National Institute for Public Health
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S.E. Schoustra (author), WUR
J. Zhang (author), WUR
B.J. Zwaan (author), WUR
A.J.F. Debets (author), WUR
P. Verweij (author), Radboudumc
D. Buijtenhuijs (author), RIVM
A.G. Rietveld (author), RIVM

Contact:
Anton Rietveld
Centrum Voeding, Preventie en Zorg\Voedselveiligheid
anton.rietveld@rivm.nl

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Synopsis

New insights into the development of resistance in *Aspergillus fumigatus*

In this study we investigated a number of factors that may contribute to the development of resistance to azoles in the fungus *Aspergillus fumigatus* in plant waste of the bulb-growing industry. Fungicidal agents called azoles are used in the bulb growing industry, as well as in many other applications. We show that *A. fumigatus* already develops resistance to these agents at very low concentrations. We also demonstrate that different types of azoles can result in resistance development. Resistant Aspergillus was found to be present throughout the year in bulb plant waste.

A. fumigatus is a fungus that grows on decaying plant material. It produces large amounts of spores, and these spores spread very effectively through the air. Humans inhale these spores on a daily basis. This does not pose a risk to healthy persons, but it can cause serious lung infections in patients with a weakened immune system. The azoles used to combat Aspergillus infections (medicinal azoles) are similar to the azoles that are used in agriculture and industry. The effectiveness of medicinal azoles is diminishing, as increasingly *A. fumigatus* strains are found that are resistant to these agents.

The resistance develops through adaptations of the fungus in response to azole exposure. The resistance mechanisms found in Aspergillus strains from plant waste were identical to Aspergillis strains cultured from patients. It is therefore considered plausible that patients obtained a resistant infection by inhalation of resistant spores from the environment. Controlling the development and proliferation of resistant Aspergillus strains in plant waste material has the potential to reduce the infection burden of the resistant fungus in patients. We investigated whether the development of resistance could be prevented by disturbing the life cycle of the fungus. The results showed that disturbance could not prevent the development of resistance.

Our studies indicate that storage of decaying plant waste the bulb-growing industry facilitates the selection of azole resistance in *A. fumigatus*, when residues of azoles are present. A preventive measure can therefore be to avoid the storage of plant waste in the bulb-growing industry. Whether this also applies to plant waste storage in other industries remains to be investigated.

Keywords: *Aspergillus fumigatus*, resistance, azoles, plant waste, bulb farms, control measures.

Publiekssamenvatting

Nieuwe inzichten in ontwikkeling van resistentie bij *Aspergillus fumigatus*

In dit onderzoek is gekeken naar factoren die de ontwikkeling van resistentie van de schimmel *Aspergillus fumigatus* beïnvloeden in plantenafval uit de bollenteelt. Anti-schimmelmiddelen, de zogenaamde azolen, worden gebruikt in de bollenteelt en in vele andere toepassingen. Resistentie van *A. fumigatus* ontstaat al bij zeer kleine hoeveelheden van deze azolen. Ook blijkt dat alle gebruikelijke typen azolen deze resistentie kunnen veroorzaken. Resistente *A. fumigatus* komt het hele jaar voor in het onderzochte plantenafval.

Aspergillus fumigatus is een schimmel die groeit op dood plantenmateriaal. Deze schimmel maakt grote hoveelheden sporen die in de lucht komen die wij vervolgens kunnen inademen. Voor gezonde mensen vormt dit geen gevaar, maar voor patiënten met een verzwakt immuunsysteem kan dit zorgen voor ernstige longinfecties. De azolen waarmee we *A. fumigatus* bestrijden (medicinale azolen) lijken erg op de azolen die in de landbouw en voor andere toepassingen gebruikt worden. Deze medicinale azolen werken echter steeds minder goed, omdat in patienten steeds vaker resistente *A. fumigatus* wordt aangetroffen.

Deze resistentie ontstaat door aanpassingen van de schimmel als die wordt blootgesteld aan azolen. Het resistentiemechanisme dat gevonden wordt in *A. fumigatus* in plantenafval in de bollenteelt is gelijk aan het resistentiemechanisme van *A. fumigatus* dat werd gevonden bij patiënten met Aspergillus-infecties. Het is daarom plausibel dat patiënten een infectie kunnen oplopen door het inademen van resistente sporen uit de omgeving. Beheersing van resistente *A. fumigatus* in plantenafval in de bollenteelt kan mogelijk infectie van patiënten met de resistente schimmel beperken. We hebben onderzocht of de ontwikkeling van resistentie afgeremd kon worden door de levenscyclus van de schimmel te verstoren. Verstoring van de levenscyclus bleek geen invloed te hebben op de ontwikkeling van resistentie.

Deze studie laat zien dat opslag van plantenafval in de bollenteelt gunstig is voor de selectie van resistente *A. fumigatus.* Een voor de hand liggende preventieve maatregel zou daarom zijn de opslag van plantenafval in de bollenteelt te voorkomen. Of dit ook geldt voor voor opslag van plantenafval in andere bedrijfstakken is nog niet voldoende onderzocht.

Kernwoorden: *Aspergillus fumigatus*, resistentie, azolen, plantenafval, bollenteelt, beheersmaatregelen.

Contents

Summary

Background
Over the last years an increase in the frequency of resistant strains of the fungus *Aspergillus fumigatus* to azoles has been observed, both in the environment and in hospitalized patients. *A. fumigatus* is a common plant waste degrading soil fungus of which spores are abundant in the air. When inhaled, these spores can cause invasive aspergillosis in humans with a compromised immune system, posing a threat to such patients. In previous research we and others have gathered evidence that resistant *A. fumigatus* spores develop in plant waste containing environmental azoles. These environmental azoles form a group of fungicides with similar chemical structures as the azoles being used to treat fungal infections in patients (medical azoles). Resistance to environmental azoles yields cross-resistance to medical azoles. Thus, the development and spread of resistant isolates from the environment is a growing problem that hampers successful treatment of patients with Aspergillus diseases. Therefore, there is an urgent need to reduce azole-resistant *A. fumigatus* spores from environmental origin. However, the factors that play a role in the development and maintenance of azole resistance in *A. fumigatus* in the environment remain unclear.

Thus, besides the use of azoles in the clinical setting (medical azoles), similar azoles are used in agriculture and industry for numerous purposes (environmental azoles). This led to the hypothesis that there are certain so-called environmental 'hotspots' where azole-resistant *A. fumigatus* could emerge and propagate, in this way forming a source of azole-resistant air-borne spores. These hotspots of azole-resistant *A. fumigatus* are environments favorable for the abundant growth and sporulation of *A. fumigatus* in the presence of environmental azoles.

Based on this hypothesis, a two-phased research programme was set up. In the first phase, a series of expert meetings brought forth the characteristics for, and a list of potential hotspots. These potential hotspots were sampled to test for the presence of *A. fumigatus*, azole residues and resistant *A. fumigatus* spores.

The phase I research was finalized and reported in 2017[1]. This led to the identification of several hotspots and the main results can be summarized as follows:

- Plant waste of bulb farms, industrial wood-chopping storage and industrial green-waste storage contained both azoles (mostly tebuconazole and prothioconazole-desthio) and high amounts of resistant *A. fumigatus* spores, pointing out these three locations as clear hotspots.
- Several plant waste processes did not yield resistant *A. fumigatus,* even when various azoles were present. This indicated that the ability of *A. fumigatus* to grow in plant waste and the

[1] https://www.rijksoverheid.nl/documenten/rapporten/2017/12/07/rapport-azole-resistance-selection-in-aspergillus-fumigatus-final-report

concentration and type of (agricultural/industrial) azoles present are important parameters in defining a hotspot.

This report: research project phase II

Based on the results of phase I research, phase II research was set up in order to obtain a better understanding of what characterizes a hotspot, which factors influence the development of resistant *A. fumigatu*s, and which the genetic mechanisms underlie this resistance. The phase II research is described in this report and where relevant, related to the results from phase I.

Phase II consists of two parts: 1) a longitudinal study in which hotspots were sampled at three bulb farms during a period of 16 months, and 2) a laboratory study in which an experimental model of a hotspot was designed in order to be able to study the impact of specific variables on resistance under controlled conditions. Out of the three hotspots identified in phase I, plant waste material from bulb farms was chosen as a case for further study in phase II as it contained the highest levels of azole-resistant *A. fumigatus*.

In the longitudinal study, the following factors were analysed:

1) presence of azole fungicides and azole residues;
2) abundance and fraction of *A. fumigatus* resistant to one representative environmental azole (tebuconazole) and one medical azole (itraconazole);
3) genetic characterization of the *cyp*51 gene and its promotor to assess the mutations underpinning the resistance mechanism.

In the experimental laboratory hotspot model various treatments were setup to study the effect of parameters influencing the development of azole resistance in *A. fumigatus* in more detail. Importantly, conditions in this experimental setup were consistent with those in the field situation. The following parameters were studied:

1) Azole concentration and azole combinations: how is resistance development affected by different concentrations and combinations of azoles?;
2) Disturbed and undisturbed incubation: does disturbance of incubation by repeatedly adding fresh material affect resistance development?;
3) Competition resistant and non-resistant strains: how is resistance development affected by competition between resistant and non-resistant strains?;

With this systematic approach, our study aimed to create focussed information for the formulation of possible measures to control the development and spread of azole resistant *A. fumigatus* while potentially maintaining a sustainable and responsible use of environmental azoles.

Results longitudinal study

The results of the longitudinal study can be summarized as follows:

- Both *A. fumigatus* (resistant as well as sensitive) and azoles are ubiquitously present in the sampled plant waste material throughout the whole 16 month period.

- Of the isolated *A. fumigatus*, roughly 50% is resistant to the two indicator azoles tebuconazole (agricultural azole) and itraconazole (medical azole).
- The total azole concentrations found in the environmental samples are around 10% of the levels used for clinical applications.
- The genetic characterization of azole-resistant *A. fumigatus* strains showed that resistance mechanisms found in the hotspot locations were the same as those cultured from patients with Aspergillus diseases.
- In all hotspots identified under phase I of the research, the same genetic resistance mechanisms were found as those found in clinical strains.

Results experimental hotspot model in the laboratory
The results from the experimental laboratory hotspot model can be summarized as follows:

- Under experimental conditions, spore production was remarkably high, showing that *A. fumigatus* can proliferate very well in bulb plant waste material.
- Azole-resistance developed at all concentrations and combinations of azoles tested, including very low concentrations.
- Disturbance of the fungal cultures by the addition of fresh material did not affect the development of resistance.
- Resistant strains were not displaced by sensitive strains when grown in the presence of background levels of azoles.

Conclusions
This study confirms that heaps of environmental azole-containing decaying bulb plant waste are significant hotspots for development, propagation, and sporulation of azole-resistant *A. fumigatus*.

In the three hotspots identified in phase I (see above), the same resistance mechanism was found in *A. fumigatus* isolates. With respect to resistance development of *A. fumigatus*, further investigation is needed to see whether the results of this study with bulb waste material may similarly apply to the other two hotspots that were identiefied in phase I. Moreover, it is important to analyse whether based on current insights other, yet uncharacterized, hotspots may identified.

Based on the results of the experimental hotspot model in the laboratory, we can draw the following conclusions:

- A very low environmental azole selection pressure is sufficient to yield resistance, including cross resistance to medical azoles.
- Different regimes of disturbance of the decaying plant waste material are not likely to prevent resistance development and maintenance, since our results show that azole resistance emerges regardless of disturbance.
- Sensitive strains added to compost do not outcompete resistant strains at low azole concentration.

The findings in this study indicate that reducing the use of azoles in agriculture offers no short term solution to the problem of azole

resistance since even very low azole concentrations are sufficient to maintain azole resistance.

Sampling in the phase I study showed that some mature (actively processed) compost does not contain *A. fumigatus*, even though the starting material (feedstock) did contain *A. fumigatus.* This indicates that controlled professional composting procedures exist that diminish *A. fumigatus*.

Recommendations

Waste removal

Our results show that plant waste storage at bulb farms without active composting procedures provides very favorable conditions for *A. fumigatus*. Therefore, longer term and unsupervised storage of plant waste material at professional production sites should be avoided. Regular waste removal and further handling by professional composters could be considered.

Waste treatment

Upon removal of plant waste from bulb farms, waste processing protocols should be tuned to diminish growth of A. fumigatus. Phase I of this research and personal communications with bulb producers during phase II have shown that large variations exist in the current practice of plant waste processing to mature compost, some of which lead to lower counts of *A. fumigatus.* We recommend to explore existing waste treatment protocols as a starting point for the testing of feasible and effective protocols. The effectiveness of diminishing *A. fumigatus* needs to be monitored.

Other hotspots

The resistance mechanisms described in this study are also found in strains isolated from the other hotspots described in phase I: industrial wood chippings and green waste storage (Verweij, 2017). Whether the findings from the case study with plant waste material from the bulb industry also apply to these other hotspots remains to be explored. We therefore recommend to 1) explore storage conditions for feedstock in wood chippings and green waste composting industry that diminish the growth of A. fumigatus, and 2) analyse whether additional hotspots may exist based on current knowledge.

Understanding transmission dynamics

We found further confirmation for a link between hotspot and resistant infection of patients due to the presence of identical resistance mutations in environmental and clinical *A. fumigatus* isolates. Our study did however not address the actual spread of spores from hotspots to humans. At the international symposium on azole resistance in *A. fumigatus* hosted by the Royal Netherlands Society of Arts and Sciences (KNAW) on the 31st of Januari and the 1st of Februari 2019 the consensus expert opinion was that transmission of resistant spores from hotspots to patients is sufficiently plausible. Developing and implementing methods to qualitatively and quantitatively analyse airsamples (at hotspots and along main wind direction routes) will help assess both infection risk and effectiveness of meaures to turn hot- into coldspots.

1 Introduction

Triazoles are important in combating fungal infections in humans (Latgé, 1999). In recent years, there has been a worrying increase in the resistance of the fungus *Aspergillus fumigatus* to triazoles in patients. *A. fumigatus* is a thermophilic saprotrophic fungus that thrives on decaying plant material, where it can produce large numbers of spores that easily become airborne. This fungus causes a number of diseases in humans of which invasive aspergillosis is the most severe in immuno-compromised patients. Resistance to azole treatment reduces the efficacy of azole therapy and has detrimental consequences for the treatment of these patients (Verweij et al., 2016a).

Therefore, there is a high need for the reduction of azole-resistant *A. fumigatus* spores that reach patients. However, the factors playing a role in the development and maintenance of azole resistance in *A. fumigatus* and its transfer to patients are still unclear (Verweij et al., 2009, van der Linden et al., 2011).

BOX 1. First phase of the research (completed in 2016) that forms the basis of the studies described in this report.

Phase I of this research (completed in 2016; Verweij, 2017) yielded the following main insights:

- A series of meetings of experts in this discipline resulted in a list of locations that could be designated as potential hotspots. A hotspot is an environment in which
  (a) the biotic and abiotic conditions facilitate the growth of the fungus and from which the fungus can spread;
  (b) this growth can take place for prolonged periods and the fungus can complete all the stages of its growth cycle; and
  (c) azoles are present, in different concentrations and combinations.
- These hotspots were sampled for the presence of azoles and their residues and for *Aspergillus fumigatus*; if the fungus was found, the fraction of fungi that were resistant to azoles (both medical and agricultural azoles) was also analyzed (Table 1).
- A high level of resistant Aspergillus was repeatedly found in plant waste from the bulb-growing industry, industrial storage of mixed waste wood chippings and green waste (Table 1).
- New resistant forms of Aspergillus were found to arise in plant waste materials which contained azoles.
- The manner in which the different materials are composted affects the survival of the (resistant) Aspergillus.
- The types of fungicidal agents selected for use in the environment can affect the emergence of resistance to medical triazoles.

Table 1. Main findings of phase I of this research project, taken from (Verweij, 2017). Expert meetings resulted in the formulation of a list of potential hotpots that were sampled for the presence of (resistant) Aspergillus fumigatus and for the presence of azole residues.

Potential hotspot	A. fumigatus growth	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
Private compost*	Yes	Yes	No
Bulb waste	Yes	Yes	Yes
Green waste	Yes	Yes	Yes
Wood chopping storage	Yes	Yes	Yes

* the presence of resistant *A. fumigatus* in the absence of azole residues is considered due to the background presence of resistant *A. fumigatus* in the materials that were added to the heap, rather than an indication that private compost is a source of resistance.

Azoles with similar mode of action as the medical triazoles are widely used as antifungals in agriculture and industry and as a result, agricultural and industrial plant waste contains azole residues (Snelders et al., 2012). A perspective for action to fight resistant strains of *A. fumigatus* is essential for a sustainable use of triazoles as medicines in clinical applications and (tri)azoles in agricultural and industrial applications in the long term. Therefore, a research plan comprising two phases was designed to create a perspective for action.

The first phase of this research was reported in 2016 ['Azole-resistance selection in *A. fumigatus*' (Verweij, 2017)] and is summarized in Box 1.

The second phase is reported here. The objective of the research project phase II was to create a sound basis for the formulation of potential control measures to prevent the further development and spread of resistance and to ensure that, where necessary or desirable, the responsible use of azoles can be continued in the long term (Figure 1). Insight into the emergence mechanisms leading to resistance is important for the design of tailor-made control measures that will avoid the risk of overregulation, unnecessary or ineffective measures.
The research reported here was commissioned by the Dutch Ministry of Agriculture, Nature and Food Quality. It was a collaborative project between the Wageningen University and Research (Laboratory of Genetics) and Radboud University Medical Centre (Radboudumc) (Medical Microbiology), in active conjunction with the partners involved in phase I and the commissioning authority. The National Institute for Public Health and the Environment (RIVM) had a coordinating role in the study.

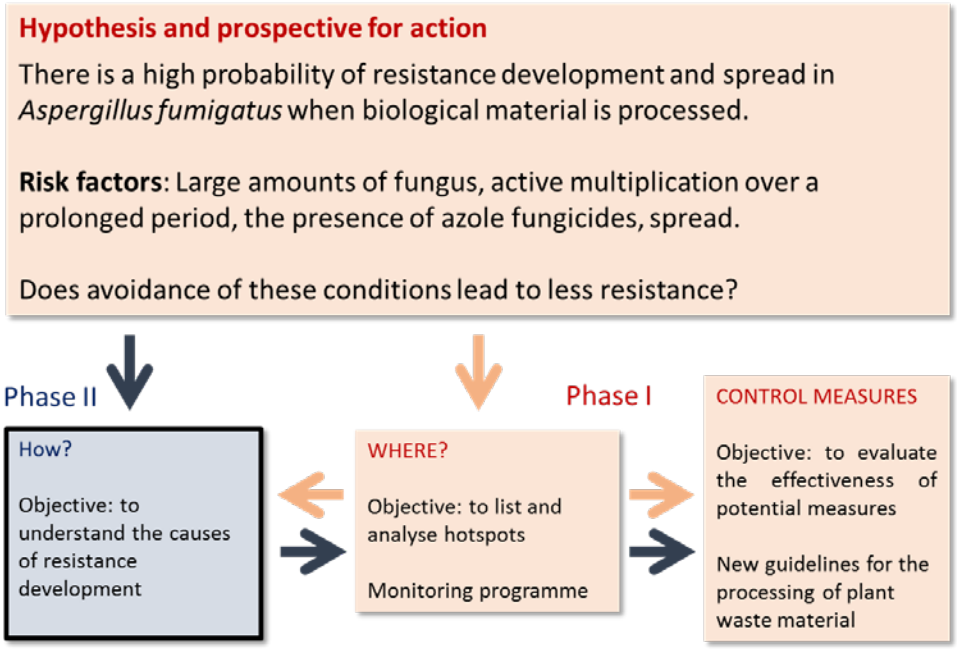


Figure 1. Overview of how the research carried out in phases I and II is connected and provides scientific substantiation for a perspective for action to prevent the further emergence and spread of azole-resistance in Aspergillus fumigatus.

Based on the results from the phase I study (Box 1), the storage of plant waste from the bulb-growing industry was selected as a representative hotspot for further study. The objective of the study was to systematically study potential mechanisms and conditions that contribute to resistance development, with particular attention for two key aspects:
-the identification of fungicides and concentrations of these fungicides that cause the emergence and maintenance of azole resistance mutations in *A. fumigatus* and,
- resistance development mechanisms, compensation mechanisms in *A. fumigatus* and the importance of the sexual cycle herein.

To study these issues, we set up a project consisting of (1) a longitudinal field study where we sampled at three locations over a period fo 16 months and (2) laboratory experiments using an experimental hotspot model.

2 Project set-up and methods

2.1 Longitudinal field sampling at three hotspot locations

The goal of the field study is to understand how resistant strains develop over time and what types and levels of fungicides are present in these hotspots. This information will allow us to create conditions in the laboratory experimentthat are consistent with the field situation. In addition to this, the analysis of resistance mechanisms found in the field is relevant to study potential similarities to resistance mechanisms found in the experimental setup as well as in the medical setting.

Based on results from phase I (Verweij, 2017), the heaps with decaying plant waste material found at bulb farms have been chosen as representative hotspots (Box 1; Table 1).

Synopsis of the approach.
We selected three bulb-grower farm sites in the north of the province of Noord Holland at which the farmers pile up their plant waste during the year. They start to accumulate plant waste material starting in July and continue to pile up for a full year after which composting takes place. The heaps become around six to 10 meters high and around 50 by 50 meters wide.

We sampled heaps of this material every few weeks during 16 months by taking samples at various depths out of heaps of decaying plant material. A total of 127 samples was collected, of which all have been analyzed so far for presence of *A. fumigatus* and the fraction of isolates resistant to representative azole fungicides. For 35 representative samples, we also determined a chemical profile of azole fungicides and other compounds. For 100 representative azole resistant *A. fumigatus* isolates, we performed a genetic characterization of the *cyp51* gene to assess the genetic mutation(s) possibly underpinning the resistance mechanism.

A. Selection of sites
Three sites were selected for sampling based on similarity for important factors so that they could be seen as independent replicates. The sites are located in the same area. All three are farms of flower bulb farmers using common conventional methods (not organic growers). The farmers apply azole fungicides as they see fit and as their practice requires. They store their plant waste on site. In July, they start to accumulate their waste, building a storage heap of decaying plant waste material (‘pre-compost’). Starting in April, they mix their plant waste material with straw for aeration to stimulate composting and subsequently the heap is regularly turned. In June, composting would be completed and the compost is then used on-site, exchanged with other farmers or sold.

B. Sampling of decaying plant material
Every month, several samples were taken at each of the sampling sites and in various places and depths of the growing pre-compost heap.

Depth and type of plant material that was in the heap was recorded. Per sample 10 grams of material was collected and stored at 4 ^{o}C until further analysis. A total of 127 samples was taken combined over all three sites over a period of 16 months (between July 2016 and December 2017).

C. Chemical analysis of decaying plant material
For 35 representative samples, we performed a chemical analysis to document the presence of azole fungicides and azole residues. Samples were analysed using GC-MS/MS and LC-MS/MS by the Eurofins laboratory, Zeeuws Vlaanderen (http://www.labzvl.nl/en).

The samples were ground with a vegetable cutting machine. The homogenized samples were weighed into a Teflon jar. A (homogenized) sub-sample was extracted with acetone, followed by extraction with dichloromethane / petroleum ether. A portion of the extract was evaporated to dryness, for subsequent re-dissolution.

LC-MSMS determination was performed in methanol re-dissolution acidified by 0.02% HAC. The quantitative determination of fungicides was carried out using the liquid chromatography-mass spectrometry (triple quad LC-MSMS), with turbo ion spray ionization. The content was calculated by using a calibration line. The identification took place on the basis of the Multiple Reaction Monitoring (MRM). Quantification was performed using the external standard method.

The sample was re-dissolved in iso-octane / toluene (9: 1). The quantitative determination of fungicides was carried out using the capillary gas chromatography-mass spectrometry, GC-MS-TQ (Quadro-Pole Triple-Detector) in EI mode and gas chromatography-electron capture detection, GC-ECD. Identification was done on the basis of mass-2 transitions to GC-MS-TQ and on the basis of retention time on GC-ECD and peak shape.

D. Isolation of A fumigatus from samples: abundance and fraction resistant to TEB and ITR
Samples of 1 gram decaying plant waste material were added to 10 mL sterile saline with 0.05% Tween and screened for the presence of *A. fumigatus* by plating 50 µl of dilutions of samples on MEA (malt extract agar) supplemented with two antibiotics (streptomycin 10 µg/ml and tetracycline 15 µg/ml, Sigma Aldrich, Germany) to suppress growth of bacteria and incubated at 48°C which is selective for *A. fumigatus*. Ten randomly selected colonies, that all showed Aspergillus morphology, from each decaying plant waste material-heap were genetically identified as *A. fumigatus* by sequencing of the ß-tubulin and carboxypeptidase-5 genes (32) and phenotypically by their capacity to grow at 48°C.
The fraction of resistant fungal individuals to tebuconazole (TEB, an agricultural fungicide, Sigma Aldrich, Germany) and itraconazole (ITR, a medical drug, Sigma Aldrich, Germany) in these samples was measured via plating dilutions of the fungal spore suspension on MEA (Malt Extract Agar medium) supplemented with two antibiotics to prevent bacterial growth and two indictor azoles TEB or ITR 4mg/L (Sigma Aldrich, Germany). After four days of incubation at 48°C, the number of resistant

colonies was recorded, and the fraction of resistance was calculated. Two colonies from each plate were stored at -80℃ for sequencing analyses to elucidate the resistance mechanism.

E. Analysis of resistance mechanisms (cyp51A mutations)
In resistant *A. fumigatus* isolates from patients, the genetic mechanism for resistance typically involves changes in the *cyp51* gene (Hagiwara et al., 2016, Camps et al., 2012) (Figure 2). This gene is central to the ergosterol metabolism of the fungus and genetic changes in isolates from patients consist of additions of tandem repeats in the promotor region combined with point mutations in the structural region of the gene. The number of tandem repeats increases in the promotor region correlates positively with the expression level of the gene, while the point mutations in the protein coding region change the three-dimensional and folding structure of the enzyme the gene codes for.

Figure 2. Schematic representation of the cyp51 gene of A. fumigatus. Genetic changes conferring resistance to azole fungicides typically involve additions of tandem repeats in the promotor region (increasing expression levels) and point mutations in the structural region of the gene (changing the protein structure of the cyp51 enzyme.

Azole resistant strains collected from either the environment or from patients share highly similar resistance mechanism involving the gene *cyp51A*, which is a key gene in the sterol biosynthesis pathway that the medical triazoles are targeting (Snelders et al., 2009). This pathway is crucial for the production of cell membrane components and central to the metabolism of *A. fumigatus*. Genetically, high resistance is based on a tandem repeat in the promoter region in combination with one or more specific point mutations in the coding region of the *cyp51A* gene. A point mutation or tandem repeat alone yields low levels of resistance; only the combination of both results in the fully resistant phenotype. It is highly unlikely that two genetic changes occur at the same time; this will only happen with a very low probability (Hartl et al., 1997).

Single resistant colonies collected as described above were inoculated in 3 ml Malt extra broth and grown overnight at 37°C. Mycelial mats were recovered and subjected to a DNA extraction protocol. The full coding sequence of the *cyp51A* gene and the promoter region, were determined by amplification and subsequent sequencing as previously described (Mellado et al. 2004; Mellado et al. 2007). For analysis, the *cyp51A* sequence under accession number AF338659 in GenBank was used for comparison to detect mutations. cyp51A-PF(5'-ATGGTGCCGATGCTATGG-3') and cyp51A-PR (5'-CTGTCTCACTTGGATGTG-3'). The presence of the tandem repeat (TR) in the promotor region of the gene was investigated by amplifying part of the promoter region of the cyp51A gene using appropriate primers (5'-TGAGTTAGGGTGTATGGTATGCTGGA-3' and 5'-AGCAAGGGAGAAGGAAAGAAGCACT-3'). The cycling program consisted of a 2-min denaturation step at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 60°C, and 45 s at 72°C and a final elongation step of 5

min at 72°C(Snelders et al. 2012). The amplified DNA fragments were purified with a QIAquick PCR purification kit (Qiagen). DNA sequencing of the forward strand of each fragment was performed Eurofins sequencing company. The resulting sequences were aligned in CLUSTALW46 using the program BioEdit47.

F. Isolation of sexual spores from environmental hotspots
Laboratory evidence exists that completion of the sexual part of the life-cycle of *Aspergillus fumigatus* could facilitate the emergence of the tandem repeat (TR) in the promotor region (Zhang et al., 2017a) (see figure 2). To assess the potential role of the sexual cycle, we examined the heaps of decaying plant material for the presence of sexually derived spores. The presence of these spores is indicative of the completion of the sexual phase of the life-cycle within the decaying plant material.

Heat-shock procedure has been shown to kill *A. fumigatus* asexually derived conidiospores, but not sexually derived ascospores (O'Gorman et al., 2009, Kwon-Chung & Sugui, 2009). In order to investigate whether sexual reproduction occurred in the pile of plant waste material or not, we screened the existence of ascospores in decaying plant waste material samples. Samples of 1 gram decaying plant waste material were added to 10 mL sterile saline with 0.05% Tween and exposed to 70^{o}C heat shock for 1h. Control experiments showed that under these conditions asexually derived conidiospores did not survive. The number of surviving ascospores were recorded by plating 50 µl samples on MEA (malt extract agar (Zhang et al., 2015a)) supplemented with two antibiotics (streptomycin10 µg/ml and tetracycline15 µg/ml, Sigma Aldrich, Germany) and incubated at 37^{o}C for 48h.

G. Resistance mechanism characterized across different hot spots
In Phase I of the research, three hotspots for the development and persistence of azole resistant *A. fumigatus* were identified (Box 1, Table 1), of which plant waste from bulb farms was selected for the in-depth longitudinal study. The other two hotspots that were indentified were industrial wood chippings storage and industrial green-waste storage. These materials are feedstocks for industrial composting. To verify the similarity of hotpots, we now characterized twelve resistant *A. fumigatus* isolates from the other two hotspots for their resistance mechanism, in the same way as for the plant waste of bulb farms described above under E.

2.2 Experimental hotspot model in the laboratory

Synopsis of the approach. Based on the longitudinal study, we designed a laboratory set-up. We took a large batch of decaying plant material, and homogenized and sterilized it before putting it in glass test tubes, two grams of plant material per tube. We then inoculated the tubes with *A. fumigatus*. After a few days, abundant fungal growth took place with up to 10^{10} fungal spores harvested from 2 grams of plant material.

After assessing that fungal growth was very well possible in this set up, we then designed a large experiment in which three relevant factors were varied (also see Table 2). In other words, all possible combinations

were tested of all three factors, which were all performed in triplicate. This adds up to a total of 504 experimental variations.

Table 2. Code and variables tested of the treatments in the laboratory experimental hotspot model.

1. Type and concentration of azoles
a. Only background levels of azole fungicides (control; see Table 3)
b. TEB low (0.05mg/kg)
c. TEB high(1mg/kg)
d. PRO low(0.05mg/kg)
e. PRO high(0.1mg/kg)
f. TEB low(0.05mg/L) + PRO low (0.05mg/L)
g. TEB high(1mg/L) + PRO low(0.05mg/L)
2. Disturbed or undisturbed incubation
a. Undisturbed for 3 months, sampling every 2 weeks
b. Sampling and transfer to fresh plant material every 2 weeks of a fraction of the fungal population
3. Persistence of resistant strains when sensitive strains are present
a. Only resistant strain (TR34/L98H)
b. Only sensitive strain
c. Both strains combined

Each of the factors was chosen such that findings could potentially be translated to concrete control measures (*perspective for action*):

1. Variations in azole concentration: **high** *versus* **low** (as observed in the longitudinal field study) and **combination of azoles** using two environmental azoles found in 90% of decaying plant waste material samples (tebuconazole and prothioconazole-desthio).
   Rationale for this treatment: There is some debate that the concentration of azoles may affect the likelihood of resistance to arise and/or may affect resistance levels (Gisi, 2014). This treatment will allow us to directly test this.
   Potential control measures based on the outcomes could be to include changes in the use of azole compounds, both in their quantity and also whether single or multiple azoles should be applied at any given time.
2. **Disturbed** and **undisturbed** incubation; role of the sexual cycle: we mimic the decaying plant waste material storage situation of prolonged storage in large heaps on the farm or in the field (undisturbed) with the scenario of repeatedly adding of fresh new materials while turning over the heap (disturbed). In the laboratory model, the disturbed and undisturbed treatments were set up by either transferring cultures to fresh tubes every two weeks (disturbed) *versus* incubation without periodically transferring to fresh tubes (undisturbed).
   Rationale for this treatment: Disturbance may inhibit the sexual cycle, which may prevent the emergence of resistance since no tandem repeats are formed in the promotor region (Figure 2) (Zhang et al., 2017a). Disturbance can be achieved by turning of

the plant waste material and in the laboratory by transfer to fresh tupes disrupting the fungal structure.
Potential control measures based on the outcome of this experiment could be a change to current practice of accumulation of plant waste material over prolonged periods of time. A change that could be potentially effective is to turn the material regularly.

3. **Combinations of sensitive and resistance strains**: the effect of competition in the decaying plant waste material was tested by mixing resistant strains and sensitive strains.
Rationale for this treatment: Strains that have acquired a resistance mutation commonly exhibit a growth disadvantage relative to sensitive strains when growing in the *absence* of azoles, a phenomenon known as a cost of resistance (Verweij et al., 2016b, Schoustra et al., 2006). Should this cost of resistance be large, growing in conditions without azoles, sensitive strains could potentially outcompete resistant strains. In this way, it is tested whether the restriction of azole presence would lead to the elimination of resistant strains due to the presence of azole sensitive strains.

A. Growth media, growth conditions and fungal inoculum
The laboratory hotspot model was set up by transferring decaying plant waste material from pre-composting storage heaps sampled for the longitudinal study. The material was homogenized, placed in glass tubes, and sterilized. We then added various amounts and combinations of azole fungicides to the tubes and added fungal inoculum.

Moreover, before the start of the experiment, we determined the background level of azole compounds in the decaying plant material we used, having selected material with the lowest observed amounts of azoles. This is listed in table 3. Note that the material we used contained the azole fungicide prochloraz at at concentration of 0.08 mg/kg, which is considered very low.

*Table 3: Fungicides and their concentrations as found in the plant material used to set up the laboratory hotspot model (before any of the two azole compounds TEB and PRO were added for the various experimental conditions- see step 4). Azole fungicides are marked with *.*

Prochloraz *	0.08 mg/kg
Captan	0.052 mg/kg
carbendazim	0.074 mg/kg
Fluazinam	0.014 mg/kg
PyraclostrobineThiofanaat-methyl	0.02 mg/kg

Detailed step-by-step protocols are described below.

Step 1: The decaying plant material collected from sampling sites were cut into small pieces and mixed well using a blender.

Step 2: Place 2 gram of homogenized decaying plant waste material into a glass tube.

Step 3: Sterilize the decaying plant waste material in glass tubes to eliminate any existing *A. fumigatus* and other microbes using the condition of 121°C for 20 minutes.
Step 4: Apply fungicides from a water-based stock with (or without) azoles. Based on the fungicide types and concentrations found in the 38 decaying plant waste material samples, we found that tebuconazole (TEB) and prothioconazole-desthio (PRO) were present over 90% of the samples of decaying plant waste material. Therefore, we selected these azole fungicides as representative fungicides to add in various combinations to our tubes with decaying plant waste material.

The types of azole fungicides and their concentrations and combinations used for various laboratory condition are listed in Table 2. After applying the azole fungicides from a 10X water-based stock solution, the glass tubes were vortexed to homogenize.

Step 5: Adding fungal inoculum. We used two different color mutants of *A. fumigatus* isolates in the evolutionary experiment. *A. fumigatus* strains used in this experiment are from the Laboratory culture collection and isolated from soil in the pre-azole era. This allowed the detection of possible cross contamination between tubes, and importantly, also a quick scan for the ratio between resistant and sensitive strains as the ratio at the starting point. To inoculate, 50µl fresh sensitive (S, orange), resistant (R,TR34/L98H, green), mixture of sensitive and resistant (S+R) *A. fumigatus* were applied into a glass tube, resulting in an total inoculum of 10^5 spores per tube (Table 2).

B: Incubation conditions
Glass tubes were incubated at 37 ^{o}C either in an undisturbed or disturbed way. In the **undisturbed incubation** treatment, every two weeks replicate tubes of each of the treatments were taken from the incubator and spores were harvested (Table 2).
In the **disturbed incubation** treatment, spores and other fungal material was harvested every two weeks by adding 5 ml of saline-tween (0.8% NaCl and 0.05% tween-80 in water) and vortexing for at least 10 seconds. Fifty µl of harvested material was transferred to fresh tubes containing the same decaying plant waste material and azole concentrations as the tube spores were harvested from (Table 2).

All replicates are destructively sampled. In order to collect the same number of replicates per treatment and time point, we started the experiment with the same number of tubes for each parallel treatment. (a+b+c+d+e+f+g)x3x(disturbed+undisturbed)x3(sensitive+resistant+R/S)X4 time points=504 tubes.

C: Isolation and characterization of fungal strains at the end of the laboratory experimental hotspot cycle
Collection of fungal material from the incubation tubes. Samples of 2 gram decaying plant waste material were added to 5 mL sterile saline with saline-tween (0.8% NaCl and 0.05% tween-80 in water) and vortexed. This yields a spore suspension to be used for further analysis.

Total spore yield after selection. The total number of *A. fumigatus* was calculated by plating 50 µl of serial dilutions of the spore suspension on

MEA supplemented with 1% triton and counting resulting colonies after two days of incubation at 37 C.

Level of resistance after selection. We placed 5 µl of spore suspensions in the center of Petri dishes containing MEA supplemented with 4 mg/l of tebuconazole. On this medium, only resistant types within the fungal population harvested from each tube will grow. The size of the resulting colony is representative of the resistance levels of these resistant strains within the population. As a control, we also included the used sensitive strains. After four days of incubation, we determined the diameter of the colony on each plate, which is a measure for the mycelial growth rate (MGR) of each evolved population. We measured the diameter in millimeter in two randomly chosen perpendicular directions and took the average.

Fraction resistant strains in the mixed fungal populations after selection. Harvested spore suspensions (may) contain a mixture of sensitive and resistant types and within the resistant class variation may exist. We determined the fraction resistant strains by plating serial dilutions of spore suspensions on MEA 4 mg/l of TEB or ITR. TEB is an indicator azole used in agriculture and was used during selection, ITR (Itraconazole) is a medical azole allowing detection of cross-resistance to medical azoles as a result of resistant selection for environmental azoles. After four days of incubation at 37°C, the number of resistant colonies were recorded, and the fraction of resistance was calculated by using the total spore yield of each population.

D. Sexual reproduction in the laboratory hotspot model environment
In order to verify whether the material and setup of the laboratory hotspot model can provide enough nutrition and space for the occurrence of sexual reproduction in *A. fumigatus,* we tested the occurrence of sex in sterilized decaying plant waste material with background decaying plant waste material material from agricultural field in a farm PA. Spore suspensions of super-mater strains of *A. fumigatus* (AfIR964/974) were mixed as ratio of 1:1 and inoculated into the side of decaying plant waste material glass tube. After three weeks of incubation at 30 ^{o}C of darkness. The fruiting sexual fruiting body (*cleiostothecia*) were harvested and screened for the evidence of viable sexual spores, which would show the occurrence of sexual reproduction under laboratory hotspot conditions.

3 Results

3.1 Longitudinal field sampling at three bulb farms

Results summary of this part:

- Both *A. fumigatus* and azoles are ubiquitously present in the sampled decaying plant material;
- Roughly half of the isolated *A. fumigatus* is resistant to azoles;
- Total azole concentrations in the sampled material are around 10% of the level used for clinical applications;
- Resistant strains found in bulb waste are also resistant to medical triazoles (i.e. agricultural azoles can cause cross-resistance to medical azoles).
- At the genetic level, resistance mechanisms found in the hotpot locations are the same as in clinical environments.
- Sexual reproduction is possible in bulb waste, given the presence of ascospores.

A. Sampling sites and samples taken

Samples were collected from three farms as decribed, indicated here as PA, PH and V. Plant waste from the fields is accumulated in so-called storage heaps (Figure 3).

Figure 3. Heap of decaying plant material where sampling took place.

B. Concentration of azole fungicides in samples

For 35 samples, we determined the chemical profile including concentrations of azole fungicides.

Azole fungicides were detected in all samples, at concentrations ranging from 0.01 to 1 mg/kg, with one outlier of 3.7 mg/kg. Figure 4 shows the total azole concentration detected over the 16-month sampling period, averaged per month and per sampling location. We found that azoles are present all year round in relatively low concentrations, on average 10 times lower than concentrations used in medical drugs for clinical applications.

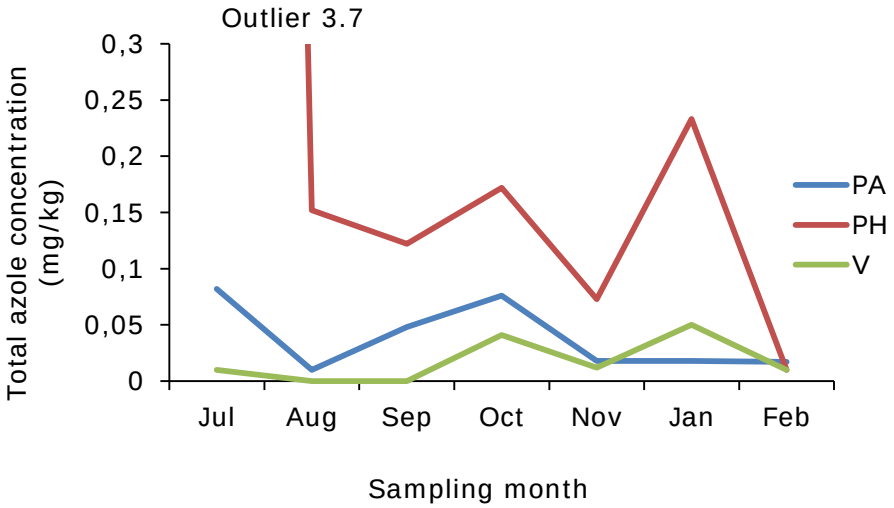


Figure 4. Results of chemical analysis showing total azole concentration over part of the sampling period averaged per sampling location.

C. Abundance of *A. fumigatus* in samples

For each of the 127 samples, we measured the abundance of *A. fumigatus* by plating out serial dilutions of the samples on Petri dishes with general fungal growth medium and incubating at 48 ^{o}C, which are under conditions that are selective for *A. fumigatus*.

Figure 5 shows the average abundance of *A. fumigatus* per sampling site for each month. Results show that density of *A. fumigatus* is consistent throughout the sampling period, and stable on average around 10^5. Sample by sample variation exists, probably as a result of the heterogeneous nature of the decaying plant material with respect to allowing fungal growth. Nevertheless, between sampling sites similar fungal densities were found. Analysis of samples of mature compost (so plant waste material that is processed into compost) is free of *A. fumigatus* (not shown in the graph).

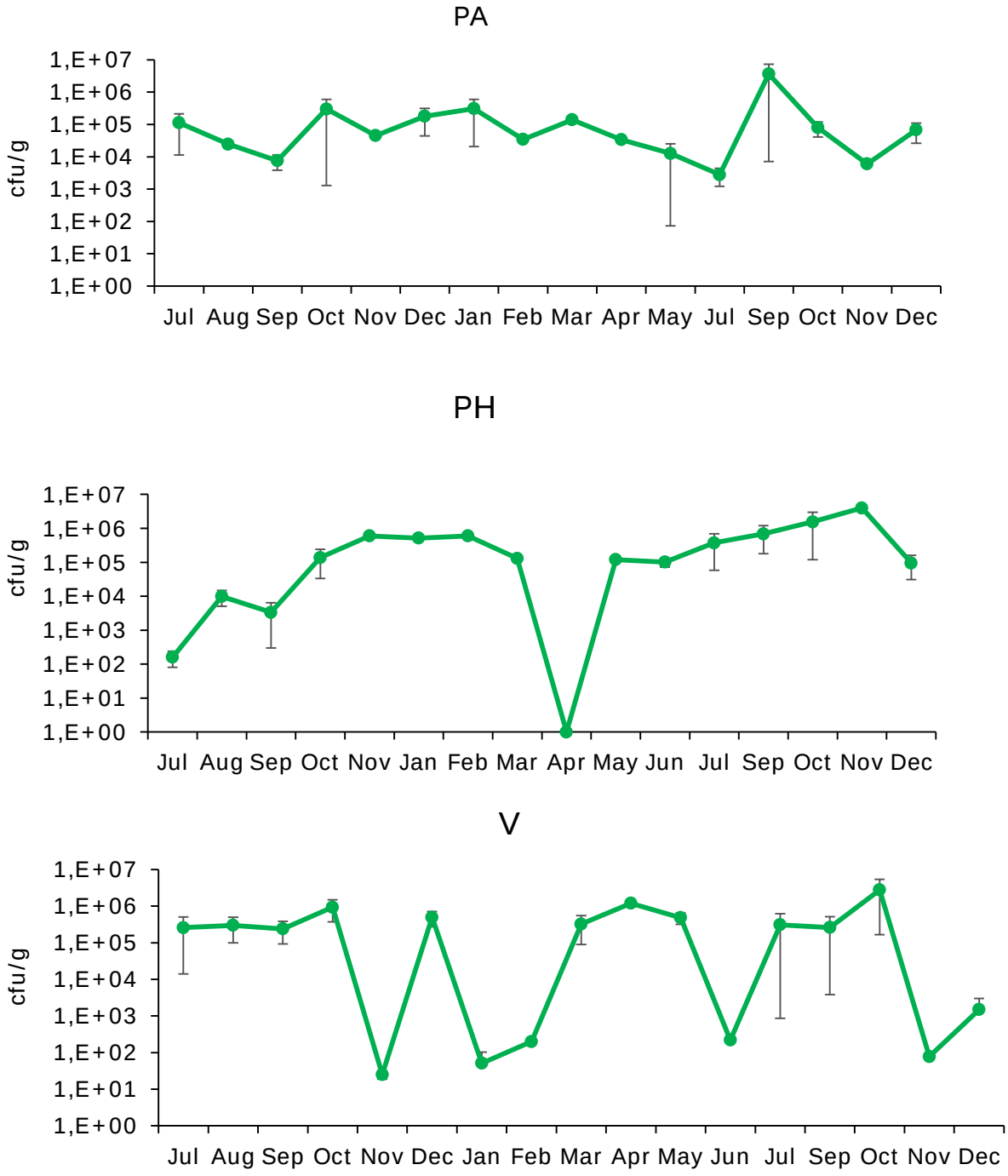


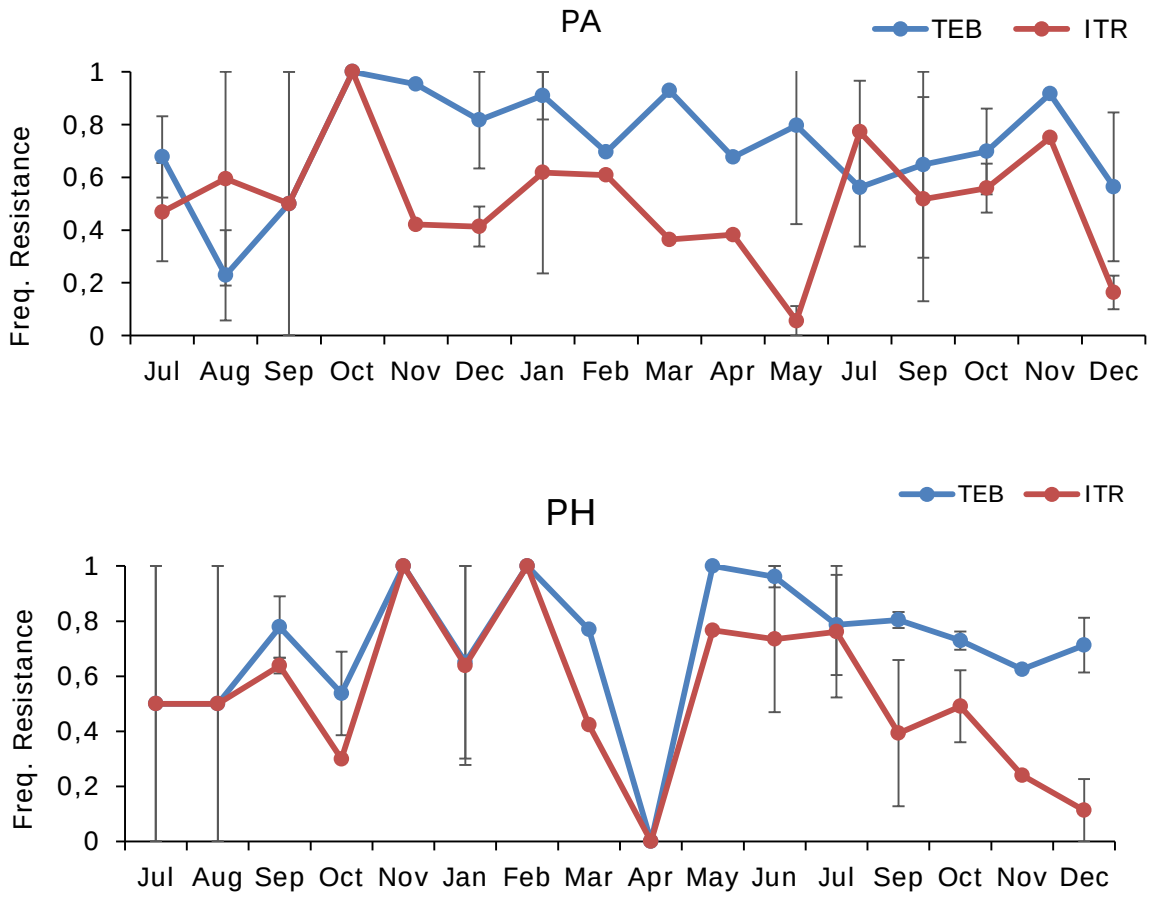
Figure 5. Total abundance of A. fumigatus over a 16 month period. Points show averages over all samples of each sampling site taken in the same month. Error bars show standard errors of the mean (SEM).

D. Fraction resistant isolates to two indicator azole fungicides

We measured the fraction resistant *A. fumigatus* by plating dilutions of samples on growth medium containing tebuconazole and on growth medium containing itraconazole. Tebuconazole is an azole fungicide used in agricultural applications and was widely detected in nearly all of the samples. Itraconazole is a medical drug, that was not detected in

the samples we collected but is one of the most commonly used medical azoles. We call these two compounds indicator azoles in the rest of the report.

Figure 6 shows the average fraction resistant of samples taken in the same month at the same location, differentiated per indicator azole. Results are similar for the two indicator azoles with around half the *A. fumigatus* present being resistant to both compounds. While it is highly unlikely the fungus had been exposed to the medical drug itraconazole, this means that the presence of environmental azoles also results in cross-resistance to medical azoles. The fraction resistant is stable throughout the sampling period at around 50%.



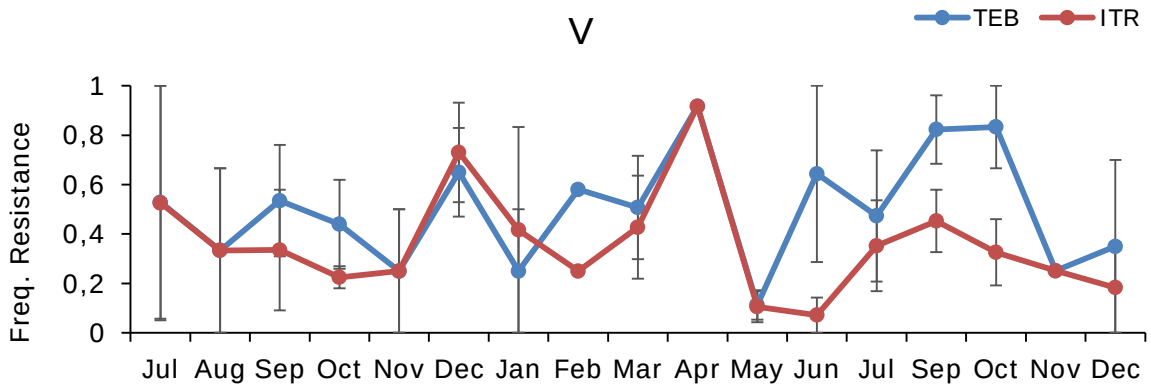


Figure 6. Fraction of resistant A. fumigatus against two indicator azole fungicides (the agriculturally used tebuconazole and medically used itraconazole) over a 16 month period. Points show averages over all samples assayed on each indicator azole. of each sampling site taken in the same month. Error bars show standard errors of the mean (SEM).

E. Genetic analysis of resistant strains: screen for changes in the *cyp51* gene and its promotor region

Table 4 shows the results of the genetic analysis of the *cyp51* gene of 117 randomly picked resistant *A. fumigatus* isolates. In all 117 isolates genetic changes were detected in the *cyp51* gene relative to sensitive wild-type strains. This shows that resistant strains isolated from hotspots carry the same resistance mutations as isolates collected from clinical sources. In clinical strains that carry resistance, the resistance mechanisms TR34-L98H and TR46-Y121F/T289A are most frequently reported (Verweij, 2017).

Table 4: Genetic analysis of the cyp51A gene and promotor region of 117 randomly selected resistant A. fumigatus isolates from the three different sampling sites (PA, PH, V).

Tandem repeat	**Point mutation**				
		PA	PH	V	TOTAL
TR34	L98H	7	2	6	15
TR34	L98H/S297T	1	1	0	2
TR46	Y121F/T289A	23	27	32	82
TR46	Y121F/M172I/T289A/G448S	2	1	2	5
TR46	Y121F/T289A/S363P/I364V/G448S	1	7	0	8
TR92	Y121F/M172I/T289A/G448S	0	2	2	4
TR138	Y121F/M172I/T289A/G448S	0	1	0	1
					117

F. Isolation of sexual spores from environmental hotspots

We found that 20% (26/127) of samples contained sexually derived ascospores, which indicates the potential occurrence of sexual reproduction in the hotspots sampled. The number of sexually derived spores (*ascospores*) in 1g of decaying plant waste material ranged from 20 to more than 10,000. All three farms contained samples with ascospores, suggesting ascospores are not location specific. In all

samples, the samples from farm V accounted for 46% and farm PH for 35%, farm PA for 20%.

G. Resistance mechanism characterized across different hot spots

In the reseach completed under phase I in 2016, we found that not only decaying plant material from bulb waste was a potential hotsport, but also green waste and wood chippings. We revisited these hotspots by characterizing azole resistant isolates from these locations for their resistance mechanism. We found tandem repeat (TR) variation as well as point mutations (Figure 2). For green waste TR34/L98H (24), TR34/L98H (12), TR34/L98H/L343H (1); for wood chippings we found TR34/L98H (18). This shows that resistant strains from the other two hotpots identified under Phase I of the research also share the same resistance mechanism as resistant strains collected from bulb waste material and clinical environments.

3.2 Experimental hotspot model in the laboratory

Results summary of this part: using a laboratory hotspot model, we assessed the effect of several parameters on the development and maintenance of resistance.

Main results:

- Under testing conditions, spore production was remarkably high showing that *A. fumigatus* can proliferate very well in plant waste material;
- Resistance to azoles developed under all concentrations and combinations of azoles tested;
- Disturbance (or not) of the fungal cultures by the addition of fresh material did not affect resistance development;
- Resistant strains are not displaced by sensitive strains when growing in the presence of back ground levels of azoles for a period of three months.

Figure 7. Pictures of the experimental laboratory hotspot model. Each glass tube is filled with decaying plant material, azole fungicides (or not) are added and fungal inoculum. Tubes are incubated at 37 ^{o}C

In order to be as realistic as possible we used decaying plant waste material collected at the field sites described above as the basis of our laboratory conditions. This plant waste material used to set up the laboratory selection experiment contained low background levels of azole fungicides (Table 3).

To verify that *A. fumigatus* (the sensitive strain, resistant strain, and the mixture of the two strains) could grow under our experimental conditions, we harvested all fungal spores from glass tubes after various incubation times. The results (Figure 8) show that on their own and in combination the two strains grow very well in our experimental hotspot set up. In fact, the spore yield is remarkably high; around two orders of magnitude higher than on standard laboratory medium. No differences between total spore yield after three months of incubation for each of the azole treatments was found (ANOVA: $P>0.05$), while resistant strains reach a higher yield than sensitive strains under the conditions used (ANOVA, $P<0.05$). That was observed even for the control which may be explained by the fact that the used decaying plant waste material contained azole fungicide residues (see Table 3).

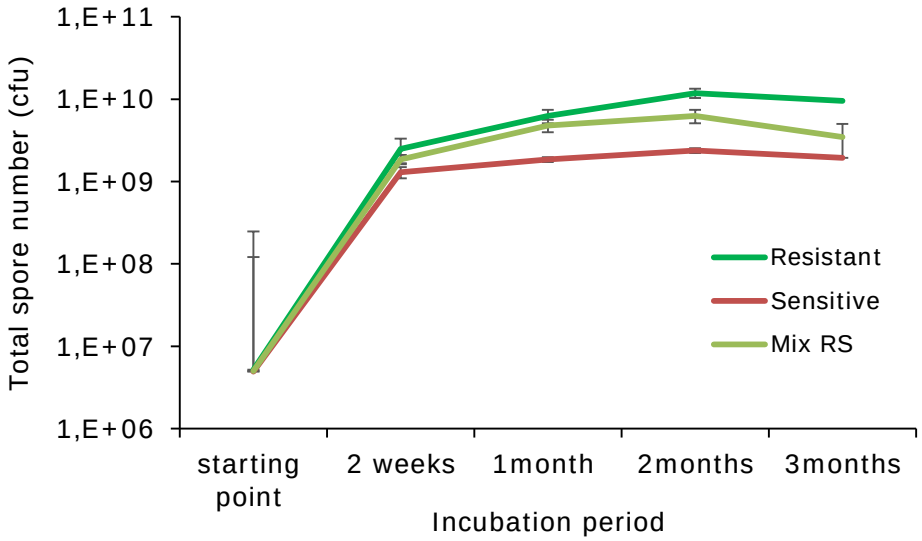


Figure 8. Total spore yield of in the resistant strain, the sensitive strains and the mix of the two strains, averaged over all azole treatments. Error bars show standard errors of the mean (SEM).

B. Growth rate of A. fumigatus strains after three months of incubation under the various azole treatments and with or without disturbance

In different glass tubes and using replicates, we let *A. fumigatus* grow under seven different azole treatments and two different transfer regimes (Table 2). After three months, we harvested spores from all tubes that were initiated with the sensitive strain (only the sensitive strain). We then used the resulting spore suspensions to inoculate Petri dishes with medium supplemented with the same azole concentrations corresponding to the applied three-month treatment. We measured growth rate on the Petri dishes, including the growth rate of the original sensitive strain. We then calculated the relative growth rates of the evolved strains by dividing all growth rates by the growth rate of the sensitive strain grown on the same medium with the same azole concentration (Figure 9). For instance, a growth rate relative to the sensitive strain of 1.1 means that the strain that had grown for three months in tubes now had a 10% higher growth rate than the original strain. These relative growth rates are indicative of strains having become resistant to the azoles applied during their laboratory growth.

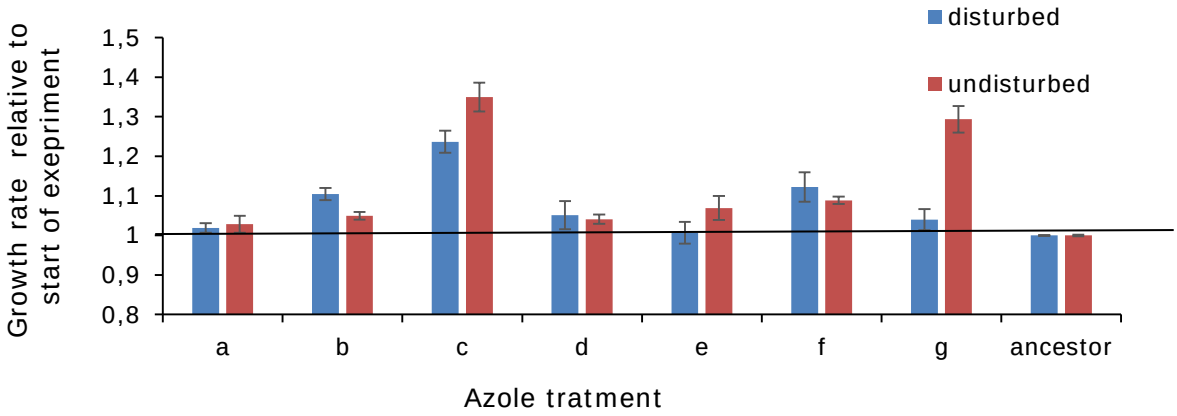


Figure 9: Relative growth rate of mixed populations of fungi that had grown under the different experimental azole treatments, either with or without disturbance during the three months incubation, measured on Petri dishes containing the same azole concentrations as used during the three months of incubation. A relative growth rate greather than 1 is indicative of the emergence of resistance. Error bars show standard errors of the mean (SEM). The x-axis shows the treatment conditions used during the laboratory experiment (Table 2) a:Only background levels of azole fungicides; b:TEB low; c:TEB high; d:PRO low; e:PRO high; f:TEB low + PRO low; g:TEB high + PRO low ; ancestor: sensitive strain before exposure to our experimental treatments. Under all condition tested, the relative growth rate of evolved strains is greather than 1, showing that resistance evolved under all conditions tested.

Results show (Figure 9) that under all azole treatments and both when cultures are transferred every two weeks or grew without disturbance, evolved strains grow better than the original sensitive strain. This shows that the initially sensitive strain had adapted to the imposed (azole) environmental conditions, indicating that at least a fraction of the total population had developed fungicide resistance. No differences were found between populations that grew with or without disturbance over the three months of incubation.

To verify that strains indeed developed resistance, we placed spore suspensions on plates supplemented with a high concentration of tebuconazole (4 mg/l, indicator concentration for resistance). Here, the growth will be dominated by the most resistant types in the possibly heterogeneous spore suspension.

Results (Figure 10) confirm that all populations contain resistant types as shown by the consistently higher growth rates of the evolved strains compared to the sensitive ancestor strain. Even the control treatment, where only background levels of azoles are present, now contain individual resistant spores. Previous experiments have shown that no resistance evolves when growing in the absence of azoles (Zhang et al., 2015b, Zhang et al., 2017b). The highest level of resistance is different for the treatments; some treatments have yielded higher resistance than others. In c treatment (TEB high) and g treatment (TEB low and PRO low), post hoc testing ($p<0.05$) showed the disturbed and un-disturbed treatments are significantly different, however, reverse effect in c and g settings. In all, the results show that resistance to triazoles

develops under all variations of azoles and disturbed and undisturbed tested.

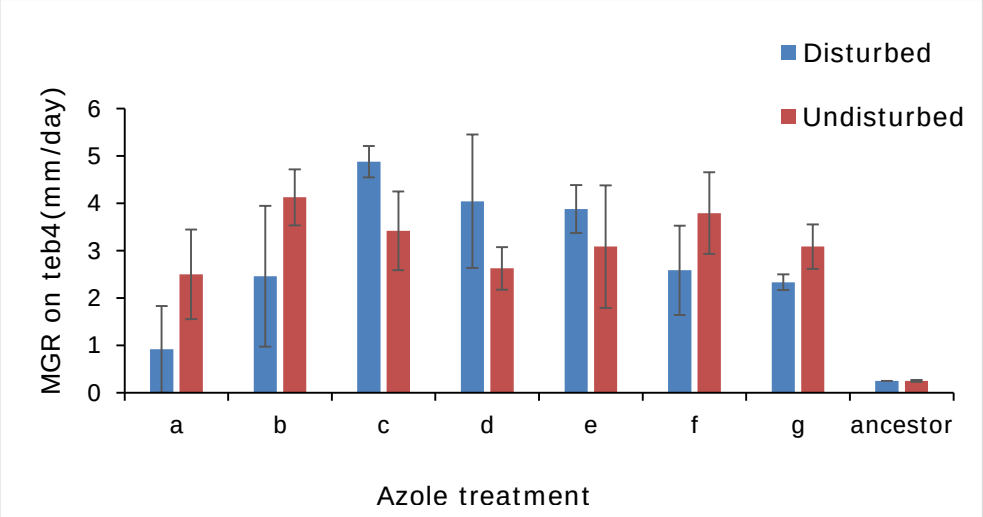


Figure 10: Mycelium growth rate (indicative of resistance levels) of populations on indicator fungicide TEB 4 mg/L, which evolved under the seven fungicides combination pressure with and without disturbance (Table 2). Error bars show standard errors of the mean (SEM). a:Only background levels of azole fungicides; b:TEB low; c:TEB high; d:PRO low; e:PRO high; f:TEB low + PRO low; g:TEB high + PRO low. Results show that under all variations tested - levels of presence of azoles and disturbance or not – resistance to azole fungicide develops.

Finally, to assess what fraction of the spores in the spore suspension is highly resistant, we plated serial dilutions of the spores suspension on Petri dishes containing medium with high levels of tebuconazole and itraconazole (4 mg/l), as well as on plates without azoles. On the plates without supplementation all viable spores will germinate and on tebuconazole plates, only highly resistant spores. Results are in Figure 11 (TEB-4) and (ITR-4) and show that fractions of resistant spores within the populations are all in the same range for each azole treatment used during the three months of incubation.

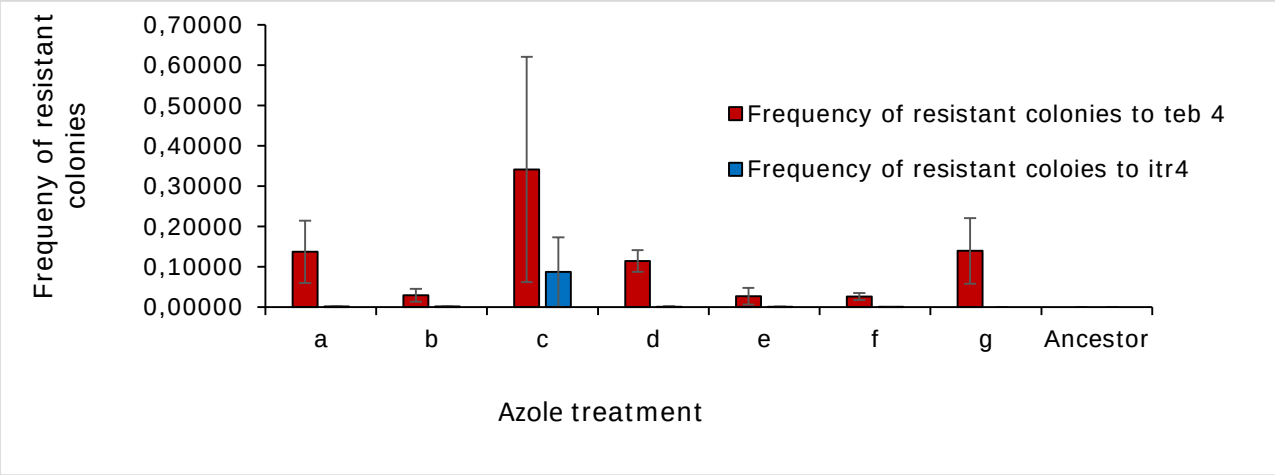


Figure 11. Fraction of highly resistant spores in spore suspensions harvested after three months, assayed using colony counts on medium supplemented with TEB and ITR at 4 mg/l. Error bars show standard error of the mean (SEM). Azole treatments are a:Only background levels of azole fungicides; b:TEB low; c:TEB high; d:PRO low; e:PRO high; f:TEB low + PRO low; g:TEB high + PRO low.

C. Competition between sensitive and resistant strains.

To test the degree to which resistant strains are outcompeted by sensitive strains in the conditions of our laboratory hotspot model, we mixed resistant and sensitive strains, and subsequently counted their ratio before and after incubation in the glass tubes under the various azole treatments (Table 2). The results (Figure 12) show that under all conditions, the resistant strain gains in frequency over the sensitive strains. This means that even the very low azole concentrations of the control treatment with only background levels of azoles is sufficient to provide resistant strains a selective advantage.

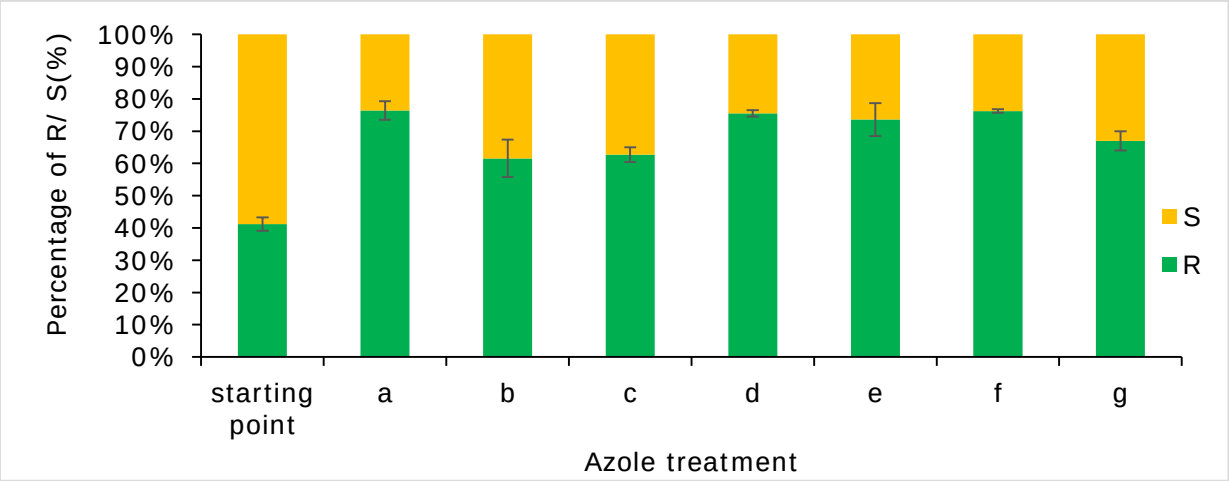


Figure 12 : Ratio of resistant and sensitive under seven different azole treatments before and after three months of incubation. Error bars show standard errors of the mean(SEM). R: resistant; S: sensitive. Azole treatments are a: CONTROL - only background levels of azole fungicides; b:TEB low; c:TEB high; d:PRO low; e:PRO high; f:TEB low + PRO low; g:TEB high + PRO low.

D. Sexual reproduction in the laboratory hotspot model environment

In total 20 decaying plant waste material glass tubes (the laboratory hotspot model) were inoculated with genotypes where outcrossing can be detected by visual inspection (super-maters; (Zhang et al., 2017a)). After three weeks, we observed the presence of sexual fruiting bodies in 50% (8/16) of the glass tubes. In total we observed 17 cleistothecia, either via visual inspection or through a microscope (Figure 13). This is in line with the percentage of samples from field sampling that show presence of sexually derived spores (*ascospores*). Combined with previous observations that sexual crosses can increase the number of tandem repeats in the promotor region that is crucial for the development of clinical cyp51 gene based resistance (Zhang et al 2017) (Zhang et al., 2017a), this current finding provides strong evidence for the occurrence of sex in the decaying plant waste material and the potential importance of the completion of the sexual part of the life-cycle in the emergence of azole resistance.

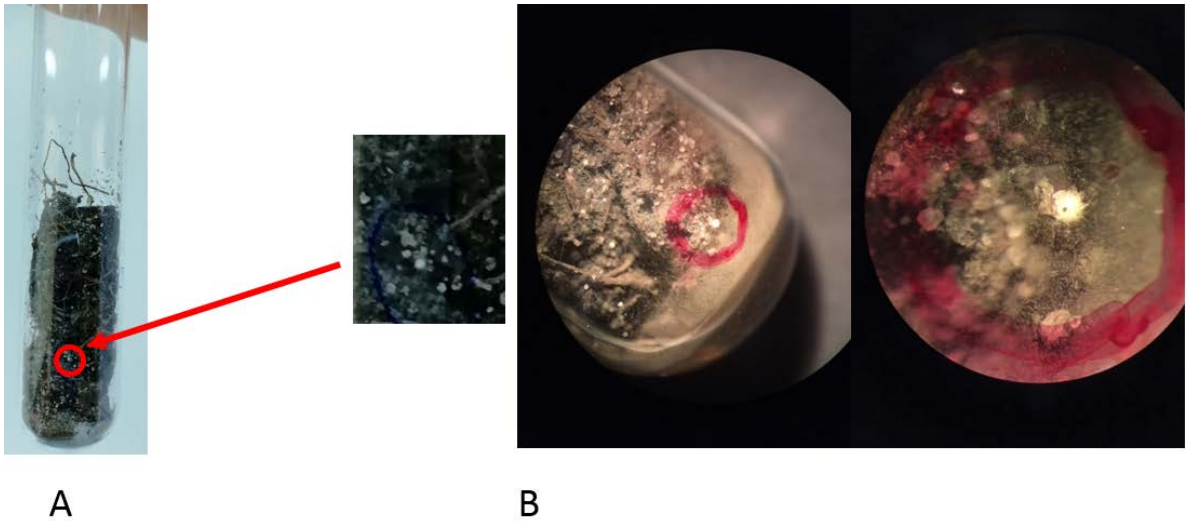


Figure 13: Sexual fruiting body (cleistothecia) observed from the decaying plant waste material glass tube. A: In glass tube observed from outside. B: under the microscope with 40x, 100x

4 Main conclusions

Longitudinal study

- The study described here forms further support for the hotspot hypothesis. *A. fumigatus* is abundantly present in decaying plant waste material on the sampled bulb farms, year round.
- Low levels of azoles are present in the plant waste collected at sampling sites, year round.
- The fraction resistant *A. fumigatus* at sampling sites is consistently around 50%
- Identical resistance mechanisms were found in environmental strains and strains isolated from patients.

Experimental study

- In the lab under experimental conditions, a remarkably high numbers of fungal spores was produced per gram of plant waste material.
- A very low azole selection pressure is sufficient to yield resistance, including cross resistance to medical triazoles.
- Disturbance of decaying material does not influence resistance development.
- Sensitive strains do not outcompete resistant strains, regardless of the presence or absence of azoles or their concentration.

5 Experimental limitations and uncertainties

5.1 Longitudinal field sampling at three bulb farms

The large temporal variation in both fungal counts and resistance levels suggest that hotpots are very heterogeneous – in some parts not all the necessary conditions for being hotspots may be present. For instance, in eight of our 127 samples no presence of *A. fumigatus* was detected. Further research could show whether this was because of sampling bias or because some intrinsic difference in that sample, for instance other physicochemical conditions. More intensive sampling at one location at one time could shed light on this; this could be part of further validation studies. Nevertheless, the fact that over the sampling period we consistently detected *A. fumigatus* clearly shows that the sites we sampled are hotspots.

While the sites we chose for sampling represent the most common practice of plant waste handling and azole use, we observed that some other variations in processing of (azole containing) organic waste exist on bulb farms. The variation and heterogeneity may differ between waste handling practices.

5.2 Experimental hotspot model in the laboratory

Azole concentrations in the laboratory experiment are not uniformly distributed over the decaying plant waste material in the tubes, and while this mimics the situation in heaps of decaying plant material at farms, it is difficult to reach true standardization comparably to the homogenous distribution of nutrients in for instance a MEA filled Petri dish. This at least in part explains the variation between replicate tubes of the same treatment. Moreover, we checked concentrations of azole fungicides before and after we applied azole stocks to the decaying plant waste material and found that the achieved concentrations are lower than what we had set out to achieve. Also, differences between high and low concentrations were smaller than planned.

Fungi grow as mixed populations containing both sensitive and resistant types. Within the resistant fraction, genetic variation may exist. We do not have full insight in all of this variation. Highly resistant strains are the most rare and by chance we may have missed these when sampling the populations to select strains that show resistance for further analysis. Because of this, the analysis of the laboratory selection experiment may have yielded an underestimation of the potential for resistance development.

Within mixed populations that were initiated from sensitive strains, we observed a low fraction of resistant types (ranging from 0.002 to 0.34%). While in percentage this fraction is low, in absolute terms, this still amounts to 10^4 spores per gram of decaying plant waste material. This low level could however be an underestimation since finding the correct conditions to isolate individual resistant types is not straightforward and the actual numbers of resistant types is likely higher than we have reported.

6 Discussion

6.1 Longitudinal field sampling

Overall, the results confirm the notion that heaps of azole containing decaying plant material can be significant hotpots for resistance development and maintenance, supporting the findings of phase I of the study (Box 1, Table 1) (Verweij, 2017). We detected azole fungicides throughout the entire 16 months sampling period at levels that inhibit growth of *A. fumigatus* by 10 to 50% compared to azole-free conditions. This level of growth reduction provides a strong selection pressure for resistance. We further detected *A. fumigatus* during the entire period at levels of around 10^5 fungal spores per gram of material. Roughly half the isolates was resistant to our two indicator azole fungicides, one of which is a commonly used agricultural azole (tebuconazole) and one a medically used triazole (itraconazole). The latter indicates that agricultural azoles can induce or at least support cross-resistance to medical triazoles.

We found evidence of a link between resistance against azoles present in the decaying plant material and resistance to medical azoles in clinical strains isolated from patients: the striking similarity of the resistance mechanism at the genetic level between environmental strains and clinical strains strongly suggest that the patients treated for aspergillus related diseases became infected by inhaling triazole-resistant *A. fumigatus* propagules from environmental sources. This supported by the fact that other potential resistance mechanisms exist. We have consistently found genetic changes in the *cyp51* gene that are typically found in isolates from patients and are also found in all *A. fumigatus* isolates tested that were isolated from the hotspot sampling sites. This may explain how patients contract a triazole-resistant *A. fumigatus* lung infection while not having been treated with triazoles before.

The detection of sexually derived spores shows that *A. fumigatus* can complete the sexual part of the life-cycle under hotspot conditions. For high level resistance tandem repeat variation in the cyp51 promotor region is required (and observed in isolates from the hotspot). Previous work has shown that the sexual part of the life cycle can generate this tandem repeat variation (Figure 2). The finding of sexually derived spores further underlines that azole-containing plant waste material is a hotspot for azole resistance development.

In the longitudinal study, we found strong support for the notion that large-scale waste piles of environmental azole-containing plant material creates a hotspot for growth and development of azole resistant *A. fumigatus*, from which it may spread via airborne conidiospores through the air and may be inhaled by future patients.

The results of the longitudinal study are also relevant for future studies and monitoring: given the relatively stable presence of azoles and (resistant) *A. fumigatus* throughout the year, long periods of sampling on other sites should not always be necessary. Also, testing the efficacy

of potential control measures does not necessarily require months of monitoring.

6.2 Experimental hotspot model in the laboratory

The remarkably high numbers of spores that the fungi can produce when growing on plant waste, as shown in the experimental hotspot, suggests that the types of sites we sampled are significant hotspots for the spread of resistant (and sensitive) *A. fumigatus*.

The experiments using the experimental hotspot in the laboratory aimed at testing the impact of three parameters for their effect on resistance development.

Effect of azole concentration. Our experiments using the experimental hotspot model demonstrate that resistance can arise in a sensitive strain under various azole concentrations and that even azole concentrations that are as low as 1% of a medical dose are sufficient to select for resistance. This resistance is not only to the agricultural azole fungicides that were used as selection pressure, but also cross-resistant against a medical azole was observed. All tested incubation regimes, including all azole treatments and both disturbance or no disturbance, led to similar levels of resistance development. There is no significant correlation between the amount of azole applied and observed resistance levels. Reducing the use of azoles in agriculture does therefore not provide a quick solution to the problem of azole resistance since even low azole concentrations in the environment will cause continued selection pressure.

Disturbance/no-disturbance. Disturbance or not during incubation does not affect the levels of resistance. In all treatments, resistance evolved. This implies that disturbance does not hamper the sexual cycle of the fungus and/or that this potential effect is countered by the fact that disturbance also promotes prologned growth through the addition of fresh material such that the fungus has the opportunity to evolve resistance. We found that the sexual part of the life-cycle can indeed be completed under our experimental conditions, including under our treatment of disturbance. Therefore, disturbance of waste piles (potentially preventing the sexual part of the life-cycle) does not seem to be an effective way to reduce growth of *A. fumigatus* or reduce the fraction of resistant strains.

Combinations of senstive and resistant strains. Sensitive strains do grow in the presence of background levels of azoles but do not diplace resistant strains under these conditions. Control measures where sensitive strains are deliberately added to waste material to outcompete resistant strains are therefore unlikely to be successful.

All of the above indicates that, for a short term effective control of resistant Aspergillus, large scale accumulation of plant waste material on bulb farms may need to be avoided all together. From earlier research we have learned that certain controlled professional composting procedures exist that diminish *A. fumigatus.* Our sampling in this study

confirms earlier findings that once decaying plant material is processed to mature compost it does no longer contain *A. fumigatus.*

7 Recommendations

The common procedure found at several bulb farms to store plant waste material on piles for prolonged periods creates favorable conditions for the emergence of resistant *A. fumigatus*. The high numbers of fungal spores found in the material indicate that decaying plant material is an important reservoir for (resistant) *Aspergillus fumigatus*. Potential steps to influence the emergence of resistant Aspergillus that were tested in the experimental setup described here, were not effective under the tested conditions. Therefore, with the current knowledge, our recommendation is that this waste storage on-site should be avoided.

Waste removal

- Our results show that plant waste storage at bulb farms without active composting procedures provides very favorable conditions for *A. fumigatus*. Therefore, longer term and unsupervised storage of plant waste material at professional production sites should be avoided. Regular waste removal and further handling by professional composters could be considered.

Waste treatment

- Upon removal of plant waste from bulb farms, waste processing protocols should be tuned to diminish growth of A. fumigatus. Phase I of this research and personal communications with bulb producers during phase II have shown that large variations exist in the current practice of plant waste processing to mature compost, some of which lead to lower counts of *A. fumigatus.* We recommend to explore existing waste treatment protocols as a starting point for the testing of feasible and effective protocols. The effectiveness of diminishing *A. fumigatus* needs to be monitored.

Other hotspots

- The resistance mechanisms described in this study are also found in strains isolated from the other hotspots described in phase I: industrial wood chippings and green waste storage (Verweij, 2017). Whether the findings from the case study with plant waste material from the bulb industry also apply to these other hotspots remains to be explored. We therefore recommend to 1) explore storage conditions for feedstock in wood chippings and green-waste composting industry that diminish the growth of *A. fumigatus*, and 2) analyse whether additional hotspots may exist based on current knowledge.

Understanding transmission dynamics

- We found further confirmation for a link between hotspot and resistant infection of patients due to the presence of identical resistance mutations in environmental and clinical *A. fumigatus* isolates. Our study did however not address the actual spread of spores from hotspots to humans. At the international symposium on azole resistance in *A. fumigatus* hosted by the Royal Netherlands Society of Arts and Sciences (KNAW) on the 31st of Januari and the 1st of Februari 2019 the consensus expert opinion was that

transmission of resistant spores from hotspots to patients is sufficiently plausible. Developing and implementing methods to qualitatively and quantitatively analyse airsamples (at hotspots and along main wind direction routes) will help assess both infection risk and effectiveness of meaures to turn hot- into coldspots.

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