of current practices.

When analyzing the full process of azole fungicide application, waste management and composting, it appeared that the management of organic waste that contains azole residues is an important factor. Active professional composting was found to reduce the burden of (resistant) *A. fumigatus*, but high counts where found at sites where the organic waste was (only) collected and stored. Organic waste greatly facilitates the growth of *A. fumigatus* and presence of azole residues favors the growth of resistant isolates. Creating conditions that do not favor growth of *A. fumigatus* or avoiding exposure to azole fungicides that cause cross-resistance to the medical triazoles might lead to reduction of the resistance burden. Sampling of the various sites showed that in some samples *A.*

fumigatus was absent, for instance in very dry material or when hydrolyzed. This indicates that alteration of the conditions might help reduce the burden of (resistant) *A. fumigatus*.

Surveillance of clinical *A. fumigatus* isolates showed resistance frequencies exceeding 10% in most centers. There is variation between centers and some centers consistently show high resistance rates. Although this might indicate different levels of exposure to airborne resistant *A. fumigatus* spores, many other factors may play a role such as laboratory practices and differences in patient populations. The general surveillance does indicate that the environmental resistance mutations, i.e. TR_{34}/L98H and TR_{46}/Y121F/T289A are high dominant accounting for >80% of Cyp51A-mediated resistance mutations. We did not investigate the genetic relatedness of isolates between environmental hot spot and clinical isolate. Linking clinical resistant *A. fumigatus* isolates to specific sources / hotspots would help to develop preventive interventions, but it remains unclear if genetic markers are present that can differentiate between origins of resistant strains.

Our current understanding of azole-resistance selection in the environment, based on this study, is shown in Figure 5.

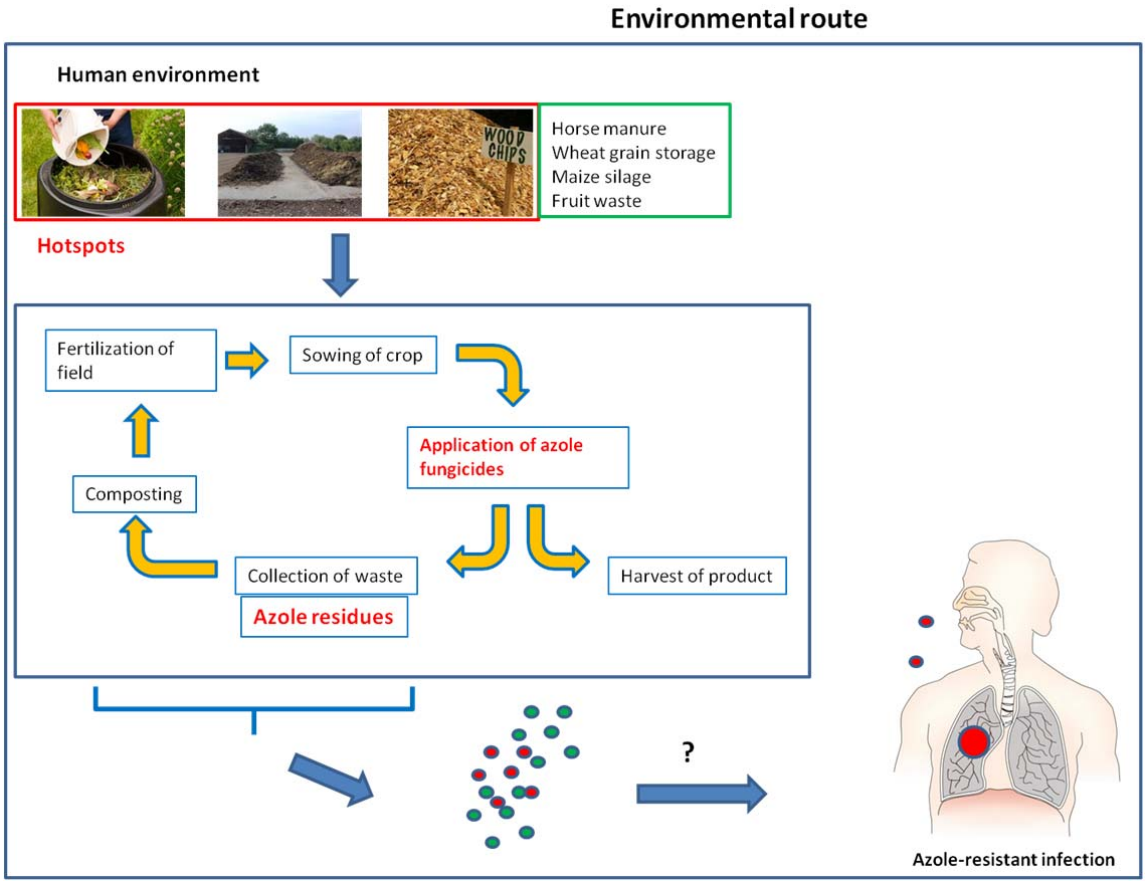

Figure 5. Schematic representation of azole resistance selection as cause of triazole-resistant aspergillosis. In the environment that has been altered through human activities sites were sampled that might fulfill the hotspot criteria. Several sampled sites did not meet the criteria (green box), but three hotspots were identified, which harbored high levels of azole-resistant *A. fumigatus* (red box).

Our research indicated that the collection and storage of organic waste facilitates growth of resistant *A. fumigatus* if azole residues are present. These may serve as sources for airborne resistant *A. fumigatus* spores which are subsequently inhaled by humans. How *A. fumigatus* spores are dispersed from the hotspot and infect humans remains unclear. Green dots represent azole-susceptible spores, and red dots represent azole-resistant spores.

Recommendations

Our studies indicated that growth of *A. fumigatus* in the presence of azole fungicides creates a situation that favors the selection of resistant *A. fumigatus* in the environment. However, many questions still remain which would require further research. Therefore only general recommendations can be made:

1. Antifungal stewardship.

Azole fungicides represent an important group of compounds used for the control of fungal crop disease and for the preservation of a diversity of materials such as wood and coatings. The medical triazoles are an important class of medicines for the management of aspergillus diseases. The ultimate aim would be to preserve this drug class for both applications and to strongly reduce the risk of induction of resistant *A. fumigatus* at the same time. A general recommendation is to use the azole compounds wisely. An antifungal stewardship program designed to improve the appropriate use of azoles, including indications for appropriate use, selection of the optimal drug, the dose, duration and route of administration would help to retain the desired effect but reduce inappropriate and unnecessary use. Such programs are being implemented in hospitals and could also be promoted for applications outside medicine.

2. Substitution.

Our laboratory studies indicated that resistance mutations that develop through exposure to an azole affect the activity of other azole compounds if these have a similar molecule structure. Using similar molecules in the environment and in medicine is the basis of the resistance problems observed in aspergillus diseases. Five fungicides, all from the triazole class, were previously identified to have a similar molecule structure to that of the medical triazoles. Substitution of these fungicides by other molecules might reduce the risk of cross resistance and reduce the burden of resistant aspergillus disease. Further studies would be needed to ascertain the efficacy of the alternative agents against the target pathogen, to make sure the alternative is authorized for use, and to confirm that exposure to the "dissimilar" azoles indeed does not result in cross resistance to the medical triazoles.

3. Organic waste handling.

A. fumigatus is a saprophyte that grows on dead plant material. We found that the fungus grew abundantly on the compost obtained from the sampled hotspots and that the presence of azole residues selected for resistance. We also demonstrated that *A. fumigatus* can complete both the sexual and asexual life cycle in compost samples. The collection and storage of organic waste therefore is an important risk for resistance. We recommend to reassess the handling of organic waste focusing on

altering the conditions that reduce or preclude the grow of *A. fumigatus*. Absence of *A. fumigatus* in several of the sites we have sampled indicates that modification of storage conditions or composting process may strongly reduce the ability of *A. fumigatus* to grow. Ongoing research supported by the Ministry of Economic Affairs will help to identify and understand the relative importance of a number of factors that facilitate resistance selection in compost.

4. **Surveillance of azole-resistant aspergillosis.**

Surveillance of triazole resistance is ongoing in Dutch hospitals and is used to detect trends in resistance frequency and in the distribution of resistance mutations. Continued surveillance is important to assess the effect of preventive measures if they are to be implemented in the environment. Issues related to the diagnosis and treatment of patients with triazole-resistant aspergillus diseases are addressed through medical research and national guidelines (SWAB).

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Attachment 1. Participants of the project team and Expert group

Het project was executed in commission of the Ministry of Health. The RIVM acted as delegated mandator.

- National Institute for Public Health and the Environment (RIVM)
    - Dr. A.G. Rietveld

The project team:

- Radboudumc
    - Prof. Dr. P.E. Verweij
    - Dr. W. Melchers
- Centre for Agriculture and Environment (CLM)
    - Dr. P.C. Leendertse
    - Ir. E. Hoftijser
- Wageningen University (WU)
    - Prof. Dr. B.J. Zwaan

Expert from different fields were invited to participate in expert meetings.

Invited experts :

- National Institute for Public Health and the Environment (RIVM)
    - Dr. C. Graven (specialist pesticides)
- Centre for Agriculture and Environment (CLM)
    - Ir. F. van der Schans (specialist livestock farming)
- Wageningen University and Research centre (WUR)
    - Dr. S. Schoustra (specialist evolutionary biology)
    - Dr. F. Debets (specialist fungal genetics)
- Board for the Authorisation of Plant Protection Products and Biocides (Ctgb)
    - Dr. D. Heemsbergen (specialist azoles, biocides)
- KWR Watercycle Research Institute (KWR)
    - Dr. P. Van der Wielen (specialist Aspergillus and water)
- Utrecht University, Faculty of Veterinary Medicine (UU)
    - Prof dr. Johanna Fink-Gremmels (specialist fungal disease in animals)
- CBS-KNAW Biodiversity Center
    - Dr. J. Houbraken (Aspergillus taxonomy)
- Alb. Groot B.V,
    - A. Conijn (specialist bulb diseases)

Attachment 2. Source analysis: incidence of azole-sensitive (S) and azole-resistant (R) *A. fumigatus* in various types of organic waste material with or without azole fungicide residues.

Sample #	Sample ID	Origin	Type*	CFU/gr *A. fum*: S	CFU/gr *A. fum*: R	% R	Ascospores[§]	Azole residues
18	GRA.D G 291015	Wheat grain dry	R	<20	<20		No	Yes
19	GRA.N G 291015	Wheat grain wet	R	<20	<20		No	Yes
20	GRA.D B 291015	Wheat grain dry	O	<20	<20		No	No
1	KIP 1 G 2009	Poultry manure	R	<40	<40		No	No
16	KIP B 291015	Poultry manure	O	<20	<20		No	No
4	JVM 1 G 2409	Cattle manure	R	<40	<40		No	Yes
15	VEE 1B 9-10-15	Cattle manure	O	<20	<20		No	Yes
5	PAA 1 G 2409	Horse manure	R	<40	<40		No	Yes
6	PAA 2 B 2409	Horse manure	O	<40	<40		No	Yes
2	MAI 1 G 2409	Maize silage	R	<40	<40		No	Yes
3	MAI 2 G 2409	Maize silage	R	<40	<40		No	No
30	FRU 1G 291115	Fruit waste	R	<20	<20		No	No
14	FRU B 091015	Fruit waste	O	<20	<20		No	No
21	EXFR G 281015	Exotic fruit waste	R	<20	<20		No	Yes
22	EXFR B 281015	Exotic fruit waste	O	<20	<20		No	Yes
17	GFT 1G 291015	Household waste (GFT) compost-private	R	6.4×10^4	1.48×10^3	2.3%	No	No
29	GFT 2G 291115 duplicate sample 17	Household waste (GFT) compost-private	R	2.7×10^5	1.8×10^3	7%	No	No
26	GFT 1B 29112015	Household waste (GFT) compost-private	O	100	<100		No	Yes
37	AGF 13102016	Household waste (GFT) –private container	O	<20	<20		No	Yes
25	VGFT 1 27112015	Household waste (GFT) compost hydrolysis fresh	R	2.25×10^3	200	9%	No	No
27	VGFT 2 27112015	Household waste (GFT) 1 week hydrolysis	R	<100	<100		No	Yes
28	VGFT 3 27112015	Household waste (GFT) 3 weeks hydrolysis (end)	R	200	<100		No	Yes
7	BOL 1G 30092015	Bulb compost	R	1.96×10^4	4.3×10^3	21.9%	No	Yes
8	BOL 1B 30092015	Bulb compost 2014	O-R[¥]	1.23×10^4	2.5×10^3	20.3%	No	Yes
24	BOL BA 301015	Bulb compost	O-R[¥]	2.9×10^3	180	6.2%	60 (not *A.fum*)	Yes
9	BOL 2B 30092015	Bulb compost 2015	O-R[¥]	2.73×10^4	3.9×10^3	14.3%	No	Yes
23	BOL B2015 301015	Bulb compost 2015	O-R[¥]	9.4×10^5	2.3×10^5	24.5%	60 (not *A.fum*)	Yes
31	BOL 13102016	Bulb compost 2015	O-R[¥]	8.0×10^3	1.0×10^3	13%	60 (not *A.fum*)	Yes

32	BOL B2016 131016	Bulb compost 2016	O-R[¥]	3.0×10^5	9.0×10^5	30%	No	Yes
40	BOL 3 B 13102016	Bulb fermentation Bokashi	O-R[¥]	20	<20		No	Yes
11	COM 9 9-10-15	Green compost (1 week)	R	9.4×10^4	8×10^3	8.5%	No	Yes
12	COM 2 9-10-15	Green compost (5 weeks)	R	1.88×10^6	8.4×10^4	4.5%	No	Yes
10	COM 0 9-10-15 Location Goor	Green compost (final, 7 week)	R	2.72×10^5	3.6×10^3	1.3%	No	Yes
33	COM 3 13-10-16 Location Goor	Green compost (5 weeks)	R	3.0×10^5	9.0×10^4	30%	No	Yes
34	COM 4 13-10-16 (Location Goor)	Green compost (final, 7 week)	R	2.2×10^3	60	2.7%	No	Yes
35	COM 1 13-10-16 Location Gelder	Green compost (5 weeks)	R	7.0×10^4	2.8×10^3	4%	No	Yes
36	COM 2 13-10-16 (Location Gelder)	Green compost (final, 7 week)	R	2.2×10^3	60	2.7%	No	Yes
13	HOU 1G 9-10-15 C wood (location Goor)	Wood chippings waste /	R	6.2×10^4	3.6×10^3	5.8%	No	Yes
37	HOU 3G 13102016 C wood (location Goor(	Wood chippings waste /	R	1.0×10^4	1.3×10^3	13.0%	No	Yes
38	HOU 2G 13102016 B wood (location Goor)	Wood chippings waste /	R	3.0×10^5	6.0×10^3	20%	No	Yes
39	HOU 1G 13102016 B wood (location Gelder)	Wood chippings waste /	R	60	<20	5.8%	No	Yes

*R, regular; O, organic.

[¥]O-R represents bulb compost of an organic grower. However, azole residue analysis indicated that the organic grower mixed regular straw and grass with organic waste in the compost.

[§]Ascospores are *A. fumigatus* spores that originate from sexual reproduction.

Mismatch between resistant *A. fumigatus* in the absence of azole residues. Might be due to sampling error or background resistance.

C-wood is heavily treated wood , B wood is moderately treated wood

Attachment 3. Source analysis: fungicide measurements (only detected triazoles, diazoles and imidazoles and metabolites are shown).

Sample#	Origin	Sample and fungicide*	Concentration	Code
18	**Wheat**	**Wheat dry (R)**	**mg/kg**	GRA.D G 291015
		Epoxiconazole	<0.01	
		Tebuconazole	<0.01	
19	**Wheat**	**Wheat moist (R)**	**mg/kg**	GRA.N G 29102015
		Epoxiconazole	<0.01	
		Tebuconazole	<0.01	
20	**Wheat**	**Wheat (O)**	**mg/kg**	GRA.D B 291015
			-	
1	**Poultry manure**	**Poultry manure (R)**	**mg/kg**	KIP1G2009
16	**Poultry manure**	**Poultry manure (O)**	**mg/kg**	KIP B 291015
			-	
4	**Cattle manure**	**Cattle manure (R)**	**mg/kg**	JVM1G2409
		Tebuconazole	0.3	
		Epoxiconazole	0.048	
		Prochloraz	0.017	
15	**Cattle manure**	**Cattle manure (O)**	**mg/kg**	VEE 1 B 9-10-15
		Difenoconazole	< 0.01	
		Epoxiconazole	0.051	
		Propiconazole	0.038	
		Prothioconazole-destio	0.0205	
		Tebuconazole	0.087	
5	**Horse manure**	**Horse manure / straw (R)**	**mg/kg**	PAA1G2409
		Epoxiconazole	0.021	
		Cyproconazole	0.012	
		Prochloraz-desimidazole-amino	0.048	
		Tebuconazole	0.082	
6	**Horse manure**	**Horse manure / straw (O)**	**mg/kg**	PAA2B2409
		Tebuconazole	0.015	
2	**Maize silage**	**Maize silage (R) with Retengo**	**mg/kg**	MAI1G2409
		Epoxiconazole	0.012	
3	**Maize silage**	**Maize silage (R) without Retengo**	**mg/kg**	MAI2G2409
		Prochloraz-desimidazole-amino	0.34	
30	**Fruit waste**	**Fruit waste (O)**	**mg/kg**	
		Tebuconazole	<0.01	FRU B 91015
14	**Fruit waste**	**Fruit waste (R)**	**mg/kg**	FRU 1G 29112015
			-	

21	**Exotic fruit**	**Exotic fruit waste (R)**	**mg/kg**	EXFR G 28102015
		Imazalil	0.22	
22	**Exotic fruit**	**Exotic fruit waste (O)**	**mg/kg**	EXFR B 28102015
		Azaconazole	<0.01	
		Imazalil	0.19	
		Tebuconazole	<0.01	
17	**House hold waste (GFT) compost – privately owned**	**GFT compost Privately owned (R)**	**mg/kg** -	GFT 1G 29102015
29	**House hold waste (GFT) compost – privately owned**	**GFT compost Privately owned (R) duplicate**	**mg/kg** -	GFT 2G 29112015
26	**House hold waste (GFT) compost – Privately owned**	**GFT compost Privately owned (O+R)**	**mg/kg**	GFT 1B 29112015
		Prothioconazole	<0.01	
		Epoxiconazole	<0.01	
25	**House hold waste (GFT) compost**	**GFT compost (R) fresh**	**mg/kg**	VGFT 1 27112015
		Iprodion	0.024	
27	**House hold waste (GFT) compost**	**GFT compost (after hydrolysis) 1 week**	**mg/kg**	VGFT 2 27112015
		Difenconazole	<0.01	
		Propiconazole	0.013	
28	**House hold waste (GFT) compost**	**GFT compost, 3 weeks**	**mg/kg**	VGFT 3 27112015
		Tebuconazole	0.012	
		Difenoconazole	<0.01	
		Propiconazole	0.021	
7	**Bulb compost**	**Bulb compost (O R) 2014**	**mg/kg**	BOL1B3009
		Prothioconazole	6.4	
		Prothiconazool-destio	0.15	
		2,4,6-Trichloorfenol expressed as Prochloraz (sum)	0.11	
		Prochloraz-desimidazole-amino	0.085	
8	**Bulb compost**	**Bulb compost (O R) 2015**	**mg/kg**	BOL2B3009
		Prothioconazole	1.9	
		Prothiconazole-destio	0.073	
		2,4,6-Trichloorfenol uitgedrukt als Prochloraz (sum)	0.091	
		Prochloraz-desimidazole-amino	0.086	
24	**Bulb compost**	**Bulb compost (O R) 2015**	**mg/kg**	BOL B2015 301015
		Prothioconazole	0.43	
		Azaconazole	<0.01	
		Epoxiconazole	<0.01	

		Prochloraz (sum)	0.044	
		Prothiconazole-destio	0.026	
		Tebuconazole	<0.01	
9	**Bulb compost**	**Bulb compost (O R) 2015**	**mg/kg**	BOL1G3009
		Prothioconazole	0.033	
		Prothiconazole-destio	0.01	
		Tebuconazole	0.022	
		Prochloraz (sum)	0.18	
		Prochloraz	0.011	
		2,4,6-Trichloorfenol	0.17	
23	**Bulb compost**	**Bulb compost (O R) BA**	**mg/kg**	BOL BA 301015
		Prothioconazole	1.3	
		Prothiconazole-destio	0.033	
		Azaconacole	<0.01	
		Difenoconazole	<0.01	
		Epoxiconazole	<0.01	
		Penconazole	<0.01	
		Propiconazole	<0.01	
		Tebuconazole	<0.01	
31	**Bulb compost**	**Bulb compost (O R) BA**	**mg/kg**	BOL 13102016
		Prothioconazole	1.1	
		Prothiconazole-destio	0.026	
		Azaconacole	<0.01	
		Epoxiconazole	<0.01	
		Prochloraz (sum)	0.043	
		R	<0.01	
32	**Bulb compost**	**Bulb compost (O) BA**	**mg/kg**	BOL B 2016 13102016
		Prothioconazole	0,72	
		Prothiconazole-destio	0.026	
		Tebuconazole	<0.01	
40	**Bulb fermentation Bokashi**	**Bulb fermenation (O R)**	**mg/kg**	BOL 3B 2016 13102016
		Prothioconazole	1,9	
		Prothiconazole-destio	0.028	
		Tebuconazole	0,021	
		Epoxiconazool	<0,01	
11	**Green compost**	**Green compost (R) vers (0-1 week old)**	**mg/kg**	COM 9 9-10-15
		Tebuconazole	0.001	
12	**Green compost**	**Green compost (R) 5-6 weeks old**	**mg/kg**	COM 2 9-10-15

		Tebuconazole	0.004	
10	**Green compost**	**Green compost (R) 7 weeks old**	**mg/kg**	COM 0 910-15
		Tebuconazole	0.001	
33	**Green compost**	**Green compost (R) 5-6 weeks old**	**mg/kg**	COM 3 13-10-16
		Tebuconazole	<0.01	
		Prothioconazool	<0.01	
34	**Green compost**	**Green compost (R) 7 weeks old**	**mg/kg**	COM 4 13-10-16
		Tebuconazole	0.001	
		Prothioconazool	<0.01	
35	**Green compost**	**Green compost (R) 5-6 weeks old**	**mg/kg**	COM 1 16-10-16
		Tebuconazole	<0.01	
		Prothioconazool	<0.01	
36	**Green compost**	**Green compost (R) 7 weeks old**	**mg/kg**	COM 2 16-10-16
		Tebuconazole	<0.01	
13	**Wood chippings**	**Wood chippings waste**	**mg/kg**	HOU 1 G 9-10-15
	waste / compost	Azaconazole	0.53	
		Propiconazole	0.036	
		Tebuconazole	0.013	
37	**Wood chippings**	**Wood chippings waste**	**mg/kg**	HOU 3 13-10-16
	waste / compost	Azaconazole	0.53	
		Propiconazole	0.036	
		Tebuconazole	0.013	
38	**Wood chippings**	**Wood chippings waste**	**mg/kg**	HOU 2 13-10-16
	waste / compost	Azaconazole	<0.01	
		Propiconazole	0.022	
		Tebuconazole	<0.01	
39	**Wood chippings**	**Wood chippings waste**	**mg/kg**	HOU 1 13-10-16
	waste / compost	Azaconazole	0.53	
		Propiconazole	0.026	
		Tebuconazole	0.02	

*R, regular; O, organic

<0.01: detected but can't be quantified