Asexual sporulation facilitates adaptation: The emergence of azole resistance in *Aspergillus fumigatus*

Jianhua Zhang,¹ ² Alfons J. M. Debets,¹ Paul E. Verweij,³ Willem J. G. Melchers,³ Bas J Zwaan,¹ ∗ and Sijmen E. Schoustra¹ ∗

¹Laboratory of Genetics, Wageningen University, Droevendaalsesteeg 1, 6708PB, Wageningen, The Netherlands
²E-mail: Jianhua.zhang@wur.nl
³Department of Medical Microbiology, Radboud University Medical Centre, 6500 HB, Nijmegen, The Netherlands

Received December 18, 2014
Accepted August 3, 2015

Understanding the occurrence and spread of azole resistance in *Aspergillus fumigatus* is crucial for public health. It has been hypothesized that asexual sporulation, which is abundant in nature, is essential for phenotypic expression of azole resistance mutations in *A. fumigatus* facilitating subsequent spread through natural selection. Furthermore, the disease aspergillosis is associated with asexual sporulation within the lungs of patients and the emergence of azole resistance. This study assessed the evolutionary advantage of asexual sporulation by growing the fungus under pressure of one of five different azole fungicides over seven weeks and by comparing the rate of adaptation between scenarios of culturing with and without asexual sporulation. Results unequivocally show that asexual sporulation facilitates adaptation. This can be explained by the combination of more effective selection because of the transition from a multicellular to a unicellular stage, and by increased mutation supply due to the production of spores, which involves numerous mitotic divisions. Insights from this study are essential to unravel the resistance mechanisms of sporulating pathogens to chemical compounds and disease agents in general, and for designing strategies that prevent or overcome the emerging threat of azole resistance in particular.

**KEY WORDS:** Alternation between unicellular and multicellular growth, asexual sporulation, *Aspergillus fumigatus*, azole resistance, experimental evolution, MIC value, mycelial growth rate.

*Aspergillus fumigatus*, a common saprophytic fungus, is abundant in soil and decaying organic matter (Bignell 2014; Cuenca-Estrella 2014) and produces numerous airborne spores. As such it is generally considered to be harmless to humans and can be found in houses (e.g., bathrooms), offices (e.g., ventilation vents), and also in hospitals (Haas 2011). However, this fungus may cause a wide range of noninvasive or invasive diseases in immunocompromised patients (Latgé 1999; Rhodes 2006; Snelders et al. 2012; Kwon-Chung and Sugui 2013; Bignell 2014; Rocchi et al. 2014), where invasive aspergillosis is a major cause of mortality and morbidity (Cunha et al. 2013). Azoles are the most widely used antifungal drugs in medical treatment, especially triazoles, such as itraconazole, posaconazole, and voriconazole (Albarrag et al. 2011). These triazoles inhibit sterol 14α-demethylase, encoded by the *cyp51A* gene, thereby blocking its function in the fungal ergosterol biosynthesis pathway, resulting in ergosterol depletion and accumulation of toxic sterols (Joseph-Horne and Hollomon 1997).

Unfortunately, the emergence and spread of azole resistance has been increasingly reported, with prevalence from 0.8 to 9.5% in the various medical centers in the Netherlands during 2007–2009 (van der Linden et al. 2011). In the United Kingdom, the National Aspergillosis Centre (Manchester, UK) described an increase in azole-resistant isolates, increasing from 7% in 1997 to 20% in 2009 (Howard et al. 2006; Howard et al. 2009; Bueid et al. 2010). Indeed, azole resistance has been reported also in other
European countries, the Middle East, Asia, and most recently in Africa (Snelders et al. 2008; Arendrup et al. 2010; Lockhart et al. 2011; Mortensen et al. 2011; Pfaffer et al. 2011; Chowdhary et al. 2012; Morio et al. 2012; Gisi 2013; Bignell 2014; Chowdhary et al. 2014). Clearly, azole resistance is a growing concern as patients with azole-resistant Aspergillus fumigatus have a high probability of treatment failure, and alternative treatment options are limited (Seyedmousavi et al. 2014). To control this problem, we need to elucidate by what mechanism(s) the resistance emerges, how it can spread, and how resistant genotypes can persist in environments without azoles.

Several studies focussed on the origin of azole resistance and tested Aspergillus fumigatus strains for azole resistance isolated from both clinical and environmental settings (Mellado et al. 2007; Howard et al. 2009; Snelders et al. 2009; Arendrup et al. 2010; Mortensen et al. 2010). These studies showed that highly resistant strains were recovered from compost, flowerbeds, and agricultural fields, as well as from patients with Aspergillus diseases. In addition, recent studies indicated that azoles used in agricultural settings can select for resistance (Snelders et al. 2012; Chowdhary et al. 2013; Verweij et al. 2013; Bowyer and Denning 2014), especially the azole fungicides bromuconazole, tebuconazole, epoxiconazole, difenoconazole, and propiconazole, which are widely used in crop protection and material preservation. It is hypothesized that resistance to these fungicides is selected especially in these nonclinical environments. When susceptible patients subsequently inhale fungicide-resistant Aspergillus fumigatus spores, Aspergillus disease may develop to which the medical triazoles are ineffective due to high molecule similarity to the five “environmental” azole fungicides. However, previous studies did not allow for the identification of the factors facilitating the emergence of resistance and the responsible evolutionary mechanism(s). This is what we address in the present study.

This study focuses on the asexual stages of the life cycle of Aspergillus fumigatus and in particular on the possible role of asexual sporulation in facilitating adaptation of the fungus to azole environments. Similar to that of the model species Aspergillus nidulans, unicellular and multicellular states alternate during the life cycle (whether it be asexual, sexual, or parasexual) and selection can act on both states (Cassleton and Zolan 2002; Bruggeman et al. 2004; Mah and Yu 2006; Schoustra et al. 2006; O’Gorman et al. 2008). The asexual part of the life cycle is by far the most common in nature providing an alternation between multicellular and unicellular states by the generation of ubiquitous uninucleate asexual spores (through numerous mitotic divisions) that after dispersal and germination can generate multinucleate mycelia that again produce numerous spores that become airborne (Fig. 1A).

Previously, Verweij et al. hypothesized that asexual sporulation is essential for phenotypic expression of azole resistance mutations in Aspergillus fumigatus in clinical settings (Verweij et al. 2009). This idea is based on the fact that when a resistance mutation occurs during vegetative growth in the multinucleate mycelium, the resulting resistant nucleus is initially surrounded by sensitive nuclei in a heterokaryotic cell. All else being equal, if resistance mutations are at least partially recessive, resistance is not fully expressed into the phenotype when also sensitive nuclei are present in the mycelium. Crucially then, the formation of uninucleate asexual spores would allow resistant nuclei to escape from the heterokaryotic mycelium so that, after germination, the resistant phenotype would be fully expressed (Fig. 1). This mechanism could take place within the lungs of patients, because sporulation propagative structures have been observed in patients with cavitary Aspergillus diseases (Franquet et al. 2001). Furthermore, resistance selection during azole therapy has been reported in these patients (Camps et al. 2012). In cavitary Aspergillus diseases, such as chronic cavitary aspergillosis and aspergilloma, Aspergillus fumigatus undergoes asexual sporulation, as opposed to invasive aspergillosis, which involves only hyphal growth. This clinical observation also shows that various fungal morphotypes exist within patients and suggests that there is a link between sporulation and resistance development in patients. Therefore, it is crucial to study the role and consequence of asexual sporulation to understand the invasive infection in immuno compromised patients.

In this study, we investigated the effect of asexual sporulation on the emergence of resistance to five different azole fungicides of Aspergillus fumigatus in a laboratory evolutionary experiment. This was done by contrasting the rate of development of resistance to azoles in Aspergillus fumigatus during seven weeks, in six replicate cultures, with two different treatments: cultured either without asexual sporulation (continued mycelium growth) or serially cultured allowing asexual sporulation. For the experimentally evolved cultures of each contrast and replicate, we measured resistance levels and mycelial growth rate (MGR) (fitness). Five different azole fungicides were compared for their induced levels of azole resistance. Furthermore, we assayed whether resistance mutations were dominant or recessive in one of the evolutionary lineages, testing the underlying assumption that resistant nuclei are not (fully) expressed in the phenotype of multicellular mycelium.

Materials and Methods

Fungal Isolates and Azole Fungicides

Azole fungicides (bromuconazole [b], tebuconazole [t], epoxiconazole [e], difenoconazole [d], and propiconazole [p]) were purchased from Sigma Company (Sigma Aldrich, Germany). Aspergillus fumigatus CBS 140053 was isolated from an environmental field in Wageningen in 1992 in our laboratory at Wageningen University before these azole fungicides were applied to the environment (Snelders et al. 2012; Bowyer and...
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Figure 1. The asexual life cycle of *Aspergillus fumigatus* and hypothesis of escaping strategy.

(A) The asexual cycle starts with the formation of a mycelium that can be initiated by a single uninucleate haploid spore (either an asexual conidiospore or a sexual ascospore). Mitotic division in the mycelium takes place in the apical cells at the growing front of the colony (Casselton 2002). After one to two days, mycelial cells differentiate into aerial hyphae with a foot cell and a spore head (conidiophore) covered with conidiogenous cells (phialides). Around three days after initiation of the multicellular mycelium, these conidiogenous cells produce numerous unicellular and uninucleate asexual spores by mitotic divisions. Thus, a single spore may give rise to up to $10^9$ new asexual spores in three to four days under favorable conditions.

(B) In the multicellular mycelium with mixed nuclei, resistant mutated nuclei have limited or no effect on the resistance phenotype as they are outnumbered by sensitive nuclei. After asexual sporulation (alternation from multicellular mycelium to unicellular spores), the resistant and sensitive nuclei get separated and the former can express their superior phenotypes in an azole environment (modified with permission from P. E. Verweij [Verweij et al. 2009]).

Denning 2014). This strain is sensitive to our five azole fungicides and was used as the ancestor of the evolution experiment.

**EXPERIMENTAL EVOLUTION OF *A. FUMIGATUS* WITH AND WITHOUT ASEXUAL SPORULATION UNDER THE SELECTION PRESSURE OF AZOLE FUNGICIDES**

In a seven-week experimental evolution set-up, the culturing of *A. fumigatus* CBS 140053 with and without asexual sporulation was contrasted. For both treatments, we founded six lineages for each of the five azole fungicides and six lineages without any fungicide as a control. Seven time points for each lineage were sampled and stored in our “frozen fossil record.” Thus, the total number of samples was 505 [2 treatments × (5 azoles + control) × 6 replicates × 7 time points + ancestor = 505].

For the serial subculturing treatment allowing for asexual sporulation, parallel lineages were inoculated with $10^4$ ancestral spores in a 5 μL droplet into a bottle with 10 mL solid Malt Extract Agar (MEA) medium, which contained 1 μg/mL of one of the five azole fungicides. This amount of azole is based on the application of these fungicides estimated if a soil layer of 1 cm is considered in agricultural fields (Gisi 2013). Under this concentration, *A. fumigatus* is able to sporulate. After seven days of incubation at 37°C, all material including spores from each bottle was harvested: 2 mL of saline (distilled water with NaCl 0.8 g/L) supplemented with Tween 80 (0.05% v/v) and beads were added to the bottle that was subsequently vortexed for 5 min (Sigma Aldrich). A total of 5 μL of each suspension was used to initiate the next selection cycle and the rest was stored at −80°C after adding one volume of a 50% glycerol solution for the “frozen fossil record” (Schoustra et al. 2006, 2009). This procedure was repeated every seven days for seven rounds of selection.

For the treatment where asexual sporulation was not allowed, cultures were initiated with spores at one end of a race tube and allowed to grow undisturbed for seven weeks. A race tube is a 50 mL disposable pipette filled with 25 mL MEA medium, and for the azole treatment supplemented with 1 μg/mL of one of each of the azole fungicides, solidified horizontally, in which the mycelial growth can be tracked. The race tubes were then incubated in a 37°C incubator for seven weeks. Without disturbing the race tubes (so that the asexual spores produced behind the growth front of the mycelia could not contribute to the development of azole resistance), the growth front of the fungi was marked every week. After seven weeks of continued mycelial growth, the race tubes were opened at the weekly markings to harvest material...
RESISTANCE TESTING OF CULTURES EVOLVED WITH AND WITHOUT ASEXUAL SPORULATION AGAINST AZOLE FUNGICIDES

The resistance level of all lineages that had evolved in the presence or absence of azole fungicides over seven weeks was tested. Half the cultures had gone through seven weekly cycles of asexual sporulation, whereas the other half had grown by seven weeks of mycelial extension continuously. Resistance level was defined as the minimal inhibitory concentration (MIC) of the azole fungicide that results in 100% growth inhibition of fungal growth. The relative MIC is defined as the average MIC divided by the MIC of the azole fungicide sensitive ancestor *A. fumigatus* CBS 140053. The resistance test was performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method (Lass-Flörl et al. 2008): RPMI-1640-2% glucose medium (Sigma Aldrich) was supplemented with bromuconazole, tebuconazole, epoxiconazole, difenoconazole, or propiconazole, ranging from 0.016 to 16 mg/L in twofold concentration steps and loaded into 96-well plates. Spore suspensions used to initiate the assay were prepared in saline supplemented with Tween 80 (0.05% v/v) and counted on a Coulter counter (Beckman Coulter, the Netherlands) to standardize the concentration of spores to 1–2.5 × 10^5 CFU/mL (Pos et al. 1988). The standardized spore suspension was inoculated into flat-bottomed 96-well microtiter plates containing a series with increasing amounts of fungicide in RPMI-1640-2% G medium, and incubated at 37 °C for 48 h. Readings from the bottom of the microtiter plates were performed using a mirror to determine at which concentration we observed 100% inhibition, resulting in the MIC value estimates (Camps et al. 2012). Six replicates for each assay were used. We (arbitrarily) classified the MIC as 32 mg/L when we observed no inhibition at the concentration of 16 mg/L.

MGR OF CULTURES EVOLVED WITH AND WITHOUT ASEXUAL SPORULATION

The MGR of all cultures that had evolved under laboratory conditions in the presence or absence of azole fungicides over seven weeks was assayed. Spore suspensions of all evolved cultures were inoculated onto petri dishes with solid MEA medium containing 1 µg/mL of the azole fungicide that was used during the evolution experiment, either with an inoculation needle or with a 5 µL spore suspension. After four days of incubation at 37 °C, the MGR was determined by averaging the colony diameters (in mm) as measured in two randomly chosen perpendicular directions. The relative MGR is defined as the MGR divided by the colony diameter of the azole fungicides sensitive ancestor *A. fumigatus* CBS 140053, grown on MEA medium without azole fungicides (De Visser et al. 1997; Schoustra et al. 2006; Schoustra et al. 2009).

DOMINANCE TEST OF RESISTANCE MUTATIONS IN DIPLOIDS AND HETEROKARYONS OF ONE OF THE EVOLUTIONARY LINEAGES

*Aspergillus fumigatus* predominantly grows as a haploid. Whether azole resistance mutations were dominant or recessive in a heterozygous diploid as well as in a heterokaryon (mycelium containing multiple types of nuclei) was investigated from one of the evolutionary lineages. Spontaneous nitrate nonutilizing mutations *nia* (nitrate nonutilizing) and *cnx* (nitrate and hypoxanthine nonutilizing) were selected on the basis of chlorate resistance as selective complementing markers to facilitate heterokaryon and diploid construction (Cove 1976; Debet et al. 1990). Heterokaryons and diploids were constructed using standard methods: an azole-sensitive *nia*-strain and a highly azole-resistant *cnx*-strain (D1-7°, derived from the end culture evolved in the presence of difenoconazole) were used for selection of heterokaryons on minimal medium (MM) with nitrate as the only N-source. Asexual spores from heterokaryons were harvested and plated in sandwich plates to select for heterozygous diploids (Todd et al. 2007). We found a frequency of heterozygous diploid spores of approximately 10^-5 . Diploidy was verified by measuring the size of conidiospores—the diameter of spores of diploids is 3.2 µm (±0.2 SEM), which is 1.45 times that of haploids (2.2 µm ±0.2SEM)—the volume of diploid spores thus being three times that of haploids. The growth rates of the azole-sensitive *nia* mutant and the highly azole-resistant evolved strain D1-7° (cnx) were tested on MM 5 mM urea with 1 µg/mL concentration of difenoconazole. The level of resistance was measured as the relative MGR (see above).

STABILITY TEST OF AZOLE FUNGICIDES OVER THE EVOLUTIONARY EXPERIMENT

To verify whether the five azole fungicides were stable during the seven weeks of experimental evolution, 24 petri dishes with 1 µg/mL of each of the five azole fungicides were prepared. Of these, 21 plates were incubated at 37 °C and the remaining three plates were kept at 4 °C as the control. At the end of each week, three plates were removed from the incubator and stored at 4 °C till the end of week 7, when all plates had been removed. After this, 5 µL of a fresh ancestor *A. fumigatus* CBS 140053 spore suspension was inoculated in the middle of each plate and incubated at 37 °C for five days. The MGR of ancestor on all plates was recorded, and was compared to the MGR of ancestor on three stored control plates.
Table 1. The relative MIC and MGR of evolved cultures with and without asexual sporulation and correlation between relative MIC and MGR in the cultures with asexual sporulation.

<table>
<thead>
<tr>
<th>Azole used during experimental evolution</th>
<th>Endpoint cultures evolved with asexual sporulation</th>
<th>Endpoint cultures evolved without asexual sporulation</th>
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<tbody>
<tr>
<td></td>
<td>Relative MIC (azoles) Average ± SEM</td>
<td>Relative MGR (azoles) Average ± SEM</td>
</tr>
<tr>
<td>Bromuconazole(b)</td>
<td>4.33 ± 1.97</td>
<td>1.51 ± 0.20</td>
</tr>
<tr>
<td>Difenconazole(d)</td>
<td>4.63 ± 1.63</td>
<td>1.85 ± 0.24</td>
</tr>
<tr>
<td>Epoxiconazole(e)</td>
<td>3.33 ± 2.42</td>
<td>1.53 ± 0.23</td>
</tr>
<tr>
<td>Propiconazole(p)</td>
<td>2.33 ± 0.82</td>
<td>1.23 ± 0.18</td>
</tr>
<tr>
<td>Tebuconazole(t)</td>
<td>3.67 ± 0.81</td>
<td>1.46 ± 0.33</td>
</tr>
<tr>
<td>No azole (control)</td>
<td>1.00 ± 0.00</td>
<td>0.98 ± 0.05</td>
</tr>
</tbody>
</table>

1Six biological replicates.

STATISTICAL ANALYSIS

We used a general linear model (analysis of variance; ANOVA) that included the fixed factors “sporulation treatment” and “azole fungicide,” and their interaction, to explain the observed variation in relative MGR and relative MIC of the evolved strains (model: relative MGR ~ azole fungicide (A), sporulation treatment (S), A × S; relative MIC ~ azole fungicide (A), sporulation treatment (S), A × S). Furthermore, because the effects of the sporulation treatment depended on the azole fungicide (see Results), we performed one-way ANOVAs to test for the effect of sporulation treatment for each azole separately (model for each azole separately: relative MGR ~ sporulation treatment and relative MIC ~ sporulation treatment). Finally, a one-way ANOVA and following post-hoc Fisher’s least significant difference (LSD) tests were used to check whether the achieved level of resistance depended on the azole fungicide used for selection. This was done separately for each sporulation treatment, using the following model (relative MGR ~ azole treatment, and relative MIC ~ azole treatment). A one-way ANOVA and following post-hoc LSD tests were also used to test the difference in the induced level among five azole fungicides, for the difference in the MGR of diploid, haploid, and heterokaryons.

Results

ASEXUAL SPOORULATION ENHANCED RESISTANCE LEVEL OVER EVOLUTIONARY TIME

The resistance level was assayed of all lineages that had evolved under laboratory conditions in the presence or absence of an azole fungicide over seven weeks. Half the cultures had gone through seven cycles of asexual sporulation, whereas the other half had grown continuously by mycelial extension. Five different azole fungicides were applied. Figure 2A, B shows the average resistance level (defined as the relative MIC, of a given azole relative to the MIC of the ancestor) and evolutionary dynamics of the average of the six replicate lineages within each treatment. Our statistical analysis showed that the sporulation treatment is a key factor ($F_{1,50} = 31.696$, $P < 0.0001$; Table S1A) in explaining the differences in the relative MIC between evolved strains and that these differences also depended on which azole fungicide was used (Sporulation treatment × Azole fungicide interaction, $F_{4,50} = 2.674$, $P < 0.05$; Table S1A). When testing for the effect of the sporulation treatment for each azole separately, we found that for bromuconazole and difenoconazole, the relative MIC of cultures that had evolved with sporulation was significantly higher than the relative MIC of cultures without sporulation (for bromuconazole $F_{1,10} = 8.448$, $P < 0.05$; for difenoconazole $F_{1,10} = 22.727$, $P < 0.01$; Table S1B).

For the cultures that evolved in the presence of fungicide and with asexual sporulation, we found that the relative MIC values significantly increased at the end of our experiment compared with the treatment without azoles (control treatment) (ANOVA, $F_{5,30} = 4.854$; $P < 0.05$; post-hoc LSD test, $P < 0.05$; except propiconazole, $P > 0.05$; Table S1C). The relative MIC values started to increase right at the first cycle and leveled off around the sixth week (Fig. 2A). The overall average increase in relative MIC over all fungicides was $3.67 ± 0.90$ SEM, with the increase depending on which azole was used for resistance selection (Table 1).

For the cultures that evolved without asexual sporulation, exposure to azole fungicides also increased the relative MIC values compared to control lineages growing in the absence of azole fungicides (ANOVA, $F_{5,30} = 8.417$, $P < 0.001$; post-hoc LSD test, $P < 0.01$; except for difenoconazole and epoxiconazole, $P > 0.05$; Fig. 2, Table S1D). The relative MIC value started to increase at the first week and then all remained stable.
Figure 2. Relative MIC value (minimal inhibitory concentration corresponding to the level of resistance) and relative mycelial growth rate (MGR) of azole fungicides for 504 evolved cultures with and without asexual sporulation compared to the ancestor in the presence of one of five azole fungicides (concentration: 1 µg/mL) (b, bromuconazole; t, tebuconazole; e, epoxiconazole; d, difenoconazole and p, propiconazole; c, control: no fungicide. Lines show the average of six parallel lineages for each treatment). Error bars indicate the standard error of the mean (SEM).

(A, B) The relative MIC of cultures with and without asexual sporulation. (C, D). The relative MGR of cultures with and without asexual sporulation.

during the following six weeks. The average increase in relative MIC over all fungicides was 1.86 ± 0.56 SEM, with again the increase depending on which azole was used for resistance selection (Table 1). Crucially, although the azole resistance levels also increased in the treatment without sporulation, the average relative MIC value for cultures with asexual sporulation was twice as high as that of the cultures without asexual sporulation.

When analyzing the absolute MIC values of the evolved strains, we reach the same conclusion that asexual sporulation results in higher azole resistance levels; data and related discussion are in Figure S2.

**ASEXUAL SPORULATION INCREASED MGR AND CORRELATES WITH RESISTANCE LEVEL INCREASE**

Relative MGR, as a reliable measure for overall fitness in filamentous fungi, was assayed for all cultures that had evolved under laboratory conditions in the presence or absence of azole fungicides over seven weeks.

Figure 2C, D shows the MGR changes and evolutionary dynamics averaged over the six replicate lineages within each treatment. Statistical analysis revealed that asexual sporulation and azole type both are significant factors for explaining the differences in relative MGR and MIC (sporulation treatment: $F_{1, 50} = 94.402, P < 0.001$; azole treatment: $F_{4, 50} = 8.471, P < 0.001$). Similar to our analysis for relative MIC, there is a significant interaction between the two factors ($F_{4, 50} = 3.509, P < 0.05$; Table S1E). In the model testing the effect of sporulation treatment for each azole separately, the relative MGR of cultures evolved with asexual sporulation is significantly higher than the relative MGR of cultures without asexual sporulation (one-way ANOVA, all $P < 0.01$, except tebuconazole; Table S1F).

For the cultures that were allowed asexual sporulation, it was found that the relative MGR significantly increased at the end of the seven weeks (Fig. 2C) compared to the control (ANOVA, $F_{5, 30} = 11.267, P < 0.001$; post-hoc LSD test, all $P < 0.01$; except propiconazole, $P > 0.05$; Table S1G). The overall average increase in relative MGR over all fungicides was 1.52 ± 0.05. The relative MGR started to increase at the first cycle and appeared to level off around the sixth cycle. As reported for the MIC data, the increase in relative MGR depended on which azole was used for resistance selection (Table 1).

For the relative MGR of cultures that evolved without asexual sporulation, it was found that the relative MGR significantly changed over the course of the experiment (ANOVA, $F_{5, 30} = 9.411, P < 0.001$; post-hoc LSD test showed a significant effect of
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Figure 3. Relative MGR values of azole fungicides for evolved cultures with asexual sporulation in the presence of one of five different azole fungicides (b, bromuconazole; t, tebuconazole; e, epoxiconazole; d, difenoconazole and p, propiconazole; c, control: no fungicide). Bars show averages of six replicate lineages. Error bars show indicate the standard error of the mean (SEM).

When analyzing the absolute MGR values of the evolved strains, we reach the same conclusion that asexual sporulation results in higher azole resistance levels; data and related discussion are given in Figure S2.

In order to verify that increases in both MIC and MGR were due to the actual fixation of mutations, a sexual cross was performed between the ancestral genotype and an evolved strain with high MIC and MGR. The results show a clear Mendelian segregation of fitness among the progeny, confirming that fitness increase is due to the fixation of mutations (Fig. S3).

INCOMPLETE DOMINANCE OF RESISTANCE MUTATIONS IN DIPLOIDS AND HETEROKARYONS

Aspergillus fumigatus normally grows as a vegetative haploid, although vegetative diploids also exist at very low frequency. The dominance level of azole resistance mutations in one of the evolutionary lineages was assayed in heterozygous diploids as well as in heterokaryons. The relative MGR of the haploid-sensitive strain, the highly resistant haploid evolved strain, the heterokaryon, and the heterozygous diploid was assayed on medium with 1 μg/mL of difenoconazole (Fig. 4). The relative MGR values (± SEM over three replicates) of sensitive A. fumigatus CBS 140053, resistant D1-7, diploid, and heterokaryon were, respectively, 0.17 ± 0.007; 0.43 ± 0.012; 0.33 ± 0.0024; 0.36 ± 0.0027. The relative MGR differed significantly among the different strains (ANOVA $F_{3, 8} = 3749.663$, $P < 0.001$; post-hoc LSD test: all pair-wise contrasts: $P < 0.001$). The growth of the heterozygous diploid was 78.5% of that of the resistant strain, whereas the growth of the heterokaryon was 83.7% of the resistant strain. Both the phenotype of the diploid and the heterokaryon indicated that the mutation is incomplete dominant.
STABILITY TEST OF AZOLES FUNGICIDES OVER THE EVOLUTIONARY EXPERIMENT

The stability of the five azole fungicides during experimental evolution was tested by growing the ancestor on media with azoles that was first stored for up to seven weeks at 37°C. No significant differences in relative MGR were found between fungus inoculated on these preincubated media and those on the freshly prepared media (ANOVA, \(F_{7,120} = 0.442, P = 0.874\); Fig. S4). This indicates that all five azole fungicides were stable during the seven weeks of our experimental evolution.

Discussion

ASEXUAL SPORULATION RESULTS IN RAPID EVOLUTION OF HIGH RESISTANCE TO AZOLE FUNGICIDES IN A. FUMIGATUS

We evaluated whether asexual sporulation promotes adaptation to an azole-fungicide environment in A. fumigatus, that is, the development of resistance. We contrasted the development of resistance to five different azole fungicides between populations that went through repeated rounds of asexual sporulation with continued multicellular growth without asexual sporulation. We found clear differences in the achieved levels of resistance between cultures that had evolved with or without asexual sporulation, sporulation leading to roughly twice as high resistance levels. Generally, the relative MIC correlated well with relative MGR for all cultures. Evolutionary trajectories of cultures with asexual sporulation showed multiple steps of increase in both relative MIC and MGR, which may be indicative of the successive fixation of multiple mutations (Lenski and Travisano 1994; Schoustra et al. 2009), although exceptions have recently been reported (Lang et al. 2013). In contrast, the resistance level of the cultures growing without asexual sporulation did not further increase after taking a first (mutational) step.

EVOLUTIONARY MECHANISMS UNDERPINNING THE EMERGENCE OF AZOLE RESISTANCE

Our results show that the resistance level to azoles gradually increases when the culture goes through asexual sporulation, which suggests that asexual sporulation contributes to, and promotes, the evolution of resistance. This may be explained either by more efficient selection for resistance and/or a higher mutation supply in the sporulating cultures.

Asexual sporulation in A. fumigatus enables the phenotypic expression of the resistance by providing an alternation between multicellular and unicellular stages. This alternation provides the opportunity for mutations to be released from the multicellular mycelium and to fully express its phenotype into a newly formed mycelium founded by that spore. Life cycles with both multicellular and unicellular phases are widespread in animals, plants, and some fungi (Maynard Smith 1988; Anderson 1992; Kondrashov 1994b; Michod 2007). Grosberg and Strathmann (1998) argued that the multicellular stage reduces genetic variability and leads to inefficient selection compared to the unicellular stage (Grosberg and Strathmann 1998). For instance, the unicellular stage in the life cycle of multicellular organisms allows for the purging of deleterious mutations by exposing the individual gametes as well as the resulting zygote to selection, while at the same time this unicellular stage reduces conflicts of interest among genetically different replicators (e.g., mitochondria) within a multicellular organism (Grosberg and Strathmann 1998; Bastiaans et al. 2014). Moreover, the alternation of multicellular and unicellular stages also promotes the selection of beneficial mutations. In the case of A. fumigatus, this alternation includes a uninucleate spore stage after which a new multicellular mycelium will form. This will reduce variability in the total number of mutations per individual by either removing unique mutations or by completely fixing them in a culture.

Following this logic, we can argue that the asexual sporulation process released the mutations from the multicellular mycelium to allow for efficient selection and expression of beneficial traits. Blocking sporulation could thus reduce evolvability (Kondrashov 1994b, a). This aligns with the hypothesis that asexual sporulation is essential for phenotypic expression of azole resistance mutations in A. fumigatus. During continued mycelium growth, individual resistant nuclei may arise but these are outnumbered by sensitive nuclei. The resistant nuclei can only escape through asexual sporulation after which they end up in unicellular resistant spores that may then give rise to a resistant mycelium. Thus, exclusive mycelial growth (without asexual sporulation) prevents the emergence of resistance in A. fumigatus, at least over timescales that are relevant for the treatment of patients.

The mechanism of release of resistant nuclei from a sensitive mycelium will work most efficiently when the azole resistance mutation is recessive and therefore needs homokaryon formation for the full expression of the resistance. Therefore, we assayed whether azole resistance mutations from one of the evolutionary lineages are dominant or recessive. We constructed heterozygous diploids and heterokaryons and compared the resistance level with the haploid-sensitive and -resistant strains. The phenotype of both diploid and heterokaryon indicated that the mutation showed incomplete dominance. Thus, homokaryon formation by asexual sporulation will result in the full expression of the azole-resistant genotype, and will hence enhance the spread of the mutation(s). The extent of this benefit for resistance mutations will be dependent on the selection pressure imposed by the (azole) environment. When high concentrations of azoles are used, selection will be stronger on the resistant phenotype, and thus the benefit to a full expression of the phenotype will be higher. The significance of the contribution of the sporulation process to the emergence of azole
resistance is thus expected to depend on the precise application of the azoles in clinical and agricultural settings.

Furthermore, in terms of spores contributing to azole resistance development, asexual sporulation greatly increases the effective population size allowing for many more possible mutants to be exposed to selection and also increases the mutation supply through the additional mitoses required to generate all the spores. Typically after one week of growth, a colony of *A. fumigatus* may contain up to $10^9$ spores (Cole 1986; Latgé 1999; Dagnais and Keller 2009; Amorim et al. 2010; Gifford and Schoustra 2013; Gisi 2013). Although the number of nuclei needed to populate the mycelium is limited, upon sporulation, the formation of hundreds of millions of spores on top of the mycelium requires additional mitotic divisions, with the possibility of mutations arising during DNA replication. Therefore, the total number of nuclei produced by mitotic divisions during sporulation by far exceeds that present in the mycelium. As a result, de novo resistance mutations are more likely to occur during asexual sporulation than in the expanding mycelium. The occurrence of mutations has been demonstrated in *Aspergillus* and yeast during both meiosis and mitosis (Magni and Von Borstel 1962; Holliday 1964; Käfer 1977; Esposito et al. 1982). Thus, a large spore production is likely to generate numerous unique genotypes carrying mutations that can be tested by natural selection, for instance in environments with azoles. In addition, resistance mutations are more likely to be selected from asexual sporulation due to the single-celled nature of the spores that removes the burden of the (partial) recessivity of the mutations that shields the full expression of resistance in a multicellular mycelium.

**Other potential resistance mechanisms**

Aneuploidy is well known to underlie azole resistance in both *Candida* and *Cryptococcus*, the two other most common systemic human fungal pathogens (Selmecki et al. 2006; Sionov et al. 2010; Ni et al. 2013). We feel we can exclude aneuploidy as an explanation for emerging resistance in *A. fumigatus* because, so far, no aneuploidy has been found in *A. fumigatus*. Aneuploidy in *A. nidulans* has been associated with mitotic instability and abnormal low-fitness phenotypes (Kafer and Upshall 1973), and also in *A. niger* stable aneuploids have never been observed (Debets et al. 1993). Furthermore, in some reports, aneuploidy in pathogenic fungi such as *Candida* was linked to the emergence of drug resistance, but was limited to fluconazole therapy. The prevalence of azole-associated aneuploidy in these fungi appears not only to be due to increased azole therapy but also to the high plasticity of their genomes (Selmecki et al. 2010; Sionov et al. 2010; Kwon-Chung and Chang 2012). In contrast to the plastic *Candida* genome, the structure of the *Aspergillus* genomes appears rather stable (Gibbons and Rokas 2013). Further evidence that the observed resistance is due to changes in the DNA (mutations) rather than ploidy change comes from the observation of step-wise increases in resistance in the evolutionary trajectories. This is further supported by the results from a sexual cross of the ancestor and a resistant evolved strain, showing clear segregation of the resistant phenotype among the progeny (Fig. S3).

More generally, other parts of the *A. fumigatus* life cycle could potentially provide alternative or additional ways to generate adaptive variation through the occurrence of recombination during the sexual and parasexual cycle (O’Gorman et al. 2008; Ene and Bennett 2014). Thus, whereas the abundant asexual reproduction may be significant for mutation supply, the much less common sexual and parasexual life cycle in nature could induce diversity through recombination (O’Gorman et al. 2008; Heitman et al. 2014). Furthermore, the parasexual life cycle also could promote adaptation by changing ploidy, as it appears to do in *A. nidulans* (Schoustra et al. 2007; Anderson et al. 2015). These possibilities provide interesting avenues for future research.

**A SCENARIO FOR THE EVOLUTION OF RESISTANCE IN A. FUMIGATUS**

Based on the above reasoning, azole resistance in *A. fumigatus* could have evolved through the following scenario (Fig. 5). All cultures were started with a conidiospore sampled from an ancestral azole-sensitive colony that either may already contain rare random beneficial and detrimental mutations, or that developed mutations during asexual sporulation. This idea is supported by the fact that we found the same resistance levels (MIC value) after the first week of fungicide exposure of all cultures. After this first week, however, continued mycelial cultures did not further increase in resistance because potential beneficial mutations that appeared, for instance, during asexual sporulation on top of the mycelium were not able to spread and thus not available for selection.

In contrast, in the treatment including sporulation, all initial and subsequent mutations that occurred during sporulation could fix in those cultures and contribute to further resistance development, as seen in the stepwise increases in resistance. Continued mycelial growth ignored all these potential second and higher step mutations, because these mutations were less likely to happen in the first place, are at least partially recessive, and were trapped in a predominantly nonresistant mycelium, resulting in lower final levels of resistance at the phenotype level.

Asexual sporulation is a common reproductive mode for a diverse group of fungi that includes many medically, industrially, and agriculturally important species (Springer 1993; Adams et al. 1998; Mah and Yu 2006; Metz et al. 2011). Asexual sporulation is a conidiation process and a primary means of dispersion (Mah and Yu 2006; Metz et al. 2011) and can also occur within the lungs of patients (Franquet et al. 2001). In our study, we illustrate
Figure 5. An evolutionary model for the development of azole resistance in A. fumigatus cultures. Upper panel: The cultures allowing for serial asexual sporulation (transfer of asexual spores produced by asexual sporulation). Lower panel: The cultures without asexual sporulation (without a role for asexual spores). Yellow line: medium with azole fungicides; red dots: nuclei carrying beneficial mutations; blue dots: nuclei carrying detrimental mutations. From pink to dark red: resistance level increase. Note that asexual spores are produced in both modes of culturing, but in the lower panel (without asexual sporulation) the asexual spores produced do not germinate or contribute to the development of azole resistance. Moreover, as the hypha contain multiple nuclei, resistance via mycelial growth alone is unlikely to emerge over relevant time scales, such as during patient treatment.

RELEVANCE FOR AZOLE RESISTANCE DEVELOPMENT

Our results indicate that the risk of azole resistance selection in A. fumigatus depends on the selection pressure—that is, which specific azole was used—in an environment that allows asexual sporulation. A recent assessment by Gisi, indicated that high concentrations of azole fungicides are used for specific applications, including bulb dipping and protection of materials, such as wood (Gisi 2013). The risk of azole resistance selection related to these applications, however, depends on the presence and exposure of sporulating A. fumigatus, which remains to be investigated. The second major application that, according to these principles, carries a high risk of resistance selection is azole therapy in animals and humans (Gisi 2013) as also azole therapy involves high exposure levels at the site of infection. Asexual sporulation of A. fumigatus was found in animal and human lungs with cavitary Aspergillus diseases such as chronic cavitary aspergillosis and aspergilloma, where vegetative hyphae are exposed to air (Franquet et al. 2001) (Adams et al. 1998). Clinical case series indeed show that resistance emerges exclusively when both conditions are present: during azole therapy of patients with cavitary lesions (Campis et al. 2012). In contrast, azole resistance selection during azole therapy has not been reported in patients with invasive aspergillosis (Dannaoui et al. 2004). Despite high azole exposure, the risk of azole resistance development in these patients appears much lower. Our results now support the interpretation that this is due to the absence of asexual sporulation in the patients.

Nevertheless, azole resistance has been reported in patients with invasive aspergillosis. Patients are believed to inhale already azole-resistant spores that then colonize the patient, rather than that the fungus with which they are infected develops resistance during azole therapy (Verweij et al. 2013). It indicates that these azole-resistant unicellular spores are more adapted to the patients’ “ecosystem” than multicellular mycelium, which strongly supports the view that a regular unicellular life stage could promote the removal of deleterious mutations and the selection of beneficial mutations. Overall, it is essential that future research focuses on testing the role of asexual reproduction of the fungus in patients exposed to medical triazoles, as this may lead to new management strategies that avoid or overcome resistance selection.

Different azole fungicides have different activity against the ancestor (Fig. S4, MGR(b) = 3.9 ± 0.05 mm/day; MGR(d) = 3.7 ± 0.05 mm/day; MGR(e) = 4.2 ± 0.08 mm/day; MGR(p) = 5.6 ± 0.11 mm/day; MGR(t) = 4.2 ± 0.11 mm/day; and MIC(b)
(d) = 2; (e) = 8; (p) = 8; (t) = 4. The initial fitness of the ancestor to five azole fungicides was inversely proportional to the rate of adaptation of the ancestor to five azole fungicides (Fig. S5), which may explain the differences in the selection level of the different fungicide azoles against A. fumigatus. Azole resistance in our lineages is incomplete dominant, and explaining this observation requires knowledge of the mechanism(s) involved. From the evolutionary trajectory and sexual cross analyses, it appears that the resistant strain contained two mutations (Fig. S3). Epistatic interaction between these mutations may explain the incomplete dominance. In relation to this, it would be of interest to test the predominant highly resistant mutant TR34/L98H found in hospitals for dominance.

The clear correlation between MIC and MGR is also useful for future studies on the development of resistance in fungi. For susceptibility testing, the MIC value of azoles against Aspergillus is influenced by variable factors such as inoculum size, endpoint, and reading time, which can easily result in a one to two wells difference in the MIC interpretation (Gehrt et al. 1995; Llop et al. 2000; Espinel-Ingroff et al. 2001; Albarrag et al. 2011). Also it is difficult to detect relatively small differences in the level of resistance. In contrast, MGR is much more accurate and easier to measure and can thus be used as a reliable proxy to determine the resistance level. However, for the cultures of the treatment without asexual sporulation, we did not find a correlation between MIC and MGR, which is likely explained by the lower levels of MIC and MGR increase during the evolution experiment. Probably there should be random mutations that affect MIC but not MGR at one particular level of drug.

Conclusions and Future Outlook

Our results demonstrate that the full life cycle of A. fumigatus needs to be taken into account to explain the emergence and possible persistence of azole resistance. In addition, the selection pressure appears to be a key factor. As we observed differences in selection pressure for the various azole compounds, the implications of both the dose and the molecule structure should be considered in future studies. Although knowing the exact identity of the adaptive mutations in A. fumigatus was not required to address the hypotheses in this article, it will be an essential avenue of future research, which will further unravel the evolutionary dynamics of A. fumigatus adaptation both in the human setting and in the environment. Understanding the key factors that facilitate resistance selection in A. fumigatus is essential to design strategies that prevent or overcome this emerging threat.

Acknowledgments

We gratefully acknowledge funding from the China Scholarship Council to JZ and a Marie Curie Fellowship to SES (FP7-PEOPLE-2012-IOF-328888). We thank B. Koopmanschap and M. Slakhorst for technical assistance. We further thank D. Aanen and A. de Visser (Wageningen University) for discussion.

Data Archiving

The doi for our data is 10.5061/dryad.tk43n.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1A. Results of an analysis of variance (ANOVA) for the relative MIC data with type of azole (Azole) and sporulation treatment (Sporulation) as fixed factors (relative MIC ~ azole treatment × sporulation treatment; *P values lower than 0.05; ††P values lower than 0.01.
Table S1B. Results of an analysis of variance (ANOVA) for the relative MIC data with sporulation treatment (Sporulation) as a fixed factor (model for each azole separately: relative MIC ~ sporulation treatment; *P values lower than 0.05; ††P values lower than 0.01.
Table S1C. Results of an analysis of variance (ANOVA) and following post-hoc LSD test for the relative MIC data with azole treatment as a fixed factor for the data with sporulation to test which azole treatment leads to a different MIC than the treatment without azoles (model: relative MIC ~ azole treatment; *P values lower than 0.05; ††P values lower than 0.01.
Table S1D. Results of an analysis of variance (ANOVA) and following post-hoc LSD test for the relative MIC data with azole treatment as a fixed factor for the data without sporulation to test which azole treatment leads to a different MIC than the treatment without azoles (model: relative MIC ~ azole treatment; *P values lower than 0.05; ††P values lower than 0.01.
Table S1E. Results of an analysis of variance (ANOVA) for the relative MGR data with type of azole (Azole) and sporulation growth treatment (Sporulation) as fixed factors (relative MGR ~ azole treatment × sporulation treatment; *P values lower than 0.05; ††P values lower than 0.01.
Table S1F. Results of an analysis of variance (ANOVA) for the relative MGR data with sporulation treatment (Sporulation) as a fixed factor (model for each azole separately: relative MGR ~ sporulation treatment; *P values lower than 0.05; ††P values lower than 0.01.
Table S1G. Results of an analysis of variance (ANOVA) and following post-hoc LSD test for the relative MGR data with azole treatment (Azole) as a fixed factor for the data with sporulation to test which azole treatment leads to a different MGR than the treatment without azoles (model: relative MGR ~ azole treatment; *P values lower than 0.05; ††P values lower than 0.01.
Table S1H. Results of an Analysis of Variance (ANOVA) and following post-hoc LSD test for the relative MGR data with azole treatment (Azole) as a fixed factor for the data without sporulation to test which azole treatment leads to a different MIC than the treatment without azoles (model: relative MGR ~ azole treatment; *P values lower than 0.05; ††P values lower than 0.01.
Table S1I. Results of difference in the induced level between five different azole fungicides by an analysis of variance (ANOVA) and following post-hoc LSD test for the relative MGR data of cultures with sporulation with different azole treatment (model: relative MGR ~ azole treatment; *P values lower than 0.05; ††P values lower than 0.01.

Figure S2. Absolute MIC increase (MIC values of evolved strains with the value of the ancestor subtracted) and absolute increase in mycelial growth rate (MGR) for 504 evolved cultures with and without asexual sporulation compared to ancestor in the presence of one of five fungicides (concentration: 1 μg/mL). b, bromuconazole; t, tebuconazole; e, epoxiconazole; d, difenoconazole; p, propiconazole; c, control, no fungicide. Lines show the average of six parallel lineages for each treatment. Error bars indicate the standard error of the mean (SEM).
Figure S3. Segregation of MGR among the progeny from a sexual cross between the ancestral genotype (parental type 1; P1) and an evolved strain (parental type 2; P2) with high MIC and fitness.
Figure S4. The mycelial growth rates of the ancestors on plates with 1 μg/mL of one of each of five azoles fungicides that had been incubated for different amounts of time 37 °C prior to inoculation with fungi.
Figure S5. The relationship between the initial fitness of the ancestor in the azole environment and the rate of adaptation of ancestor to azole fungicides.