THE POPULATION STRUCTURE OF AZOLE-RESISTANT *ASPERGILLUS FUMIGATUS* PRESENT ON COMMERCIAL AGRICULTURAL PRODUCTS

by

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ABSTRACT

Aspergillus fumigatus is the primary causal agent of the respiratory illness aspergillosis. Resistance to triazoles has been developing as a result of their use in clinical and agricultural environments. The goal of this research is to characterize triazole resistance in *A. fumigatus*, particularly in the United States. An extensive review of literature found that two *cyp51A* alleles were found to be primarily responsible for pan-azole-resistant phenotypes throughout the world: TR_{34}/L98H and TR_{46}/Y121F/T289A. These alleles and others were found in *A. fumigatus* primarily isolated from soil, air, and plant debris. Eight types of commercial products were surveyed to identify azole-resistant *A. fumigatus* in the United States. The majority of pan-azole-resistance was found in strains isolated from compost and flower bulbs. DAPC and cluster analysis revealed that 3 clusters are formed from these isolates based on the presence of a *cyp51A* tandem repeat and the T248N/E255D allele.

INDEX WORDS: emerging diseases, aspergillosis, *Aspergillus fumigatus*, antifungal resistance, Sanger sequencing, *cyp51A*, azole resistance, population genetics and genomics, microsatellite markers, population structure.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Fungi are serious pathogens of both humans and plants. One study showed that fungi are the root cause of approximately 65% of animal and plant host extinction events, and that fungi are responsible for approximately 33% of crop losses (1, 2). Another study showed that over 1.5 million people die from fungal infections yearly, and another 150 million are affected by severe invasive fungal diseases (3). These numbers are rising due to increasing numbers of patients afflicted with AIDS, cancer, respiratory illnesses, and those requiring organ transplants (4, 5). One fungal disease that has become more common is invasive aspergillosis caused by *Aspergillus fumigatus* (6). Out of the 1,000,000 patients already at risk every year, approximately 250,000-300,000 will develop invasive aspergillosis (1, 5). The primary class of drugs used for treatment of invasive aspergillosis is triazoles (7). Triazoles inhibit the 14-α-demethylase enzyme (*cyp51A*) in the ergosterol synthesis pathway, causing a depletion of ergosterol from the fungal cell membranes and an increase in toxic sterol byproducts (8). However, an increasing number of patients have presented with pan-azole resistant strains of *A. fumigatus* before treatment with azoles (9). The TR_{34}/L98H and TR_{46}/Y121F/T289A alleles in the *cyp51A* gene encoding 14-α-demethylase are the most common alleles granting pan-azole resistance (9). Several studies have supported that these alleles arose in agricultural settings as a result of long-term use of triazoles to combat plant-pathogenic fungi (10, 11). A recent review paper highlighted that the alleles mentioned above as well as other alleles conveying azole resistance are found in agricultural environments where azoles are commonly used (11). *A. fumigatus* has

also been found on food and commercial products that originate from the environment, such as various nuts, compost, and tulip bulbs (12-16). However, no studies have identified if azole-resistant *A. fumigatus* is present on products in the United States shown to commonly carry *A. fumigatus*, or if products which come from fields in the United States treated with azoles carry azole-resistant *A. fumigatus.* The purpose of this study is to identify if azole-resistant *A. fumigatus* is present on agricultural commercial products commonly treated with azoles in Georgia.

LITERATURE REVIEW

Aspergillus fumigatus

Aspergillus fumigatus is a terrestrial fungus found nearly everywhere in the soil and in the air (17). The fungus is present throughout the world and is known to exist on every continent except for Antarctica (17). *A. fumigatus* mainly survives in the soil as a saprophyte although it is also commonly present in the air. *A. fumigatus* is also known as the most common fungus to cause aspergillosis, an opportunistic fungal disease of humans and animals (4).

This fungus obtains nutrients as a saprophyte by degrading many organic substrates, but primarily plant matter (18). *A. fumigatus* is well equipped to degrade plant cell walls with a genome containing genes which encode cellulases, hemicellulases, pectinases, glycosyl hydrolases, and many other plant cell wall degradative enzymes (18, 19). However, *A. fumigatus* lacks genes that encode proteins able to degrade lignin, which suggests that *A. fumigatus* is not able to survive and reproduce on woody plant material, instead using the digestible material of green plants (18).

A. fumigatus plays a notable role in carbon and nitrogen recycling in soil and compost heaps (18). This fungus can utilize a wide array of nitrogen sources and contributes to the worldwide nitrogen cycle using nitrogen assimilation (20). The ability to metabolize many sources of nitrogen also contributes to its pathogenicity. Studies have shown that mutants deficient in genes used for nitrogen transport lose their ability to be virulent (20). *A. fumigatus* has also been proven to utilize a variety of carbon sources. When mutants lacking the enzymes used for growth on acetate and fatty acids were created, they were found to have severely reduced pathogenicity of animal models (21). This study also found that when deprived of traditional sources of carbon, *A. fumigatus* was able to utilize lipids as a source of carbon intake (21). The versatility of *A. fumigatus* in its ability to degrade different forms of carbon and nitrogen have been viewed as an adaptation to different environments inside hosts, but it also contributes to its survival outside of a host as well.

"*Aspergillus fumigatus* and Aspergillosis" written by Jean-Paul Latgé summarizes the morphological characteristics of *A. fumigatus* (6). *A. fumigatus* spreads by readily producing conidia that are easily made airborne. These conidia are produced on conidiophores which can each hold thousands of conidia. Each conidiophore releases conidia in response to simple environmental disturbances or wind currents. The conidia are green, echinulate, and approximately 2.5-3 µm in diameter. Their small size causes buoyancy and allows them to remain airborne indefinitely once they are released from their conidiophores. The conidia are borne from green phialides, which are 6-8 µm by 2-3 µm in size. The phialides attach directly to swollen, club-shaped vesicles which are 20-30 µm in diameter. Colonies of *A. fumigatus* begin as white and fluffy and turn green over time as sporulation begins (4). Sporulation usually occurs within 36-48 hrs of incubation at 30-37°C (4). The underside of the colonies shows a distinct

puckered star pattern when grown on PDA (personal observation). *Aspergillus* species are very fast-growing fungi, and *A. fumigatus* is the fastest growing species among them. One study found that *A. fumigatus* can grow faster at 40°C than any other airborne fungus (18).

One of the most unique features of *A. fumigatus* is its thermotolerance. *A. fumigatus* can easily grow in temperatures up to 55°C and can tolerate temperatures up to 70 or 75°C (6, 22). It is also able to grow in temperatures as low as 12°C and its conidia can tolerate short periods of freezing (23). *A. fumigatus* expresses features of both thermophilic and thermotolerant fungi because it can grow in temperatures below 20°C and can tolerate temperatures above 60°C, which makes it able to survive and thrive in a variety of different areas (24).

A. fumigatus is most abundant in areas where decomposition increases temperatures, such as in compost piles and waste treatment plants (15). One study found that the concentration of *A. fumigatus* in a compost heap was 10^6 CFU/g compost and that *A. fumigatus* spores made up most of the fungal microflora in the air samples surrounding the compost piles (25). The thermotolerance of *A. fumigatus* also assists in its ability to survive in the human body. Its preferred temperature is 37°C, which coincides perfectly with the average temperature of the human body. *A. fumigatus* has several specific genes that contribute to its ability to survive in extreme environments (26). These genes only have orthologues in bacteria and archaea, suggesting that *A. fumigatus* has a unique thermotolerance not related to that of other fungi (26).

Many studies have found *A. fumigatus* to be prevalent in the outdoor air, compost piles, sewage sludge, woodchips, and commercial potting mixtures, manures, and mulches (15, 17). *A. fumigatus* has also been found on commercial products such as pine nuts (12), pecans (13), walnuts (12, 14), hazelnuts (14), and tulip bulbs (16). *A. fumigatus* has been isolated from fields

that grow commercial products such as peanuts (27, 28), watermelons, grapes, apples, peaches, wheat, herbs, cantaloupe, hemp, corn, soy, and tulips (28).

A. fumigatus is an opportunistic human and animal pathogen. Although its primary ecological niche is as a saprophyte, *A. fumigatus* can cause an infection if the fungus encounters an immunocompromised host. There are many factors which contribute to this species' ability to infect a human or animal host as described by Kwon-Chung and Sugui (23). The small diameter of the conidia allows the fungus to bypass the mucus membranes and cilia in the nose and travel between alveoli into the lower airways. There, the conidia become embedded, germinate, and form hyphae in immunocompromised hosts. *A. fumigatus* is not able to survive if the immune system is fully-functioning for many reasons including its lack of specialized virulence factors (18). However, in immunocompromised individuals, *A. fumigatus* conidia have time to alter their physiology so they can better adapt to the host environment. *A. fumigatus* was found to have differential gene expression which allowed adaptation to low levels of nitrogen, glucose, and iron, alkaline stressors, and other unfavorable conditions (29). This fungus is also known to produce a wide array of secondary metabolites, including but not limited to fumagillin, gliotoxin, fumitremor-gin, verruculogen, fumigaclavine, helvolic acid and sphingofungins (6, 26). A notable secondary metabolite produced is gliotoxin, which has immunosuppressive qualities and has been theorized to serve as a way for *A. fumigatus* to collect nutrients (26, 29, 30). The ubiquity of *A. fumigatus* across the world allows access to many hosts. One survey found that humans around the world can inhale hundreds of *A. fumigatus* conidia on average per day (6).

Aspergillosis

Aspergillosis is a wide spectrum of respiratory conditions caused by the inhalation of spores from *Aspergillus* species (4).The vast majority of cases worldwide (90%) are caused by the thermotolerant fungus *Aspergillus fumigatus*, followed by *Aspergillus flavus* (10% of bronchopulmonary cases), which is mostly reported in Asia, Africa, and the Middle East (6, 31). The most common method of introduction to the body is inhalation into the respiratory tract, but introduction can also occur through other paths such as through damaged skin, cornea, ears, and gastrointestinal tract (4, 6). The immune system of a healthy host can usually clear out *A. fumigatus* spores quickly. Macrophages present in the lungs and nose ingest and kill the spores before germination can occur (32). Cells, which germinate and form hyphae, are destroyed extracellularly by neutrophils (32). However, aspergillosis can still occur in hosts with high immune system function (atopic), normal immune system function (immunocompetent), and low immune system function (immunocompromised). The classification of aspergillosis disease can range from allergic bronchopulmonary aspergillosis (ABPA) to chronic pulmonary aspergillosis (CPA), to invasive aspergillosis (IA), which is the most severe and highly lethal (3, 5, 6). A review by Latgé best describes the different aspergillosis diseases (5).

There are two forms of aspergillosis that commonly affect atopic individuals, or those who are afflicted with allergies: allergic bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitization (SAFS). ABPA is the most severe form of aspergillosis that can affect an atopic patient or a patient with cystic fibrosis, and affects nearly 5 million people annually (3). SAFS is another form of aspergillosis that affects millions of people with asthma a year, and results in chronic asthma that does not respond to steroid treatments. In immunocompetent individuals, aspergillosis most commonly affects patients with tuberculosis or

other cavitary chronic lung diseases. Chronic, noninvasive forms of aspergillosis are all classified as chronic pulmonary aspergillosis (CPA). CPA was estimated to affect 3 million people annually in 2017, but this disease is underrecognized so incidence cannot be accurately estimated (3). Invasive aspergillosis (IA) is the most severe form of aspergillosis, which affects immunocompromised patients, patients under immunosuppressive treatments or those undergoing to a surgery or a transplant. IA is estimated to affect 250,000 people worldwide and the incidence is increasing (3); one study found that IA hospitalizations rose 3% in the United States between 2000 and 2013 (33), and another found that from 2013 to 2017, four times as many patients were admitted for IA per year as in 1996 (34, 35). This is likely due to the rise in immunosuppressive treatments for hematologic malignancies, stem cell transplants, and solid organ transplants, leading to neutropenic patients (6, 33). There has also been a shift in incidences of IA from neutropenic to non-neutropenic patients, such as those admitted for severe viral infections like influenza and Corona Virus 2019 (COVID-19) (36). In cases of IA that do not respond to treatment, mortality rates are between 50% and 100% (37). There are three classes of antifungals available to treat aspergillosis: triazoles, polyenes, and echinocandins, however, aspergillosis diseases are primarily treated using azole antifungals (7).

Azole drugs

Azoles are a class of antifungals that are used both to treat human diseases and plant diseases. The actions of azoles are best described by Georgopapadakou & Walsh (8). Azoles are a broad-spectrum class of fungicides that can be used on both filamentous fungi and yeasts. Azoles work by inhibiting the action of the 14-α-demethylase enzyme during the C14-demethylase stage of ergosterol biosynthesis. The nitrogen ring of the azoles can form a

stoichiometric complex with the heme iron of 14-a-demethylase, immobilizing and inhibiting its action. Binding 14-α-demethylase leads to the depletion of ergosterol and the buildup of lanosterol and other 14-methylated sterols. Ergosterol helps maintain the integrity and fluidity of fungal cell membranes. When ergosterol synthesis is stalled and methylated sterols are introduced, membranes are less stable than their counterparts containing mainly ergosterol. This mode of action is fungistatic, where the growth of fungi is inhibited but the fungi are not killed directly.

Azoles are divided into two different groups: imidazoles and triazoles. Imidazoles are azole drugs whose chemical structure contains two nitrogen rings, and triazoles contain three nitrogen rings (38). Azoles are commonly used in both agriculture as a fungicide and in medical treatment as an antifungal.

Fromtling describes the common uses of triazoles and imidazoles in treating fungal diseases in humans (39). Imidazoles are primarily used as topical antifungals. However, imidazoles are used less commonly than triazoles. Some imidazoles are limited in what they can be used for because of their side effects, potency, solubility, or spectrum of activity. In clinical settings, triazoles are more widely used for systemic infections because they have a reduced toxicity and a broader range of activities. Some examples of triazole drugs are fluconazole, itraconazole, isavuconazole, voriconazole, and posaconazole. Fluconazole is mainly used for the treatment of invasive yeast diseases (40). Voriconazole, isavuconazole, and posaconazole are used to treat invasive aspergillosis (7). Itraconazole is now mainly used for treatment of skin and nail fungal infections by dematiaceous fungi, but it was used primarily for invasive aspergillosis infections in the past (7, 40). Voriconazole is the main triazole that is recommended to treat aspergillosis, with supplementary treatments using isavuconazole (41). Posaconazole and

itraconazole are only recommended for use after all other therapies have failed as of 2016 (41). Itraconazole, voriconazole, and posaconazole are also approved for prophylaxis treatment in patients at high risk of fungal infections (41). Azole drugs have been shown to cause severe side effects. Benitez and Carter best described the results of long-term use of azole therapy (42). Long-term use of azoles has been reported to cause hepatotoxicity and some hormonal changes. Voriconazole specifically has been linked to causing pancreatitis, peripheral neuropathies, periostitis, phototoxic reactions, and squamous cell carcinoma. However, pre-existing illnesses in the patients make it difficult for health care professionals to differentiate which symptoms are caused by the illness being treated and which are caused by their antifungal medication.

Azoles are also used in agriculture. Examples of environmental triazoles are propiconazole and tebuconazole (43). Azoles in agriculture are used far more frequently in Europe than in the United States, where only 5% of total crop areas are treated using an azole fungicide (44). However, azole use on crops in the United States has been increasing. Tetraconazole use in the United States has spread from being used strictly in the northern plains in 1999 to being distributed through the California Central Valley, the upper Midwest, and the Southeast as of 2014 (45). Tebuconazole use in the United States has increased from less than 0.5 million pounds in 2000 to 2.0 million pounds in 2016 (45). Propiconazole use has also increased from just over 0.5 million pounds in 2000 to 2.5 million pounds in 2016 (45). Azoles are the fungicide of choice for some crop producers across the United States for reasons described by Hof (46). Azoles are inexpensive when compared to other fungicides with the same mode of action. Azoles have a broad spectrum of activity which allows them to be used to treat a wide variety of plant diseases such as powdery and downy mildews, rusts, leaf spots, and flower blights (44, 47). They also can be applied to crops during different stages of infection because of

their systemic fungistatic effect on the fungi. They are labeled and distributed as both plant protectants and for post-infection use. Azoles are also incredibly stable molecules and can resist changes to their molecular structure. This allows them to persist in the environment for a long time, which decreases the chance of recurring infections to crops.

The most widely used azoles in the United States are tebuconazole and propiconazole (45). Tebuconazole has been approved to prevent infection by many fungal pathogens on a variety of different crops, including but not limited to cereals, ornamentals, vegetables, fruits, and grape vineyards (48). Propiconazole is also licensed for use in the US on many different crops, most notably peanuts, pecans, almonds, and tree nuts (49).

Mechanisms of azole resistance

Azole-resistant *A. fumigatus* was first discovered in the clinic in 1997 when analyzing isolates collected in the 1980s from patients who had received long-term itraconazole therapy. (50). A later study confirmed that itraconazole resistance can indeed develop as a result of azole therapy in patients (51). Azole resistance that develops in the clinic is generally caused by nonsynonymous nucleotide substitutions in the gene *cyp51A* leading to amino acid changes such as G54E, P216L, and F219I (52). Several nucleotide positions have been categorized as hotspots for the development of azole-resistance: 54, 98, and 220 (53). These single nucleotide polymorphisms (SNPs) in *cyp51A* are able to convey azole-resistance by causing changes in the 14-α-demethylase azole-binding site so that azoles have a lower affinity for the enzyme. It is important to note that these SNPs likely arose independently in patients because aspergillosis is not able to be transferred between patients (52, 53).

Patients who have undergone long-term treatment of azoles are most likely to serve as breeding grounds for these SNPs. This is because the use of azoles leads to a high selection for azole resistance and the high temperatures of the human body are conducive to an abundance of asexual growth and reproduction (9, 52, 53). Patients who have either aspergillomas or aspergillosis in cavity lesions are even further prone to resistance development because growth in the open space in their lungs leads to reproduction via sporulation rather than by hyphal growth. This increases the chance for spontaneous mutations to occur because more incidences of reproduction occur to produce spores (54-56).

However, pan-azole-resistant strains of *A. fumigatus*, which are resistant to multiple azole drugs, were detected in azole-naïve patients with aspergillosis in 2007 (57). The TR_{34}/L98H allele of *cyp51A* was discovered to underlie pan-azole-resistance in these strains (57). A later study found this allele in over 90% of isolates they had acquired from IA patients (58). Since inter-human transmission of aspergillosis has not been documented and these patients had IA, which has a low rate of sporulation and thus spontaneous mutation, the authors concluded that this allele could have originated from the environment rather than from a patient (54, 58). The same authors began environmental sampling around hospitals to see if the TR_{34}/L98H allele was present, and they found that it was present in 89% of resistant strains (10, 54). More alleles containing tandem repeats were discovered both in patients and in nature. Two studies found that the same *cyp51A* alleles conveying resistance were found in both the environment and in patients, including TR_{34}/L98H, G448S, and TR_{46}/Y121F/T289A (59, 60). These strains displayed resistance to not only medical azoles, but agricultural azoles as well (59, 61-63). More recent studies have found that azole-resistant environmental isolates also displayed cross-resistance to common classes of environmental fungicides with different modes of action:

benzimidazoles, succinate dehydrogenase inhibitors (SDHIs), methyl benzimidazole carbamates (MBCs), and quinone outside inhibitors (QoIs) (28, 64, 65). This combination of evidence suggests that the alleles found in patients originated in the environment rather than in a patient.

Alleles such as TR_{34}/L98H and TR_{46}/Y121F/T289A combined tandem repeats in the promoter region of the *cyp51A* gene with single nucleotide polymorphisms in the gene itself. The tandem repeats caused overexpression of the gene while the single nucleotide polymorphisms decreasing the affinity of the azoles for the 14-α-demethylase enzyme. In the TR_{34}/L98H allele, expression of CYP51A has been shown to increase 8-fold (54). The combination of these two modes of action causes azole therapy to become nearly useless against these mutants. The TR_{34}/L98H allele conveys pan-azole resistance and an especially high itraconazole resistance profile (9, 66). The TR_{46}/Y121F/T289A allele also causes pan-azole resistance but especially high levels of voriconazole resistance and varying itraconazole resistance (9, 67). Studies have shown that the Y121F and L98H mutations only convey moderate resistance to triazoles and that the entire series of mutations is required for complete resistance (66, 68). There have been other less-common varieties of these mutations recently reported in both the environment and in patients, such as TR_{53}, $TR_{46}{}^{3}$/Y121F/M172I/T289A/G448S, $TR_{46}{}^{3}$and $TR_{46}{}^{4}$ (69, 70).

Several non-*cyp51A*-based methods of resistance have been reported. These mutations can affect efflux pumps, cholesterol import, *cyp51B*, and mutations of *hapE* (9). *hapE* encodes a subunit of the CCAAT-binding complex transcription factor, and the P88L mutation causes the transcription factor to overexpress *cyp51A* (71). These mutations have been shown to convey resistance to fluconazole, voriconazole, and itraconazole (9). However, non-*cyp51A*-based mutations may also have associated fitness costs that decrease their prevalence in the

environment (72). Less is known overall about these mutations and whether they have similar environmental origins to the *cyp51A* mutations.

Commercial agricultural products of interest in the United States

Both azole-susceptible and azole-resistant *A. fumigatus* isolates have been found in every continent except Antarctica, with most of the resistant isolates originating from The Netherlands (17). Some surveys have found the incidence of azole-resistance to be as high as 30% while others have found none (17, 73). The TR_{34}/L98H alleles were present in 5.9 to 100% of azole-resistant isolates tested in Europe, and 10% of azole-resistant isolates tested in the United States (17, 53, 74, 75). The TR_{46}/Y121F/T289A alleles were less common in most areas of Europe but were still present in up to 28.5% of azole-resistant isolates in Germany and 10% of azole-resistant isolates in the United States (17, 73, 75). The TR_{34}/L98H mutations are present in 75-100% of azole-resistant isolates in Asia and 22.5% of the azole-resistant isolates in Australia (17, 76-78). There is an established connection between areas with high levels of azole usage and an increased number of azole-resistant isolates of *A. fumigatus*. Surveys have found these resistant isolates in the air within and outside of hospitals (10, 79) and in agricultural fields (11, 27, 28, 80).

The Agrian website was used to find products commonly treated with tebuconazole (47). Fruit, peanuts, and tree nuts are regularly licensed for treatment with tebuconazole in the United States (48, 81). Tebuconazole is used to prevent powdery mildew, black rot, and botrytis rot in table grapes (82). Tebuconazole is used to treat apples for apple scab, powdery mildew, and cedar apple rust. Tebuconazole is also used on watermelon to prevent gummy stem blight and powdery mildew. Tree nuts are also treated for various blights using tebuconazole. Soil and plant

debris from the agricultural fields growing these commercial products have also contained both azole-resistant and azole-susceptible isolates of *A. fumigatus* (11, 28). *A. fumigatus* has been previously found on nut products such as pine nuts (12), pecans (13), walnuts (12, 14), and hazelnuts (14).

Commercial potting soil and compost have been found to contain high amounts of sensitive and resistant *A. fumigatus* isolates (11, 83). Azole-resistance has been found on both flower bulbs and flower bulb waste, and flower bulb waste has even been named a hotspot of resistance (84-86). Other hotspots such as wood chippings and green waste have also been identified as hot spots (85). Flower bulbs, most commonly tulips, are commonly treated with azole fungicide dips (47, 84). High concentrations of azole-resistant *A. fumigatus* were also isolated from soil which grew tulip bulbs in The Netherlands and the Northeastern US (28). Overall, the products that are most likely to harbor azole-resistant isolates of *A. fumigatus* are likely to be treated with azoles, historically found to have *A. fumigatus* on the surface, and have had resistant isolates of *A. fumigatus* found in soil used to grow them.

RESEARCH OBJECTIVES

Azole-resistant *A. fumigatus* is incredibly dangerous in a world where the immunocompromised population is rising. Documenting azole resistance in different environments is helpful to determine the extent of the resistance and to elucidate its origin and cause. However, these azole resistance surveys are mainly based in Europe and only two studies to date have looked at environmental azole-resistant *A. fumigatus* in the United States. Of these two, neither had considered the possibility of azole-resistance being transferred from agricultural fields via the products that originated there. My research aims to fill this gap by (1) creating a

comprehensive review of environmental azole-resistant *A. fumigatus* in the world, (2) surveying retail plant matter in Georgia to find azole-resistant strains of *A. fumigatus*, (3) identifying molecular resistance pathways and azole resistance phenotypes of the azole resistant isolates, and (4) analyze the population structure of isolates collected from this study with others from clinical and agricultural environments.

Identifying retail products that have the potential to carry and expose susceptible individuals to *A. fumigatus* will help our understanding of how susceptible people are exposed to these environmental strains, and what we can do to prevent exposure. These results will guide where surveys of azole-resistant *A. fumigatus* should be conducted and will provide vital information on where azole-resistant *A. fumigatus* could present a potential danger.

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CHAPTER 2

AZOLE-RESISTANT *ASPERGILLUS FUMIGATUS* IN THE ENVIRONMENT: IDENTIFYING KEY RESERVOIRS AND HOTSPOTS OF ANTIFUNGAL RESISTANCE[1]

[1] Burks C, Darby A, Gómez Londoño L, Momany M, Brewer MT. 2021. Azole-resistant *Aspergillus fumigatus* in the environment: Identifying key reservoirs and hotspots of antifungal resistance. PLOS Pathogens 17:e1009711.

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ABSTRACT

Aspergillus fumigatus is an opportunistic human pathogen that causes aspergillosis, a spectrum of environmentally acquired respiratory illnesses. It has a cosmopolitan distribution and exists in the environment as a saprotroph on decaying plant matter. Azoles, which target Cyp51A in the ergosterol synthesis pathway, are the primary class of drugs used to treat aspergillosis. Azoles are also used to combat plant-pathogenic fungi. Recently, an increasing number of azole-naïve patients have presented with pan-azole-resistant strains of *A. fumigatus.* The TR_{34}/L98H and TR_{46}/Y121F/T289A alleles in the *cyp51A* gene are the most common ones conferring pan-azole resistance. There is evidence that these mutations arose in agricultural settings; therefore, numerous studies have been conducted to identify azole resistance in environmental *A. fumigatus* and to determine where resistance is developing in the environment. Here, we summarize the global occurrence of azole-resistant *A. fumigatus* in the environment based on available literature. Additionally, we have created an interactive world map showing where resistant isolates have been detected, and include information on the specific alleles identified, environmental settings, and azole fungicide use. Azole-resistant *A. fumigatus* has been found on every continent except for Antarctica with the highest number of reports from Europe. Developed environments, specifically hospitals and gardens, were the most common settings where azole-resistant *A. fumigatus* was detected, followed by soils sampled from agricultural settings. The TR_{34}/L98H resistance allele was the most common in all regions except South America where the TR_{46}/Y121F/T289A allele was the most common. A major consideration in interpreting this survey of the literature is sampling bias; regions and environments that have been extensively sampled are more likely to show greater azole resistance even though resistance could be more prevalent in areas that are under-sampled or not sampled at all. Increased

surveillance to pinpoint reservoirs, as well as antifungal stewardship, are needed to preserve this class of antifungals for crop protection and human health.

INTRODUCTION

Aspergillosis is a wide spectrum of respiratory conditions caused by the inhalation of spores from *Aspergillus* species (1).The vast majority of cases worldwide (90%) are caused by the thermotolerant fungus *Aspergillus fumigatus*, followed by *Aspergillus flavus* (10% of bronchopulmonary cases), which is mostly reported in Asia, Africa, and the Middle East (2, 3). This fungus can affect both immunocompetent and immunocompromised individuals. The presentation of symptoms depends on host immunocompetency and the spore load inhaled (2). The classification of aspergillosis disease can range from allergic bronchopulmonary aspergillosis (ABPA), to chronic pulmonary aspergillosis (CPA), to invasive aspergillosis (IA), which is the most severe and highly lethal (2, 4, 5). ABPA is the most severe form of aspergillosis that can affect an atopic patient or a patient with cystic fibrosis, and affects nearly 5 million people annually (4, 5). CPA encompasses the chronic inflammatory forms of aspergillosis, including aspergilloma, which most commonly affects patients with tuberculosis or other cavitary chronic lung diseases (4). CPA was estimated to affect 3 million people annually in 2017, but it is also documented that the disease is underrecognized so incidence cannot be accurately estimated (4, 5). Invasive aspergillosis (IA) is the most severe form of aspergillosis, which affects severely immunocompromised patients, those under immunosuppressive treatments or those undergoing to a surgery or a transplant (2, 4). IA is estimated to affect 250,000 people worldwide and the incidence is increasing (5); one study found that IA hospitalizations rose 3% in the United States between 2000 and 2013 (6), and another found that from 2013 to 2017, four times as many patients were admitted for IA per year as in 1996 (7, 8).

This is likely due to the rise in immunosuppressive treatments for hematologic malignancies, stem cell transplants, and solid organ transplants, leading to neutropenic patients (2, 6). There has also been a shift in incidences of IA from neutropenic to non-neutropenic patients, such as those admitted for severe viral infections like influenza and more recently, Corona Virus 2019 (COVID-19) (4, 9). In cases of IA that do not respond to treatment, mortality rates are between 50% and 100% (10). Aspergillosis diseases are primarily treated using azole antifungals (11).

Azoles are a broad-spectrum class of antifungals that can be used on both human-pathogenic fungi and plant-pathogenic fungi (12). Human-use azoles such as voriconazole, isavuconazole, and posaconazole are used to treat invasive forms of aspergillosis and other severe fungal infections (11). Other azole drugs such as climbazole and clotrimazole are used to treat topical fungal infections (13). Azole fungicides like tebuconazole and propiconazole are used against a variety of plant-pathogenic fungi causing diseases such as powdery mildew, downy mildew, rusts, leaf spots, and flower blights (14). Agricultural-use triazoles have become more popular in recent years. Tebuconazole and propiconazole use in the United States alone increased over four-fold from 2000 to 2016 (15). Azoles are widely used in agriculture because they are inexpensive, they have a broad-spectrum systemic mode of action, and they are able to resist changes to their molecular structure so they can persist in the environment for an extended period of time (16). Contamination of soil, wastewater, and sewage sludge with azoles also happens frequently, likely due to expulsion of topical azoles from urine and the skin (13). Surface water and agricultural lands can be contaminated with azole biocides through incomplete removal from wastewater and use of sludge as fertilizer (13). The wide use and persistence of azoles in the environment can contribute to the development of azole-resistance in non-target organisms, such as *A. fumigatus.*

Azole-resistant *A. fumigatus* was first discovered in the clinic in 1997 when analyzing isolates collected in the 1980s (17). Azole antifungal drugs work by disrupting the synthesis of ergosterol, an important membrane lipid analogous to cholesterol in humans (12). The production of ergosterol in fungi is facilitated by Cyp51A, a protein that converts intermediate sterols into ergosterol (12). Azole resistance that develops in the clinic is generally caused by nonsynonymous nucleotide substitutions in the gene *cyp51A* leading to amino acid changes such as G54E, P216L, and F219I (18). These point mutations arise in patients treated with azoles, where there is high selection for azole resistance and an abundance of asexual growth and reproduction occurs (18-20). However, pan-azole-resistant strains, which are resistant to multiple azole drugs, were detected in azole-naïve patients with aspergillosis in 2007 (21). The TR_{34}/L98H genotype of *cyp51A* was discovered to underlie pan-azole-resistance in these strains (21). This allele consists of a tandem repeat of 34 bases combined with a nonsynonymous nucleotide substitution, which leads to both the overexpression of *cyp51A* and a lower binding affinity of azoles for Cyp51A, respectively (21, 22). This genotype became the dominant resistance mechanism and genotype in The Netherlands in 2008 and was then found in different unrelated patients in other parts of the world, which suggested that these alleles likely originated in environmental settings (23, 24). Cross-resistance to medical and agricultural azoles developed due to their similar structures and activities, as well as similar selective pressures for azole resistance in human hosts and in the environment when abundant reproduction by the fungus is accompanied by azole exposure (25, 26). A study by Snelders et al. (27) suggested that resistant isolates from the environment were likely moving into human hosts when they found that *A. fumigatus* isolates with the TR_{34}/L98H allele were found in clinical settings and flowerbeds outside of the hospital, and then commercial seeds, leaves, and compost. The environmental

isolates were genetically similar to the isolates found in patients (27). Since this study, there have been a plethora of sampling efforts across the world to determine where azole-resistant *A. fumigatus* is present in the environment. More alleles connected to the pan-azole resistance phenotype involving tandem repeats in the promoter region and/or nonsynonymous substitutions s in *cyp51A* have since been discovered in both the clinic and the environment, including TR_{53}, TR_{46}/Y121F/T289A, TR_{46}^3, and TR_{46}^4 (28, 29). However, it is important to note that although associations between environmental and clinical resistance have been presented, it has not been shown conclusively that patients acquire azole-resistant *A. fumigatus* from the environment.

Aspergillus fumigatus is primarily a saprophyte found on decaying plant matter and is, thus, commonly found in agricultural environments and on plant-based agricultural products (30-35). This fungus also plays a notable role in carbon and nitrogen recycling in soil and compost heaps (36). However, *A, fumigatus* is most abundant in areas where decomposition produces increased temperatures, such as in compost piles, decomposing plant material, and municipal waste treatment plants (37-39). Other studies have found *A. fumigatus* to be prevalent in the outdoor air, flower beds, commercial potting mixtures, manures, and mulches (27, 37, 40, 41). Our aim for this review is to identify reservoirs (habitats where the fungus normally lives, grows and multiplies) (42) of azole-resistant *A. fumigatus* by synthesizing published data on the environmental setting and substrate, the geographic locations, and the types of *cyp51A* alleles, if known, associated with azole-resistant *A. fumigatus* found in the environment. Additionally, we present an interactive global map showing where resistance has been detected. This will aid in characterizing and tracking the environmental settings where resistance is most prevalent and in identifying environments with azole-resistant *A. fumigatus*. Overall, this information will be

helpful in identifying factors leading to the evolution and prevalence of azole-resistant *A. fumigatus* in the environment.

MATERIALS AND METHODS

Here, we summarize the published literature on azole-resistant isolates of *A. fumigatus* originating from the environment (Fig 1.1). Studies focused on isolates only from patients or that found no azole-resistant isolates were not included in our synthesis. To assist in summarizing and synthesizing the findings, resistant isolates were assigned to one of four possible environmental settings: agricultural environments, developed environments, commercial products, or other environments (Fig 1.1; Fig 1.2A). Isolates assigned to agricultural environments were collected from horticultural or crop production settings. Isolates assigned to developed environments were collected from public parks, gardens, homes, workplaces, or hospitals. Isolates assigned to commercial products were collected from retail products or products that would have been sold in a store. Isolates that could not be assigned to one of these three categories, for example those from an isolated forest, were assigned to other environments. Resistant isolates were also assigned into categories based on the substrate sampled: air, compost, particulate debris, plant debris, plants, seeds, soil, and water (Fig 1.2B). Here, samples within the plant debris category include vegetable waste, woody debris, green waste, or other disposed plant parts. Particulate debris includes dust and swab samples from inside buildings. Resistant isolates were assigned to geographic locations of origin, including Africa, East Asia, Europe, India, The Middle East, North America, and South America (Fig 1.2C). Resistant isolates were separated by allele or phenotype, if *cyp51A* was not sequenced or no alleles that differed from wild type were present (Fig 1.2D). Isolates were assigned to the TR_{34}/L98H or

TR_{46}/Y121F/T289A categories if these alleles were present in *cyp51A*. If the isolates had a different *cyp51A* allele from wild type that was neither of the two common alleles conferring pan-azole resistance, they were assigned to the other category. Finally, if isolates were found to have a wild-type *cyp51A* or *cyp51A* was not sequenced, they were placed into the determined by phenotype category. Available information for each azole-resistant isolate of *A. fumigatus* collected from the environment, including the published reference, environmental setting, substrate, the geographic origin, azole-use in the environment, and *cyp51A* alleles, if known, was compiled to create an online interactive map (Fig 1.1, https://maphub.net/cburks817/AfumMap).

RESULTS

Environments where azole-resistant *A. fumigatus* has been detected

Among the 52 published studies where azole-resistant *A. fumigatus* from the environment was detected. 1292 total azole-resistant isolates were identified, and the specific environmental setting and substrate of 1198 of the isolates were described. For the remaining 94 isolates, either the substrate was not mentioned (27) or the division of isolates between two different settings or substrates was not clear (24, 43-45). For the resistant isolates where the substrate was clearly described, the majority were detected in soil (56.7%), followed by air (16.5%), plant debris (11.4%), and compost (9.4%), with 3.2% in particulate debris, 2.4% in plants, 0.3% in water, and 0.1% in seeds (Fig 1.2B).

Most of the studies (31 of 52; 59.6%) focused on azole-resistant *A. fumigatus* in agricultural environments and 426 (35.6%) of the resistant isolates detected in the environment originated from agricultural samples (Fig 1.2A). Most resistant isolates from agricultural environments were recovered from soil (75.6%), followed by plant debris (12%), compost (9.4%), plants (2.6%), and air (0.5%). The crops grown in the agricultural settings sampled

varied greatly; however, those that were sampled frequently included cereals, rice, maize, potato, strawberry, and flowers. A large portion of azole-resistant isolates from agricultural environments originated from fields of the crops listed above (50.2%), specifically flowers (27.7%). Seventeen of the studies (54.8%) reported the use of azole fungicides in the agricultural settings sampled.

Twenty-eight (53.9%) of the published studies found azole-resistant *A. fumigatus* in developed environments (some studies included isolates from more than one environmental category). Of the 570 (47.6%) azole-resistant isolates recovered from developed environments, most were recovered from soil (52.1%), followed by air (33.0%), plant debris (7.7%), particulate debris (6.7%), and water (0.5%) (Fig2A). Resistant isolates from developed environments were collected and identified more from outdoor environments (53.9%) than indoor environments (46.1%). Of the isolates from developed environments, over half (58%) were identified from hospital environments, with 27.1% from the inside of hospital environments and 31.9% outside, but very near hospital environments. One hundred and eighty-four isolates from developed environments (32.6%) were found in gardens and flowerpots. Only 2 (7.1%) of these 28 studies reported the use of azole fungicides in developed environments (33, 46). These 2 studies found azole residues in tea gardens, hospital potted plants (46), green waste, and household waste (33).

Four (7.7%) of the studies where azole-resistant *A. fumigatus* was detected included retail or commercial products. From these four studies, 23 (1.9%) azole-resistant isolates were recovered (Fig 1.2A). Most of these isolates (78.3%) were recovered from live tulip and narcissus bulbs originating from The Netherlands (47, 48). The remaining azole-resistant isolates from retail products were recovered from commercial compost (8.7%) (27, 48), commercial soil (8.7%) (49), and commercial seed (4.3%) (27). Most resistant isolates detected in products were

from flower bulbs (47, 48), which have been identified as a hotspot of azole-resistance (33, 47, 48, 50, 51).

The remaining 179 (14.9%) azole-resistant *A. fumigatus* isolates represented in 12 studies were sampled from other environments (Fig 1.2A). Five of these studies performed sampling in locations that did not fit within the agricultural environments, developed environments, or commercial products categories: a forest, water treatment facility, soil filled with bird excrement, and two sawmills (46, 52-54). The other 7 studies sampled compost, soil, wood, and possibly other substrates but did not include details about the origin of their samples, preventing them from being assigned to an environmental category (24, 27, 33, 49, 55-57). The studies examining wood and sawmills are especially intriguing because these areas are rarely studied as a potential for exposure to azole-resistant fungi despite the heavy treatment of wood products with azoles. (33, 52, 53). Most of the azole-resistant *A. fumigatus* isolates collected from other environments were recovered from compost (39.7%), followed by soil (32.4%), plant debris (23.5%), and air (4.5%).

Mechanisms of azole resistance in *A. fumigatus* from the environment

We were interested in determining which *cyp51A* alleles were most common among azole-resistant isolates from the environment. A total of 1190 isolates (92.1%) were included in this portion of the data analysis. Resistant isolates were excluded if the specific allele was unclear for each isolate in the study or the assignment of isolates across different environmental settings was unclear (24, 43, 58, 59). Most of the resistant isolates were found to have either the TR_{34}/L98H allele (60.7%) or the TR_{46}/Y121F/T289A allele (15.0%) (Fig 1.2D). The TR_{34}/L98H allele was found on every continent with azole-resistant *A. fumigatus* and comprises a great proportion of the resistant isolates from environmental settings. Most of the isolates with

TR_{34}/L98H alleles were found in Europe (71.1%), followed by the Middle East (8.2%), India (7.8%), East Asia (5.1%), North America (4.2%), Africa (3.3%), and South America (0.4%). This allele was found in 29.3% of the azole-resistant environmental isolates in Africa, 37.4% of the resistant isolates in East Asia, 77% of the resistant isolates from Europe, 88.9% of the resistant isolates from India, 37.1% of the resistant isolates from the Middle East, 58.8% of the resistant isolates from North America, and 4.3% of the resistant isolates from South America.

The TR_{46}/Y121F/T289A allele was also widespread and present in environmental azole-resistant *A. fumigatus* on every continent except Antarctica; however, it was far less common than the TR_{34}/L98H allele. The TR_{46}/Y121F/T289A alleles were especially prevalent in Europe (46.1%), South America (23.6%), and East Asia (14.0%), and were less prevalent in Africa (4.5%), India (3.4%), the Middle East (1.7%), and North America (6.7%). It was detected in 9.8% of the azole-resistant environmental isolates in Africa, 25.3% of the resistant isolates in East Asia, 12.3% of the resistant isolates from Europe, 9.5% of the resistant isolates from India, 1.9% of the resistant isolates from the Middle East, 23.5% of the resistant isolates from North America, and 60.0% of the resistant isolates from South America. This allele was especially common in South America, where the number of resistant isolates with this allele was far greater than the TR_{34}/L98H allele (29, 50, 60).

The remaining azole-resistant isolates of *A. fumigatus* from the environment had *cyp51A* alleles other than the two common pan-azole-resistance alleles, or the *cyp51A* sequence was not characterized (Fig 1.2D). For these, we considered isolates azole resistant based on minimum inhibitory concentration data characterized by the EUCAST susceptibility testing breakpoints: MIC values > 1 µg/mL itraconazole and voriconazole, MIC values > 2 µg/mL isavuconazole, and MIC values > 0.25 µg/mL posaconazole (61). Resistance to these antifungals often falls into

areas of technical uncertainty, where the label of resistant depends on the patient being treated, and other MIC values. In these cases, an isolate with MIC values = 2 µg/mL itraconazole, voriconazole, and isavuconazole and MIC values = 0.25 µg/mL posaconazole could be treated as susceptible (61). However, for the purposes of this review we used the 2020 strict EUCAST susceptibility testing breakpoints to label isolates as resistant or sensitive.

Sixty-four of the isolates with azole-resistant phenotypes had wild-type *cyp51A* alleles (5.4%). Isolates with wild-type *cyp51A* alleles were found in Europe (43.8%), East Asia (37.5%), South America (15.6%), and the Middle East (3.1%). Resistant isolates with wild-type *cyp51A* alleles were not reported in the environments of Africa, India, and North America.

One-hundred twenty-five (10.5%) of the isolates had another *cyp51A* allele conferring resistance. The most common alleles other than TR_{34}/L98H and TR_{46}/Y121F/T289A were the TR_{34}/L98H/S297T/F495I alleles (50; 4.2%) (27, 33-35, 53, 62-64), G54E (22, 1.8%) (44, 65), G448S (16; 1.3%) (both standalone and associated with the TR_{34}, TR_{46}, and TR_{92} alleles) (33, 47, 55, 64), and M172V alleles (11; 0.9%) (both standalone and associated with F46Y) (34, 64, 66-68).

The majority of the TR_{34}/L98H/S297T/F495I isolates originated from North America (30) (34), East Asia (16) (35, 62-64), and Europe (4) (27, 33, 53). The TR_{34}/L98H/S297T/F495I alleles were found in compost (34), soil (35, 63, 64), plant debris (33, 53), and air (27, 62). Most of the TR_{34}/L98H/S297T/F495I isolates originated from agricultural environments (33-35, 62, 63), followed by developed environments (27, 62, 64), and other environments (53).

The G54E allele was most commonly found in Africa (13) (44), then Europe (8) (44, 65), and India (1) (44). The G54E allele was found in developed environments (44) followed by

agricultural environments (44, 65). Isolates with the G54E allele were found in plant debris (44) and soil (44, 65).

The M172V allele was most often found in Europe (5) (66-68), North America (5) (34) and East Asia (1) (64). It was isolated from agricultural environments (34, 68), followed by developed environments (64, 66, 67), then other environments (67). It was most commonly found in compost (34), particulate debris (66), soil (64, 68), and air (67).

The G448S allele was most common in East Asia (13) (47, 64), Europe (2) (33), and the Middle East (1) (55). The G448S allele was found in developed environments (64), commercial products (47), agricultural environments (33), and other environments (55). Isolates containing the G448S allele were found in soil (64), followed by plants (47), plant debris (33), and compost (55). Four studies also found uncommon tandem repeat alleles: 4 isolates with a TR_{53} allele and 2 with a TR_{92} allele (29, 33, 50, 60)

Other tandem repeat polymorphisms were not commonly found in the environment. The TR_{53} allele was found in azole-resistant *A. fumigatus* in the environment in Bogota, Colombia where it is associated with flower fields and agricultural fields (29, 50, 60). The TR_{92} allele was found in flower bulb waste from agricultural fields in The Netherlands (33).

Geographic distribution of azole-resistant *A. fumigatus* and azole use in the environment

Azole-resistant *A. fumigatus* has been found in the environments on every continent except Antarctica (Fig 1.1). Most of the azole-resistant environmental isolates were detected in Europe (56.7%), followed by the Middle East (12.3%), East Asia (10.4%), Africa (6.3%), South America (5.4%), India (4.9%), and North America (3.9%) (Fig 1.2C). The number of published studies covering each region was proportional to the number of resistant isolates recovered, except in East Asia, where 17.3% of the studies covered this region, but only 10.4% of isolates

were found there. We examined the prevalence of azole-resistant *A. fumigatus* from different environmental settings within geographic regions; however, three studies were not included due to unclear assignment of isolates between settings or regions (24, 44, 62). The majority of studies covering resistant isolates from Africa, East Asia, the Middle East, North America, and South America detected resistance in agricultural settings. Most studies focused on Europe and the Middle East detected resistance in developed environments. The majority of studies that detected resistance in commercial products and other settings were focused on resistant isolates from Europe.

To investigate if the geographic distribution and abundance of azole-resistant *A. fumigatus* in the environment is associated with environmental azole applications, we compiled available data on azole fungicide applications for each country (69-72) where resistant isolates have been detected (Table 1.1). Countries in Europe and East Asia applied more azole fungicides per hectare of agricultural land, whereas India, Africa, the Middle East, and South America had lower amounts of azole fungicides applied per hectare of land (Table 1.1). It is important to note that, except for 2018 data from Iran, the data from most of these countries with low usage reported is not recent, and it may not accurately reflect current azole fungicide application levels. Unfortunately, more recent data is not available. It is also important to note that azole fungicide use is not the only method where environmental *A. fumigatus* can be exposed to azoles; contamination of soil and surface water with azole biocides is a probable yet understudied method of exposure to azoles (13). Data representing individual applications of azole fungicides would likely provide a more solid link between instances of azole resistance and fungicide use; however, that data is not easily collected and compiled. Therefore, we use country-wide data of

fungicide applications to represent collective instances of azole applications to extrapolate the effect of azole use on resistance development.

The Netherlands has by far the greatest amount of azole fungicide use per hectare of agricultural land, followed by Germany and France (Table 1.1), and the majority of the azole-resistant isolates were found in Europe (Fig 1.1, Fig 1.2C)**.** Pan azole-resistant *A. fumigatus* suspected to arise from the environment was first found in The Netherlands in 1997 (21), which is consistent with the agricultural origin theory of azole-resistant *A. fumigatus* resulting from increased use of azole fungicides (27, 73). Interestingly, Iran reported the third-greatest number of azole-resistant *A. fumigatus* isolates after The Netherlands and France but had one of the lowest amounts of azole fungicides sprayed per hectare of agricultural land (Table 1.1) (55, 74-76).

DISCUSSION

Azole-resistant *A. fumigatus*, which is a serious threat to human health, has been found on six continents and in multiple different environments around the world (Fig 1.1, Fig 1.2). We compiled data on 1292 azole-resistant *A. fumigatus* isolates reported from 52 independent studies. Most of the studies where azole-resistant *A. fumigatus* were detected were performed in Europe, where most resistant isolates were found. Over half of the azole-resistant isolates originated from soil samples. More than half of the resistant isolates came from developed environments, with the majority from sites including flower gardens and hospitals, and 35.6% of the isolates came from agricultural settings, with the majority found in sites including flowers, cereals, rice, maize, potatoes, and strawberries. This finding was unexpected since azole fungicide use is reported more often in agricultural settings, and agricultural sites were sampled

more frequently. Reasons this could be the case include plants in developed settings being sprayed with azoles before transplanting, or that there are more azoles present via deliberate azole fungicide use or unintentional contamination by azole fungicides or biocides. There was a high frequency of azole-resistant isolates found in hospital environments, which included plants, flower gardens, and samples from the air, water, and surfaces both indoors and outdoors. Similarly, the presence of azole-resistant *A. fumigatus* in hospital environmental samples is likely not due to the use of azole antifungals in the hospitals, but rather due to the introduction of foreign material such as commercial compost on the grounds (27, 46), imported tulips on the grounds (77), and other landscaping and potted plants where azoles may have been applied during the growth process (27).

A recently-published study found that an azole-resistance hotspot most often develops in the presence of decaying plant waste and high concentrations of DMI residues (33). A review paper published the following year expanded the definition of a hotspot to include "environment(s) in which: (1) the physical, biotic and abiotic conditions facilitate the growth of the fungus and from which the fungus can spread; (2) this growth can take place for prolonged periods and the fungus can complete all the stages of its growth cycle; and (3) azoles are present, in different concentrations sufficient to select in populations, and combinations" (51). Environments such as flower-bulb waste, green waste, and wood chippings have already been defined as azole-resistance hotspots (33, 51). Based on the high quantities of azole-resistant *A. fumigatus* in agricultural environments, flower gardens, and hospitals, we suggest that these locations be considered as possible azole-resistance hotspots.

Additional research focused on azole-resistance development and high frequencies of resistant *A. fumigatus* in flower production, flower waste, and flower bulbs in both agricultural

and developed environments is needed to determine the factors contributing to resistance in these settings. Whether flowers are a common site for resistance due to frequent movement of resistant isolates on plant material, selection pressure from high azole use, high use of mulch or compost, or other factors needs further investigation.

Additional studies focusing on the usage of azole fungicides and the presence of azole residues in developed environments is needed, because the amounts used or quantities present are not often measured or reported (33, 51). The greatest number of resistant isolates in developed environments came from sampling outdoors. The most common outdoor developed setting for resistant isolates was garden soil. This could be due to high amounts of azoles used on plants in nurseries before they are sold (27, 51). Alternatively, azole-resistant isolates could be introduced to these environments and even in the absence of azole applications or residues, they might increase in abundance due to genetic drift or an unknown fitness advantage. Azole-resistant isolates have been previously detected in substrates with no detectable azole residues (33). There were also high quantities of azole-resistant *A. fumigatus* found inside and on hospital grounds around the world. This finding is concerning because, while not common, incidences of nosocomial aspergillosis has been known to occur, and azole-resistant *A. fumigatus* poses an extreme health risk to immunocompromised individuals that would likely be present in higher numbers in a hospital setting (4, 78). Although unlikely, the presence of azole-resistance in hospitals environments could also indicate that aspergillosis patients are spreading the resistant isolates into the hospital environment via emission of spores. However, this is unlikely since person-to-person transmission has only been reported once, suggesting that the airborne emissions of patients are insufficient to colonize outside environments (79).

The most common *cyp51A* allele among resistant isolates is the TR_{34}/L98H which comprised almost 75% of the resistant isolates. This allele was the first linked to pan-azole resistance, and has been linked to the overuse of environmental azoles (27). South America is the one area of the world where TR_{34}/L98H was not the most frequent allele, the TR_{46}/Y121F/T289A allele was more frequently detected. This may be a result of different crops grown in that area, different azole antifungals used, introduction and proliferation of isolates with the TR_{46}/Y121F/T289A allele due to random processes, or differences in sampling.

There is a large gap in knowledge concerning azole-resistant *A. fumigatus* in retail products, where only four studies have been conducted to date. There was only one study we found that reported azole resistance in an environment with low human impact (a forest). This prompts the need for more studies focusing on environments far from human impact because the spread of azole-resistance may have spilled over into these environments as well. Several studies have found that isolates could be spread through plant material to different regions (47, 48, 77) and that isolates from different regions and environmental settings are related (27, 35, 43, 44, 46, 59, 60, 62, 74, 80-83), supporting the idea that azole resistant *A. fumigatus* might spread via contaminated plant material and dispersal. It is also likely that azole resistance had multiple independent origins since multiple different resistance mechanisms are present within different isolates. Overall, azole-resistant *A. fumigatus* is being found more frequently in more environments. Additional sampling of underrepresented environments around the world is needed so that the prevalence of azole-resistant *A. fumigatus* can be more accurately documented, and so the risks of encountering these areas can be quantified. Our comprehensive survey of published studies on azole-resistant *A. fumigatus* in environments worldwide has made

clear the overwhelming need to identify sources of resistance and areas of the environment where resistant isolates are most prevalent.

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Table 1.1. Azole fungicide application in agricultural environments

Country	Region	Year	Total azole fungicides sold (kg)	Total agricultural land area (1000 ha)	kg azoles per 1000ha	Citations
The Netherlands	Europe	2018	291,957	1,822	160.24	69, 71
Germany	Europe	2018	1,859,970	18,295	101.67	69, 71
France	Europe	2018	2,817,233	28,660	98.3	69, 71
Japan	East Asia	2018	285,763	4,420	64.65	70, 71
Denmark	Europe	2018	165,200	2,632	62.77	69, 71
Romania	Europe	2018	750,140	13,414	55.92	69, 71
United Kingdom	Europe	2018	964,482	17,687	54.53	69, 71
China	East Asia	2016	26,700,000	528,553	50.52	35, 71
Thailand	East Asia	2014	751,149	22,110	33.97	70, 71
Italy	Europe	2018	414,936	12,451	33.33	69, 71
Switzerland	Europe	2018	40,262	1,537	26.2	69, 71
Norway	Europe	2019	20,869	986	21.16	69, 71
Portugal	Europe	2017	72,751	3,603	20.19	69, 71
Ireland	Europe	2018	67,046	4,516	14.85	69, 71
Greece	Europe	2018	46,865	6,110	7.67	69, 71
United States	North America	2016	2,270,229	405,265	5.6	71, 72
Iran	The Middle East	2016	135,171	45,954	2.94	70, 71
Colombia	South America	2000	77,110	44,859	1.72	70, 71
Kenya	Africa	2001	6,350	26,839	0.24	70, 71
India	India	1999	24,494	181,021	0.14	70, 71
Iraq	The Middle East	2000	907	8,300	0.11	70, 71
Tanzania	Africa	1995	907	33,050	0.03	70, 71
Kuwait	The Middle East	1998	0	143	0	70, 71
Taiwan	East Asia	2018	Unknown	791	NA	70, 71
Brazil	South America	2018	Unknown	236,879	NA	70, 71

Fig 1.1. Azole-resistant *Aspergillus fumigatus* in the environment detected throughout A. the world, B. Europe. The figure legend indicates different environmental settings from which resistant isolates were recovered. The digital map that includes additional environmental data, *cyp51A* alleles, and links to the published articles for each isolate can be found at https://maphub.net/cburks817/AfumMap.

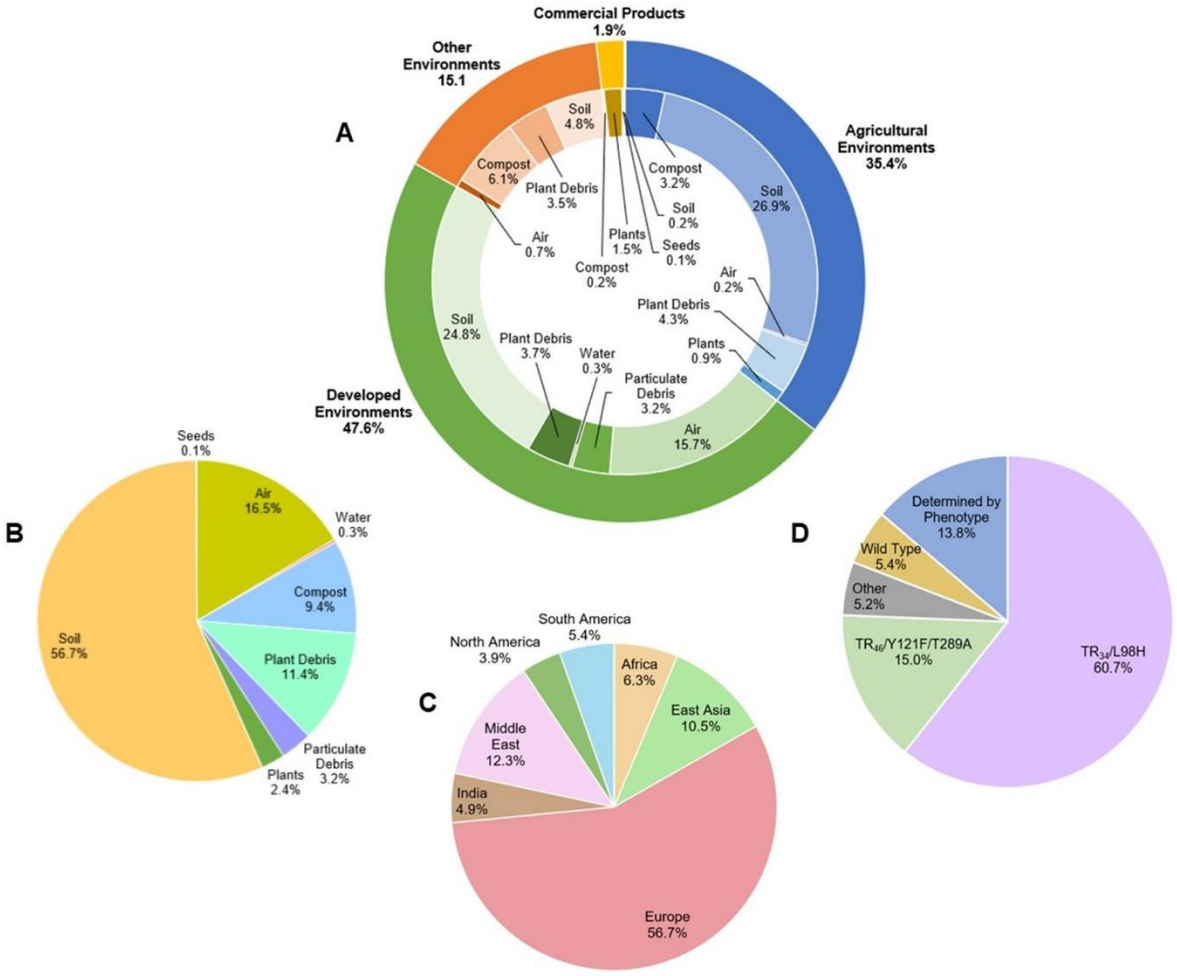

Fig 1.2. Frequency of azole-resistant *Aspergillus fumigatus* isolates detected in the environment by A. environmental setting and substrate sampled, B. substrate sampled across all environmental settings, C. geographic region, and D. *cyp51A* allele ("Other" includes less frequently detected *cyp51A* alleles and "Determined by Phenotype" includes resistant isolates that were not analyzed for *cyp51A* polymorphisms).

CHAPTER 3

MOLECULAR RESISTANCE MECHANISMS AND POPULATION STRUCTURE OF AZOLE-RESISTANT *ASPERGILLUS FUMIGATUS* PRESENT ON COMMERCIAL AGRICULTURAL PRODUCTS

[1] Burks C, Miller N, Vines D, Momany M, Severns PM, Brewer MT, 2021. To be submitted to *Applied and Environmental Microbiology*

ABSTRACT

Aspergillus fumigatus is a common environmental saprophyte as well as a human-pathogenic fungus that can infect patients with a variety of immune function; however, the disease aspergillosis can be deadly in immunocompromised individuals. Since the late 1990's resistance to azole antifungals – the first line of defense against aspergillosis - has been documented in *A. fumigatus*. This resistance was found in patients without prior exposure to azoles, leading investigators to believe that the azole resistance had developed in agricultural environments where azoles are frequently used against plant-pathogenic fungi. Previous studies in the United States have documented azole-resistant *A. fumigatus* across different agricultural environments but none have looked at commercial plant products. Several studies in other countries found azole-resistant *A. fumigatus* on commercial compost, soil, and plant bulbs. These products as well as several produce items that are commonly treated with tebuconazole and propiconazole in the field were surveyed here. Five hundred twenty-five isolates were collected and screened for tebuconazole and itraconazole resistance; and 130 isolates were chosen for further azole-resistance phenotyping. Twenty-four isolates were pan-azole resistant; these isolates were originally collected from compost, soil, flower bulbs, and peanuts. The primary genetic mechanisms underlying the azole resistance in the pan-azole-resistant isolates were the TR_{34}/L98H, TR_{46}/Y121F/T289A, and Y46F/H147Y/V172M/T248N/E255D/K427E alleles in the *cyp51A* gene. Some pan-azole-resistant isolates had a Y46F/V172M/T248N/E255D/K427E allele in *cyp51A*, indicating an alternative mechanism of resistance. To determine the genetic relatedness of azole-resistant and sensitive commercial isolates with isolates from agriculture and clinical samples, 95 isolates from this study were selected, as well as 80 clinical and environmental isolates from a previous study, for population genetics analysis. Minimum

spanning networks and discriminate analysis of principal components (DAPC) were used to visualize relatedness among the isolates to identify if populations were structured. The minimum spanning networks both showed a large amount of diversity in the population and had 2 primary branches distinguished from the other isolates that contained isolates with a tandem repeat in the promoter of the *cyp51A* allele and isolates collected from commercial products with a T248N/E255D allele. Three primary clusters were identified with DAPC, which appeared to separate out based on *cyp51A* genotype. This is consistent with previous studies that have found that pan-azole-resistant isolates are found in a unique clade. Overall, pan-azole resistance can be found in lawn and garden products in the United States. Further surveying is necessary to determine the extent to which azole-resistant *A. fumigatus* is present in these products and others.

INTRODUCTION

A. fumigatus is a saprophyte that has a widespread distribution across the world (1, 2). It is commonly located in areas where plant growth is abundant, such as in agriculture, flower gardens, and in compost piles (2-5). It primarily reproduces via asexual reproduction by means of small, lightweight spores that are easily made airborne, so *A. fumigatus* can commonly be found in the air near locations where it is growing in the soil (6). These airborne conidia are very small, about 2-3 μm in diameter, and are very easily inhaled, where they are normally destroyed by the body's defenses (6, 7). However, in immunocompromised people and people who are afflicted with chronic respiratory diseases, such as tuberculosis or Corona Virus Disease 2019 COVID-19), the fungus is able to continue to travel into the lungs, where it grows hyphae and sporulates (7). This disease is known as aspergillosis, and can range in severity depending on the immune-system function of the host, from acute bronchopulmonary aspergillosis in patients

predisposed to asthma to chronic pulmonary aspergillosis in patients with prior lung conditions, to invasive aspergillosis in patients with severely reduced immune function (8). Most cases of aspergillosis are treated primarily with triazole drugs, which all share the same mode of action and similar structures (8, 9).

Triazoles (referred to from here on as azoles) inhibit the growth of fungi by binding to 14-α-demethylase encoded by *cyp51A,* and inhibiting the production of ergosterol which is necessary to maintain structure and fluidity in fungal cell walls (10). However, in the late 1990's azole-resistant strains of *A. fumigatus* were found in patients being treated for aspergillosis with itraconazole (11, 12). This clinical azole resistance was later found to be caused by nonsynonymous nucleotide substitutions in *cyp51A* that caused itraconazole to have a lower affinity for 14-α-demethylase, decreasing its efficacy (13). Specific amino acid changes such as G54E, P216L, and F219I were found to convey azole resistance (13). Patients who withstand long-term treatment using azoles are more likely to develop strains of *A. fumigatus* containing these SNPs because their body temperatures allow abundant asexual growth and reproduction, and the presence of azoles selects for azole resistance (13-15).

Several years later, more azole-resistant strains were found in patients who had no prior exposure to azole drugs (16). Unlike the strains found before, these were resistant to multiple azole drugs and made the treatment of the disease nearly impossible (16). The first and most common molecular mode of resistance was a series of mutations in the *cyp51A* gene combining a tandem repeat of 34 base pairs in the promoter region with a L98H single nucleotide polymorphism (SNP): TR_{34}/L98H (16). In 2011, one study documented that 90% of isolates acquired from invasive aspergillosis (IA) patients contained the TR_{34}/L98H allele (17). Since IA does not present with sporulation within the patient in most cases, and inter-human transmission

of aspergillosis has not been documented, the authors concluded that the patients may have acquired isolates with this allele from the environment rather than it developing as a result of azole therapy (17, 18). A study of the same hospital where the allele had first arisen found isolates with the TR_{34}/L98H allele around the environment of the hospital during a sampling effort made 2 years after the discovery of the allele in patients, showing that this allele is found in the environment (19).

Since then, several more alleles in *cyp51A* have arisen that convey similar resistance to multiple azole drugs: TR_{46}/Y121F/T289A, G448S, and others (20, 21). These alleles were all found in patients with no prior exposure to azoles and showed a cross-resistance to agricultural use azoles, which meant that it was likely that the fungus had been exposed to azoles and developed the resistance prior to its entry into the patient (20, 22-24). Other studies have found that some clinical azole-resistant *A. fumigatus* isolates have displayed cross-resistance to classes of agricultural fungicides with no clinical-use relatives such as succinate dehydrogenase inhibitors (SDHIs), methyl benzimidazole carbamates (MBCs), and quinone outside inhibitors (QoIs) (4, 25, 26). This combination of evidence strongly suggests that these antifungal resistance alleles found in patients originated in the environment rather than in a patient.

The high presence of *A. fumigatus* and the frequent use of triazole fungicides in agricultural environments has led to many surveys for azole-resistance. Azole-resistant *A. fumigatus* has been found in the environments of multiple countries across the world, including the United States (3, 27). Many studies have investigated a multitude of different environments including agricultural fields, homes, hospitals, forests, and workplaces (3). In addition to testing for azole-resistance, many of these studies also examined the molecular mechanisms of resistance and performed population genetic analyses comparing the relatedness of their isolates

to others around the world. A very common method in *A. fumigatus* is to conduct microsatellite polymorphism analysis using 9 microsatellite markers developed by de Valk (28). Genotyping using these short tandem repeats (STRs) is highly reproducible, high-resolution, and very easily exchanged between laboratories (29). These markers are thought to behave as neutral markers and are thus not under any sort of selection (30). The 9 STRs are grouped into 3 multicolor multiplex PCRs which amplify di-, tri-, or tetranucleotide repeats, allowing for very high discriminatory power (29). A database of isolates genotyped using these STRs has been created in order to share the microsatellite data with researchers around the world (31).

Microsatellite data for *A. fumigatus* is commonly used to identify genetic diversity and population genetic structure among isolates from different environmental origins, such as clinics or agricultural environments. Many studies have compared pan-azole-resistant isolates from clinics and agricultural environments and found that these isolates cluster based on *cyp51A* allele rather than environment of origin (4, 31, 32). These analyses have found that there are usually 2 or 3 primary clusters or clades structured by pan-azole resistance and the presence of a tandem repeat in the promoter or lack thereof (31, 33-35).

Few environmental sampling studies have been performed in the United States to determine the prevalence of azole-resistance in *A. fumigatus*. Only one study has been published documenting azole-resistant *A. fumigatus* in the environment in the United States, focusing on isolates found in agricultural peanut debris waste (27). This study along with others confirming the presence of *A. fumigatus* on common food products (36-38) led to our hypothesis that azole-resistant *A. fumigatus* could be present on products that have been harvested from fields where azole fungicides are likely sprayed. Studies performed in the Netherlands and Japan have identified commercial tulip bulbs, commercial soil, and commercial compost as possible

reservoirs of azole-resistant *A. fumigatus* (3, 19, 39), but no studies in the United States have investigated the possibility of azole-resistant *A. fumigatus* being spread through commercial plant material. This study aims to identify if azole-resistant *A. fumigatus* are present on commercial products that may originate from agricultural environments that have been exposed to triazoles, such as on grapes, peanuts, pecans, almonds, apples, compost, soil, and flower bulbs. If so, we want to determine the molecular mechanism of resistance in these isolates and to determine how they are related to other isolates of *A. fumigatus* from agricultural and clinical environments.

MATERIALS AND METHODS

Collection and isolation of *A. fumigatus* from commercial products

A total of 8 commercial products were surveyed for azole-resistant *A. fumigatus* over one and a half years (September 2019 through April 2021) depending on their optimal growth and harvest seasons. Grapes, apples, almonds, pecans, peanuts, compost, soil, and flower bulbs (tulip, daffodil, *Gladiolus*, daylily, *Dahlia*, *Canna*, *Liatris*, *Caladium*, lily of the valley, *Clematis*, *Iris ensata*, and magnum elephant ears) were collected from a variety of commercial venues in the Athens, GA area, such as grocery stores and home and garden stores. Each product was collected by purchasing a pre-packaged bag at a commercial venue. A wide variety of brands, product sizes, and different commercial venues were sought out to collect the most variety. An effort was made to collect products that originated in the United States. The grapes and almonds originated from California or the greater United States. Peanuts were from the United States. Pecans were listed as from Texas, Georgia, or the United States. Apples were listed as being from Washington, New York, or the United States. The compost origins were largely unknown but

those that could be determined were from the United States. The origins of the soil were also largely unknown, but some were determined to be from the United States or Canada. Finally, flower bulbs were from the United States, the Netherlands, or China. Grapes, apples, and flower bulbs were sealed in a bin or a collection bag prior to sampling to ensure that no outside contamination from the lab environment had affected the samples. A sterile gallon Ziploc bag was half filled with each compost or soil bag purchased before entering the lab; these bags served as the sample material.

Grapes, peanuts, pecans, almonds, and flower bulbs not packed in soil were washed using the following method. The entire bag of the purchased product was placed into a sterile polypropylene 12”x15” bag and washed thoroughly with 50 mL sterile 0.05% Tween-20 by massaging the bag to distribute the liquid over the plant products. The residual liquid was collected by cutting one corner of the bag and emptying the liquid into a 50 mL conical tube. Almonds and pecans contained large quantities of debris, so the liquid was strained through sterile cheesecloth during collection. The conical tube was centrifuged at 3000 g for 5 min (40), and the supernatant poured off. Pellets from the products that were found to contain less *A. fumigatus* on the surface (grapes, pecans, and almonds) were resuspended in 1 mL of 0.05% Tween-20, and the entirety of the solution was plated onto Sabouraud dextrose agar (SDA) containing 50 mg/mL rose bengal, 50 mg/L chloramphenicol (C) and 5 mg/L gentamicin (G) using a spread plate method in 100 µL aliquots (4). Pellets from commercial products with abundant *A. fumigatus* (peanuts and flower bulbs), were resuspended in 2.5 mL 0.05% Tween-20 solution and distributed in 100 µL aliquots amongst 10 SDA+GC plates, 10 SDA+GC plates with 3 µg/mL itraconazole, and 10 SDA+GC plates with 3 µg/mL tebuconazole. Flower bulbs packed in soil were first taken from the soil and placed into a separate bag, shaking off as much

soil as possible first. They were then washed with 0.05% Tween-20 as previously stated. The liquid collected was immediately vortexed, and 3 mL was transferred into another tube. One and a half milliliters were spread plated in 100 µL aliquots amongst 5 SDA+GC plates, 5 SDA+GC plates with 3 µg/mL itraconazole, and 5 SDA+GC plates with 3 µg/mL tebuconazole. The remaining liquid in the tube was diluted 1:2 with fresh 0.05% Tween-20, and 1.5 mL was plated on 5 SDA+GC plates, 5 SDA+GC plates with 3 µg/mL itraconazole, and 5 SDA+GC plates with 3 µg/mL tebuconazole. Compost and soil were sampled using an amended sampling method (4, 27). Briefly, 2 to 4 g from each gallon ziploc bag was suspended in 0.1 M sodium pyrophosphate. The suspensions were vortexed for 30 s and allowed to settle for 1 min. Two and a half milliliters of the supernatant were plated as described above for peanuts and flower bulbs. All plates were incubated at 45°C for 2 to 4 days.

Colonies that matched *A. fumigatus* morphologically were quadrant-streaked onto SDA to obtain a pure culture. All isolates were also screened for azole-resistance by quadrant-streaking onto SDA with 3 µg/mL of tebuconazole and SDA with 3 µg/mL of itraconazole. Both sets of plates were incubated at 37°C for 2 days, after which resistance to tebuconazole and itraconazole was preliminarily assessed as resistant, intermediate, or susceptible based on a visual assessment of growth. A single spore colony was selected from the pure culture on SDA and streaked onto complete media using a sterile q-tip. Each isolate was stored by harvesting conidia from complete media slants incubated at 37°C for 2 days, suspended in 15% glycerol, frozen in liquid nitrogen and stored at -80°C.

Azole-resistance phenotyping

One-hundred-eleven isolates that were suspected to be resistant based on the preliminary screening and 19 susceptible or intermediate isolates were selected for azole-resistance phenotyping via minimum inhibitory concentration assays. These isolates were assessed using the Clinical Laboratory Standard Institute broth microdilution method (41). Briefly, conidia were harvested from 4-day old complete media slants using 3 mL sterile 0.05% tween-20. The spore suspensions were adjusted to 0.09-0.13 OD at 530 nm using a spectrophotometer, and 20 µL were added to 11 mL RPMI 1640 liquid medium (Thermo Sci Gibco, California, USA). This was distributed in 100 µL aliquots across 96-well microtiter plates containing two-fold serial dilutions of antifungals with the final concentrations ranging from 0.015625 µg/mL to 16 µg/mL. Tebuconazole (TEB; TCI America, Oregon, USA), itraconazole (ITC; Thermo Sci Acros Organics, New Jersey, USA), voriconazole (VOR; Thermo Sci Acros Organics, New Jersey, USA), and posaconazole (POS; Apexbio Technology, Texas, USA) suspended in DMSO were all tested. The plates were incubated at 37° C for 48 hr. The minimum inhibitory concentrations (MICs) were determined visually by selecting the first well which had no fungal growth; the corresponding concentration of the well was the MIC which inhibited growth. Accuracy of the MIC assays were checked using both a susceptible and a resistant *A. fumigatus* control with known MIC values for TEB, ITC, VOR, and POS, CDC internal controls B7698 and B7815, respectively. The resistant/susceptible breakpoints used were the EUCAST breakpoints defined in February 2020 (42): ITC S $\leq$ 1 µg/mL > R, VOR S $\leq$ 1 µg/mL > R, and POS S $\leq$ 0.125 µg/mL and 0.25 µg/mL > R. MIC values of 2 µg/mL for ITC and VOR and 0.25 µg/mL for POS are classified as areas of technical uncertainty (ATU) meaning that treatment with these antifungals may be used for isolates with this resistance breakpoint under certain situations, but for the

purposes of this study they will be considered resistant (42). A breakpoint cutoff for TEB was defined as > 2 µg/mL according to previous studies (4). Resistant phenotypes were further classified into 4 different categories: azole-susceptible, TEB-resistant, pan-azole-resistant, and azole-resistant. Isolates that had no resistance to any azoles tested were classified as azole-susceptible. Isolates that had a TEB-resistant phenotype but no resistance to any medical azoles were classified as TEB-resistant. Isolates that had resistance to multiple medical azoles were classified as pan-azole-resistant. Finally, isolates that were either resistant to only 1 medical azole or 1 medical azole and TEB were classified as azole-resistant.

DNA extraction

Mycelia from 102 isolates: 80 isolates from MIC testing and 22 susceptible isolates that were not MIC-tested but were from a variety of products were grown in liquid complete medium for 16-20 hrs at 30°C in a 1 g shaker (4). Tissue for DNA extraction was gathered by filtering through a 40 µm cell strainer and squeezing the residual liquid from the tissue using a sterile cotton swab. Approximately 100-200 mg of tissue was collected and set aside in 2 mL lock-lid tubes. DNA extractions were performed according to the QIAGEN DNeasy Plant Mini Kit protocol, with a few amendments (43). Briefly, buffer AP1 was placed into the 65°C water bath for at least 10 minutes to warm up. Four hundred microliters buffer AP1 and 4 µL RNase A were added to each lock lid tube and vortexed for at least 2 minutes, until all tissue was suspended. Each sample was placed in the 65°C water bath for 10 min, vortexing for 10 seconds three times throughout the incubation. One hundred thirty microliters of buffer P3 was added to each sample, vortexed, and then incubated at -20°C for 5 minutes. The samples were then centrifuged for 5 min at 18,407 g to pellet remaining solids. The supernatants were pipetted into QIA shredded

mini spin columns and centrifuged for 2 min at 18,407 g. Five hundred microliters of the flow-through fractions were transferred into new sterile 2 mL lock-lid tubes. 750 µL buffer AW1 (1.5x volume) was added to the flow-through and immediately mixed by pipetting. Six hundred fifty microliters of this solution were pipetted into the DNeasy mini spin column and centrifuged for 1 min at 18,407 g. The flow through was discarded, and the previous step was repeated with the remaining solution. The DNeasy spin column was transferred into a fresh 2 mL tube, 500 µL buffer AW2 was added, the column was centrifuged for 1 min at 18,407 g. The flow-through was discarded, and the previous step was repeated except the centrifugation lasted for 2 min rather than 1 min. The DNeasy spin column was transferred to a sterile 1.5 mL lock-lid tube, and 50 µL buffer AE was added to the membrane. The columns were incubated at room temperature for 5 min, then centrifuged at 18,407 g for 1 min. This step was repeated once, and then the DNA was stored at 4°C. The DNA was quantified using NanoDrop One (Thermo Sci, New Jersey, USA).

cyp51A sequencing

Seventy-nine isolates were selected for Sanger sequencing of *cyp51A:* 37 sensitive and 42 resistant. These isolates were first PCR-amplified using primers that were designed previously (4). The PCR reactions were performed using a mix of 12.5 µL OneTaq 2x Master Mix, 6.5 µL RNA-free sterile ddH2O, 2µL each of forward primer 5´-CGGGCTGGAGATACTATGGCT-3´ and reverse primer 5´-GTATAATACACCTATTCCGATCACACC-3´ (4). PCR reaction cycles were as follows: 98°C for 2 min followed by 30 cycles of 98°C for 15 sec, 62°C for 15 sec, and 72°C for 2:30 min, followed by a final extension at 72°C for 5 min (4). Sanger sequencing was performed (Genewiz, USA) using 4 primers: 5´-GCATTCTGAAACACGTGCGTAG-3´, 5´-GTCTCCTCGAAATGGTGCCG-3´, 5´-CGTTCCAAACTCACGGCTGA-3´, and 5´-

GCGACGAACACTTCCCCAAT-3´ (4). Sequence alignment was performed using Geneious v2019.2 (Biomatters, Auckland, NZ). Briefly, all sequences were trimmed to remove low quality base pairs with a confidence <20 from the beginning and ends of the sequences. The sequences were then sequentially pairwise aligned. The final consensus sequence was visually assessed, extracted, and the promoter region aligned with A1163 genomic sequence v43 from Ensembl. The coding sequence was translated and aligned to the *A. fumigatus* A1163 Cyp51A protein (GenBank accession EDP50065).

Microsatellite genotyping

Nine previously-developed microsatellite markers for *A. fumigatus* (STR*Af* 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, and 4C) were used to genotype 95 isolates collected in this study: 72 that had been *cyp51A*-sequenced and 23 that were included to display the full diversity of the products (28). Multiplex PCR was performed using a modified protocol for the Type-it Microsatellite PCR kit (Qiagen). Briefly, the multiplex reactions contained 5µL 2x Type-it Master Mix, 1µL 10x primer mix (2µM of each of the 6 multiplex primers), 1µL DNA template, and RNAse-free water. Thermal cycling conditions were as follows: 95°C for 5 min followed by 28 cycles of 95°C for 30 sec, 57°C for 90 sec, and 72°C for 30 sec and a final elongation of 60°C for 30 min. Amplification of several products from each multiplex was confirmed by electrophoresis on a 1% agarose gel with 1× TBE buffer. The PCR products were diluted 1:15 and then sent to the Cornell Institute of Microbiology. The facility added internal size standard Genescan-500 Liz, HiDi-formamide, and performed fragment analysis on an Applied Biosystems 3730x1 96-capillary DNA analyzer. The data were processed using the Microsatellite plugin in Geneious v.6 (Biomatters) to identify locus amplicon lengths.

Population genetic analyses

Microsatellite data for the 95 isolates genotyped here and 90 clinical and environmental isolates previously genotyped (Kang et al., 2020) was used for population genetic analyses (4). To analyze the genetic relatedness among the isolates, minimum spanning networks using Bruvo's genetic distance model (44) and Nei's 1978 distance (45, 46) were constructed with the Poppr package in R (47). Bruvo's genetic distance is often useful for analyses based on in microsatellite markers (44) and assumes a stepwise mutation model that may not be entirely accurate for an organism like *A. fumigatus* which is known to sexually reproduce (48). Moreover, each STR*Af* locus contains 11 to 37 alleles that vary in repeat number, so they may not be evolving in a stepwise manner (28). Therefore, we used Nei's genetic distance as well to incorporate a model that considers an infinite alleles model. Structure within the populations was analyzed using discriminate analysis of principal components (DAPC) in R (49). This method was used because it was not known whether the isolates would cluster based on substrate of origin, *cyp51A* genotype, or another factor. Clusters were determined using K-means clustering of principal components.

RESULTS

Isolate collection and phenotyping

A total of 525 *A. fumigatus* isolates were collected from grapes, peanuts, pecans, almonds, apples, compost, soil, and flower bulbs (Table 2.1). The majority (90%) of isolates were collected from peanuts, flower bulbs, soil, and compost. Among these isolates, 85 from pecans, flower bulbs, soil, and compost were possibly resistant to TEB, and 186 from peanuts,

compost, flower bulbs, and soil were suspected resistant to ITC based on the initial screening on media amended with TEB or ITC, respectively (Table 2.1).

Two almond, 6 pecan, 8 grape, 17 soil, 20 bulb, 44 compost, and 33 peanut isolates were chosen for MIC analysis (Table 2.1). MICs were performed using TEB, ITC, VOR, and POS antifungal drugs. Isolates B7698 and B7815 were used as susceptible and resistant controls. In total, 55 isolates (42.3%) were resistant to TEB, 24 isolates (18.5%) were resistant to ITC, 26 isolates (20.0%) were resistant to VOR, and 24 (18.5%) were resistant to POS (Table 2.2). Isolates that were resistant to at least 1 azole were found in all products except apple and grape (Table 2.2). Seven isolates from almond, flower bulb, compost, peanut, and pecan were azole-resistant. Three isolates from a peanut, pecan, and almond sample grew at concentrations of 4 µg/mL TEB and 2 µg/mL ITC. Two isolates from flower bulbs had elevated MICs to TEB and VOR. One isolate from compost had MIC values of 8 µg/mL for TEB and 2 µg/mL for ITC, and the other compost isolate had an MIC of 0.5 µg/mL for compost. There were 24 isolates total that were classified as pan-azole resistant (Table 2.3). Nine were from compost, 1 from soil, 14 from flower bulb, 1 from peanut (Table 2.3). In total, 57% of the isolates MIC-tested were azole-sensitive, 19% were TEB-resistant, 5% were azole-resistant, and 19% were pan-azole-resistant.

cyp51A genotyping

Seventy-nine isolates were selected for *cyp51A* sequencing. Among those were 23 pan-azole resistant isolates, 37 sensitive isolates, 14 TEB-resistant isolates, and 5 isolates resistant to 1 medical azole. There were 19 distinct genotypes detected that we assigned to 6 categories (Fig 2.1). Isolates with a 34 or 46 base pair tandem repeat in the *cyp51A* gene promoter were found in 9% and 14% of sequenced isolates, respectively (Fig 2.1). Six isolates contained the

TR_{34}/Y46F/L98H/V172M/T248N/E255D/K427E allele, and 1 contained the TR_{34}/Y46F/L98H/V172M/T248N/K427E allele. One isolate had the TR_{46}/Y46F/Y121F/V172I/T248N/E255D/T289A/K427E allele, 4 had the TR_{46}/Y46F/Y121F/V172M/T248N/E255D/ T289A/K427E allele, and 6 had the TR_{46}/Y46F/Y121F/V172M/T248N/E255D/ T289A/K427E/G448S allele. These isolates will be referred to as TR_{34}/L98H and TR_{46}/Y121F/T289A, respectively, when they are being grouped together by their tandem repeat alleles. The T248N/E255D allele was found in 16.5% of isolates, and Y46F/V172M/I242V/T248N/E255D/K427E was found in 7.6% of isolates (Fig. 2.1). Y46F/V172M/T248N/E255D/K427E was the most common allele among the isolates selected for *cyp51A* sequencing, found in 40.5% of isolates (Fig 2.1). Wild type (WT) and other alleles were found in 5.1% and 7.6% of isolates, respectively (Fig 2.1). The "other alleles" category included 6 distinct genotypes each found in 1 isolate in this study: A9T/Y46F/V172M/T248N/E255D/K427E, V172M/T248N/E255D, V172M/T248N/E255D/K427E, W415G, Y46F/H147Y/V172M/T248N/E255D/K427E, and Y46F/V172I/T248N/E255D/K427E (Fig 2.1).

All of the TR_{34}/L98H isolates had a pan-azole resistant phenotype, while all but 1 of the TR_{46}/Y121F/T289A isolates had a pan-azole resistant phenotype (Fig 2.2). The TR_{46}/Y121F/T289A isolate that was not pan-azole-resistant showed resistance to VOR and TEB. Isolates with the T248N/E255D and Y46F/V172M/I242V/T248N/E255D/K427E alleles were primarily sensitive to azoles, but some isolates with these alleles showed resistance to TEB. One isolate with the T248N/E255D allele was resistant to the medical azole POS (Fig 2.2). Isolates with the Y46F/V172M/T248N/E255D/K427E allele were primarily azole-sensitive but a few isolates with this allele were resistant to TEB (4), 1 medical azole (2), and 5 were pan-azole-

resistant (Fig 2.2). Isolates with the WT allele had varying levels of resistance to azoles but had no pan-azole resistance. Most isolates in the "other alleles" category were resistant to TEB or were completely susceptible, while 1 was pan-azole-resistant (Fig 2.2). Pan-azole-resistance was found in TR_{34}/L98H, TR_{46}/Y121F/T289A, Y46F/V172M/T248N/E255D/K427E, and Y46F/H147Y/V172M/T248N/E255D/K427E, overall (Fig 2.2). The majority of isolates with the TR_{34}/L98H allele and all isolates with the TR_{46}/Y121F/T289A allele originated from lawn and garden products (Fig 2.3). Fewer food products than lawn and garden products were sampled, so food products were the minority of every category, but most isolates from food products had the Y46F/V172M/T248N/E255D/K427E allele (Fig 2.3).

Population genetic structure

We conducted population genetic analyses on 95 isolates collected here and 51 environmental and 29 clinical isolates from a previous study (4). The genetic diversity and population genetic structure of the isolates were characterized by DAPC (Fig 2.4A). K=6 clusters were identified, and all isolates had a 100% assignment probability to a single cluster (Fig. 2.4B). Principle component (PC) 1 and PC2 explained 27.6% and 21.5% of the variation in the DAPC solution, respectively (Figure 2.4A). Each cluster was composed of a variety of isolates that originated from different environments. Cluster 1 contained isolates exclusively from this study: flower bulb, commercial compost, peanut, and commercial soil (Fig 2.4B). Cluster 2 contained isolates from the most diverse substrates and environments: environmental compost, clinical settings, commercial compost, grape, grape soil, peanut, peanut soil, pecan, pecan soil, commercial soil, strawberry soil, and watermelon soil (Fig 2.4B). Cluster 3 contained isolates from flower bulb, clinical settings, commercial compost, peanut, commercial soil, and

watermelon soil (Fig 2.4B). Cluster 4 contained isolates from almond, apple, flower bulb, clinical settings, commercial compost, grape soil, peanut, peanut soil, pecan soil, commercial soil, and watermelon soil (Fig 2.4B). Cluster 5 contained isolates from apple soil, flower bulb, clinical settings, commercial compost, grape, and grape soil (Fig 2.4B). Finally, cluster 6 contained isolates from environmental compost, commercial compost, flower bulb, peanut, and pecan soil.

Multiple prior population genetic studies have found either 2 or 3 clusters, clades, or groups within *A. fumigatus* (31, 33-35). Based on PC1 and PC2 we saw overlap among the isolates in clusters 2-5 (Fig 2.4A). Further analyses combine clusters 2-5 to simplify the results in a biologically informative way.

We next investigated if the *cyp51A* alleles of isolates showed patterns among the clusters. Clusters 1-5 are mainly composed of isolates with a non-TR genotype (Fig 2.5C). Cluster 1 contained primarily isolates with the T248N/E255D allele, with 1 isolate having a V172M/T248N/E255D allele (Fig 2.5D). This cluster also has a variety of resistance phenotypes, with the majority having a susceptible or TEB-resistant allele (Fig 2.5B). However, 1 isolate with the T248N/E255D allele had a resistant phenotype to posaconazole. Not all of the isolates that fall into clusters 2-5 were genotyped, so this analysis only includes 49 isolates out of the 141 in this group of clusters. These clusters include isolates displaying a variety azole-resistance phenotypes (Fig 2.5). There is 1 isolate with the TR_{46}/Y121F/T289A allele that falls into this cluster (cluster 5), while the other isolates have non-TR alleles (Fig 2.5C). The majority of the isolates in this cluster have an azole-susceptible phenotype; however, the isolate with the TR_{46}/Y121F/T289A allele has a pan-azole resistant phenotype (Fig 2.6B). Cluster 6 is exclusively made up of isolates with the TR_{34}/L98H and TR_{46}/Y121F/T289A *cyp51A* alleles and

is almost completely pan-azole resistant (Fig 2.5B, Fig 2.5C). Only 1 TR_{46}/Y121F/T289A isolate displays a resistance to just 1 medical azole. Further breakdown of the alleles finds 5 different genotypes in this cluster, incorporating many SNPs that have been found in the other clusters (Fig 2.5D). The most common alleles found in this cluster were the TR_{34}/Y46F/L98H/V172M/T248N/E255D/K427E allele found in 28% of isolates, and the TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E allele found in 29% of isolates (Fig 2.5D).

Minimum spanning networks were constructed based on Bruvo's genetic distance and Nei's genetic distance. In both networks there is a large amount of diversity present; nearly every isolate has a unique genotype. Only 2 isolates originating from different products (compost and flower bulbs) share the same STR*Af* genotype, but they also share the same *cyp51A* allele (T248N/E255D) and resistance phenotype (sensitive). There appears to be no apparent grouping based on commercial, clinical, or environmental location of isolation. However, there are two distinct branches that form based on the presence of a tandem repeat in the *cyp51A* promoter, and of sharing the same *cyp51A* allele (T248N/E255D), which correlate to clusters 1 and 6 (Fig 2.6). Both minimum spanning networks show clusters 1 and 6 branching off from the rest of the network (Fig 2.6). Isolates from cluster 1 are connected by thick, dark lines, indicating their genetic similarity despite being from different hosts (Fig 2.6). Some of the isolates in cluster 6 have slightly more genetic distance between them when compared to those in cluster 1 which is evident when looking at the diversity of SNPs in their TR_{34}/L98H and TR_{46}/Y121F/T289A alleles (Fig 2.6). In both networks there are several isolates not belonging to clusters 1 or 6 that are placed on the same branch as clusters 1 or 6 (Fig 2.6). These isolates appear to be from cluster 2. The network constructed with Nei's genetic distance also displays high genetic

distance and branching patterns suggesting past recombination within and between nodes of the network center, however the evidence of a potential trend is not as visible using the model with Bruvo's distance (Fig 2.6).

DISCUSSION

We conducted a survey of azole-resistant *A. fumigatus* from commercial food products produced in the United States where azole use is high to gain a better understanding of how widespread the population of azole-resistant *A. fumigatus* is. We also sampled commercial lawn and garden products since these substrates appear to contain high levels of azole-resistant *A. fumigatus* in the environment (19, 50). Grapes, peanuts, pecans, almonds, apples, flower bulbs, soil, and compost were identified as commercial products of interest because they either have been listed as products that are sprayed with TEB or propiconazole frequently (51), or they are products where azole-resistant *A. fumigatus* has been found detected in other regions of the world (3, 19, 39). The food products that were surveyed were produced in the United States, while the origin of the lawn and garden products was either unknown or was from the Netherlands, United States, China, Canada. We isolated 522 *A. fumigatus* isolates from the aforementioned products (Table 2.1) (4, 19, 27); however, most came from peanuts and lawn and garden products. Isolates from peanuts were only obtained from raw peanuts with their shells, not from any peanuts with either their shells removed or treated with heat (roasted or boiled) or salt. Screening of these isolates on media amended with TEB and ITC revealed that 85 and 186 isolates, respectively, grew in the presence of the antifungals (Table 2.1), so a selection of these isolates and some azole-susceptible isolates for comparison were selected for resistance phenotyping using MIC assays with TEB, ITC, VOR, and POS.

In total, almost half of the 130 isolates tested by MIC (42.3%) were moderately-resistant to TEB (Table 2.2), suggesting that exposure to TEB had selected for resistant lineages. However, only 37 of these isolates grew on TEB-amended media, indicating that either the TEB media screening may be too selective or that TEB resistance in some lineages may still be evolving. Nearly 20% of the isolates tested were resistant to ITC, VOR, or POS, and of these 25 of the isolates were pan-azole resistant (Table 2.2 and Table 2.3). Twenty-nine of these isolates grew on the ITC-amended medium, however, so did 61 of the sensitive isolates, indicating that the ITC-amended medium is not selective enough. Compost, flower bulbs, and soil were the products of origin for most of the azole-resistant and pan-azole-resistant isolates aside from one isolate originating from peanut; however, the retail nut products that we screened, including peanut, pecan, and almond, had one isolate each that were resistant to ITC with a MIC of 2 µg/mL. All pan-azole-resistant isolates from flower bulbs were found on daffodil, *Gladiolus*, daylily, *Dahlia*, and tulip from the Netherlands or an unlisted location. The two azole-resistant isolates from flower bulbs were found on tulip from an unknown location and *Canna* from the United States/Costa Rica. All isolates with a pan-azole-resistant or a medical azole-resistant phenotype, except one from compost, were also resistant to TEB (Table 2.2 and Table 2.3). This finding along with the high proportion of TEB-resistant isolates suggests that resistance to TEB, an azole widely used in the environment, may either facilitate or be associated with higher levels of azole resistance, including medical azoles. This finding is consistent with other studies reporting that both the TR_{34}/L98H and the TR_{46}/Y121F/T289A alleles may be selected for by the presence of tebuconazole (23, 52). The pan-azole-resistant isolates generally had three resistance phenotype patterns: (1) high resistance to TEB and VOR (>16 ug/ml), and lower resistance to ITC and POS (0.5-8 µg/mL), (2) high resistance to TEB and ITC (16 µg/mL or greater, except in

one isolate with an ITC value of 4µg/mL), and lower resistance to VOR and POS (0.5-8 µg/mL), and (3) high resistance to TEB (8 µg/mL or greater), and lower resistance to ITC, VOR, and POS (0.125-4 µg/mL). These phenotypes corresponded with *cyp51A* genotypes. Isolates with phenotype 1 had TR_{46}/Y121F/T289A alleles, isolates with phenotype 2 had TR_{34}/L98H alleles, and isolates with phenotype 3 had non-TR alleles, mainly Y46F/V172M/T248N/E255D/K427E with 1 isolate also having a H147Y SNP (Table 2.3). Other studies have presented similar relationships among these phenotypes and genotypes (53, 54). The H147Y SNP has been found to cause elevated MIC values to ITC, VOR, and POS, so this is likely the resistance mechanism responsible for the pan-azole resistance in this isolate (55). It is interesting that the isolates with the lowest resistance to ITC, VOR, and POS have non-TR alleles shared with azole-susceptible isolates. It is possible that pan-azole resistance is being induced by exposure to agricultural azoles in these isolates, which is supported by the high ITC MIC values respective to ITC, VOR, and POS in these isolates. The resistance mechanism in these isolates is not *cyp51A*-based and another mechanism is responsible for the resistance. It is estimated that 50% of azole resistance is non-*cyp51A* based (56). Additional studies are needed to elucidate the mechanism of resistance of these isolates.

The isolates without tandem repeats in the *cyp51A* promoter region had a variety of SNPs present in their *cyp51A* genes. Some of these SNPs have been associated with azole-resistance, including Y46F, H147Y, V172M, T248N, E255D, K427E, G448S, 1242V and A9T (13, 34, 55, 57, 58). However, only H147Y and G448S have been determined to cause azole resistance. V172I has not been reported in *A. fumigatus,* but it has been reported in *A. nidulans* (59). Further studies are required to determine the effect of these SNPs, if any, on azole resistance.

To understand how isolates from commercial products are related to isolates from the environments where they are produced and to clinical isolates in this region we genotyped 95 of the isolates collected in this study with 9 previously developed microsatellite markers (28). We found high genetic diversity among isolates collected from the same settings and substrates and isolates from different settings and substrates, indicating that populations are not obviously associated based on product of origin, environment, or geographic location (Fig 2.4, Fig 2.5, Fig 2.6). We did find that isolates with TR alleles were genetically structured, which is consistent with previous studies (31, 33-35). With DAPC (49), we identified six genetic clusters among our isolates, and environmental and clinical isolates collected from Georgia and Florida. Isolates from clusters 1 and 6 belonged to distinct branches in the minimum spanning networks based on both Nei's genetic distance (46) and Bruvo's genetic distance models (44), supporting genetic structure among isolates in those branches and the rest of the isolates (Fig 2.6). Isolates within these two clusters had similar *cyp51A* genotypes, with the majority of isolates having TR alleles in cluster 6 and the T248N/E255D allele in cluster 1, which suggests that the clusters are somewhat structured by *cyp51A* genotype and resistance phenotype (Fig 2.5D). Isolate 385 from soil was an exception, where it had a TR_{46} allele but belonged to cluster 5. This could be from recombination since it shares all 9 microsatellite markers with isolate 386 from the same sample; however, 386 is azole susceptible. The isolates found in cluster 1 were exclusively collected from commercial products in this study and all had the T248N/E255D allele, and similar clusters of isolates only from commercial products have not been reported in previous studies. More research is needed to determine if this cluster is more widely present in other environments and if these isolates cluster due to a reason other than commercial product origin or their *cyp51A*

genotype. Another explanation for this could be that these alleles could have arisen through homoplasy, which may misrepresent haplotype distance using the stepwise mutation model (60).

Surveying new environments and substrates for azole-resistant *A. fumigatus* is helpful, especially in the United States where environments have not been investigated as thoroughly as Western Europe, India, and other regions around the world, to determine how widespread azole-resistance is in the environments, to find new locations and reservoirs of these isolates, and to identify hotspots where azole resistance is prevalent. Consistent with previous studies (3, 19, 39, 50), we found that commercial flower bulbs and compost were hotspots of azole-resistant *A. fumigatus* and identified that commercial raw peanuts, almonds, and pecans are potential reservoirs of these isolates. These items and hotspots could pose a risk to people who are immunocompromised or have decreased lung function, so we suggest caution for at-risk groups when interacting with these products. Determining the population structure and diversity of clinical and environmental *A. fumigatus* isolates is important to assess risk for the development of azole resistance in different environments, and to elucidate the evolutionary origins of these mechanisms of azole resistance in the environments. Identifying the evolutionary relationship among the isolates in this study and other studies could help point to how these genotypes are arising and spreading around the globe and could eventually assist in preventing azole-resistant aspergillosis infections in susceptible patients in high-risk environments.

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Table 2.1 *Aspergillus fumigatus* isolates obtained from commercial products.

Product	Total Isolates	Growth on TEB[1]	Growth on ITC[2]	Selected for MIC[3]
Almond	2	0	0	2
Apple	2	0	0	0
Grape	35	0	0	8
Peanut	147	0	33	33
Pecan	12	1	0	6
Flower Bulb	109	42	51	20
Commercial compost	133	35	76	44
Commercial soil	85	7	26	17
Total	525	85	186	130

[1] Isolates that were able to grow on SDA amended with 3 µg/mL of tebuconazole
[2] Isolates that were able to grow on SDA amended with 3 µg/mL of itraconazole
[3] A selection of isolates chosen for further phenotypic analysis via MIC assays

Table 2.2 Azole-resistance phenotypes of select *Aspergillus fumigatus* isolates from commercial products.

Product	Isolates Assayed by MIC	Resistant Isolates (%)			
		TEB[1]	ITC[2]	VOR	POS
Almond	2	2 (100.0%)	1 (50.0%)	0	0
Apple	0	0	0	0	0
Grape	8	0	0	0	0
Peanut	33	4 (12.1%)	2 (6.1%)	1 (3.0%)	1 (3.0%)
Pecan	6	4 (66.7%)	1 (17.7%)	0	0
Flower Bulb	20	15 (75.0%)	9 (45.0%)	15 (75.0%)	13 (65.0%)
Commercial compost	44	25 (56.8%)	10 (22.7%)	9 (20.5%)	9 (20.5%)
Commercial soil	17	5 (29.4%)	1 (5.9%)	1 (5.9%)	1 (5.9%)
Total	130	55 (42.3%)	24 (18.5%)	26 (20.0%)	24 (18.5%)

[1]Resistance value set at >2µg/mL based on prior studies (Kang, 2020)
[2]Resistance breakpoints for ITC, VOR, and POS based on EUCAST 2020 breakpoints: Res to ITC and VOR >1 µg/mL, POS >0.25 µg/mL

Table 2.3 Pan-azole-resistant isolates of *Aspergillus fumigatus* obtained from commercial products.

Isolate	Product	MIC Value (µg/mL)				*cyp51A* genotype
		TEB	ITC	VOR	POS	
130	Peanut	16	16	8	2	TR_{34}/Y46F/L98H/V172M/T248N/E255D/K427E
419	Flower bulb	>16	8	>16	4	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E/G448S
432	Flower bulb	>16	4	>16	2	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E/G448S
487	Flower bulb	>16	1	>16	1	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289AK427E/G448S
496	Flower bulb	>16	2	>16	1	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E/G448S
501	Flower bulb	>16	1	>16	1	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E/G448S
449	Flower bulb	>16	1	>16	0.5	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E
483	Flower bulb	>16	1	>16	1	TR_{46}/Y46F/Y121F/V172I/T248N/E255D/T289A/K427E
448	Flower bulb	>16	4	4	0.5	TR_{34}/Y46F/L98H/V172M/T248N/K427E
433	Flower bulb	>16	>16	8	2	TR_{34}/Y46F/L98H/V172M/T248N/E255D/K427E
434	Flower bulb	>16	>16	8	1	TR_{34}/Y46F/L98H/V172M/T248N/E255D/K427E
438	Flower bulb	>16	>16	8	1	TR_{34}/Y46F/L98H/V172M/T248N/E255D/K427E
439	Flower bulb	>16	>16	8	1	TR_{34}/Y46F/L98H/V172M/T248N/E255D/K427E
506	Flower bulb	>16	>16	8	1	TR_{34}/Y46F/L98H/V172M/T248N/E255D/K427E
245	Commercial compost	16	4	4	0.5	Y46F/V172M/T248N/E255D/K427E
247	Commercial compost	16	4	4	0.5	Y46F/V172M/T248N/E255D/K427E
253	Commercial compost	16	2	2	0.5	Y46F/V172M/T248N/E255D/K427E
257	Commercial compost	16	4	4	1	Y46F/V172M/T248N/E255D/K427E
263	Commercial compost	16	2	2	0.125	Y46F/V172M/T248N/E255D/K427E
237	Commercial compost	8	2	4	0.5	Y46F/H147Y/V172M/T248N/E255D/K427E
398	Commercial compost	>16	2	>16	0.5	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E
399	Commercial compost	>16	2	>16	1	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E
206	Commercial compost	16	4	2	0.5	Not sequenced
385	Commercial soil	>16	2	>16	0.5	TR_{46}/Y46F/Y121F/V172M/T248N/E255D/T289A/K427E

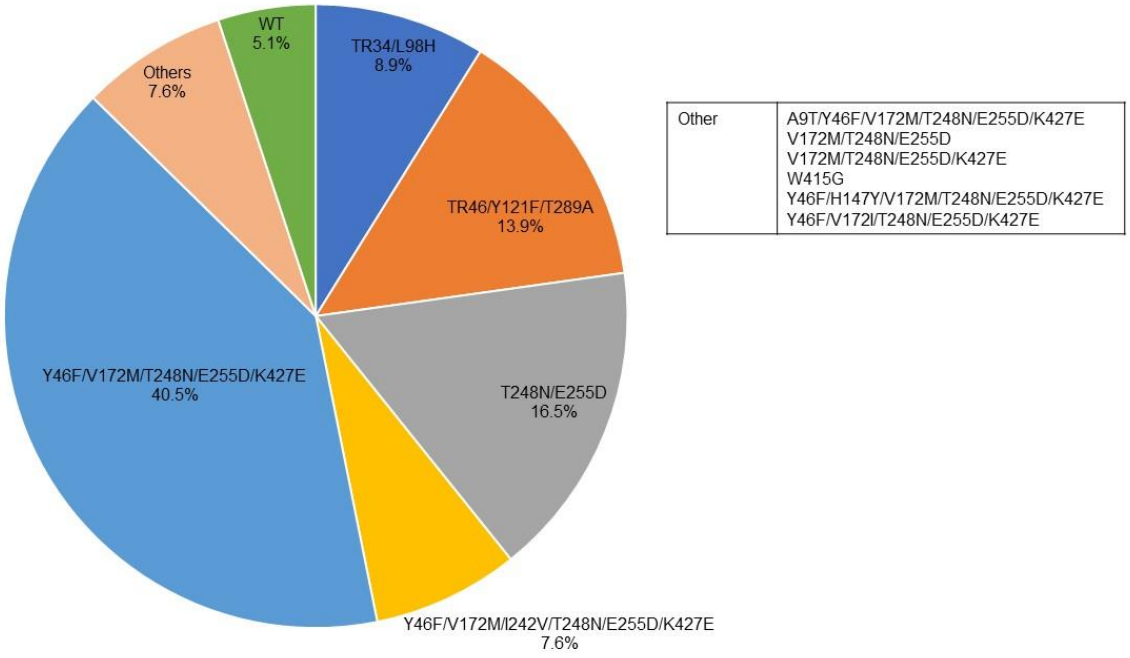
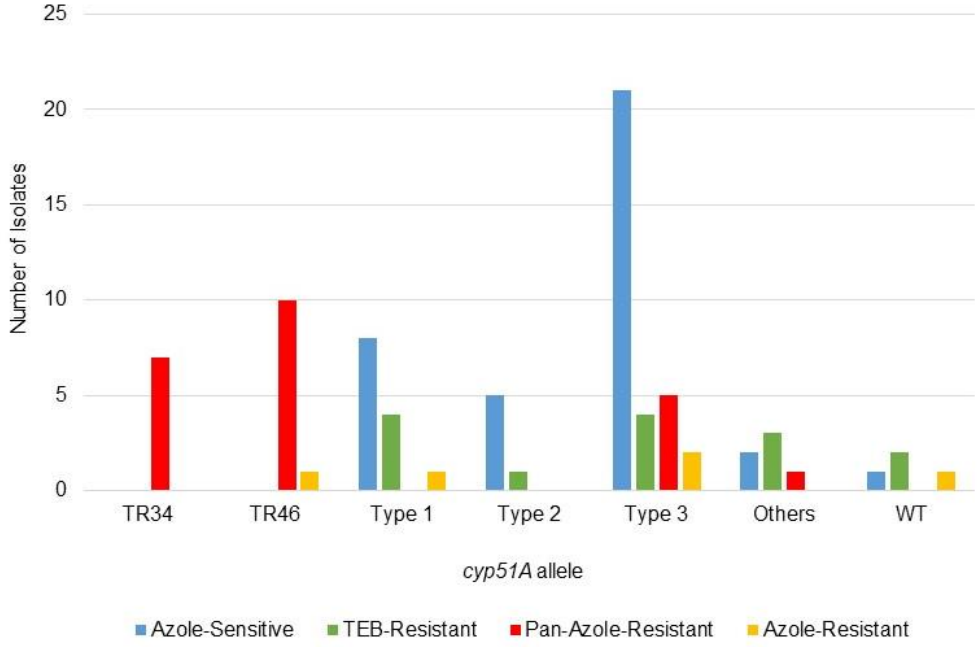
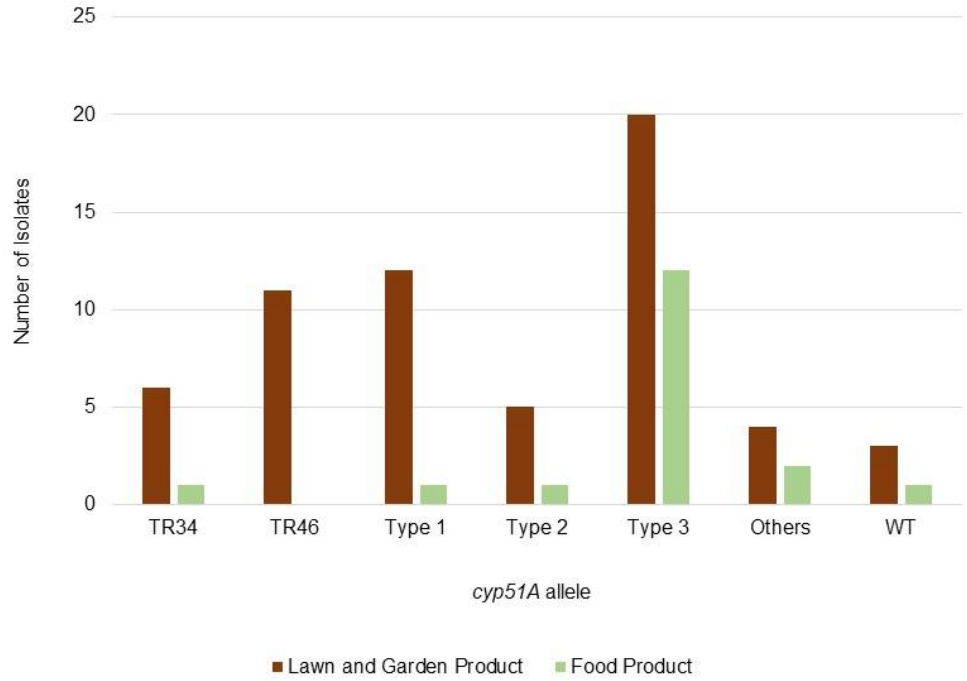


Figure 2.1. *cyp51A* genotypes of 130 select isolates of *Aspergillus fumigatus* from commercial products. TR_{34}/L98H indicates all isolates with a 34 base pair tandem repeat in the *cyp51A* promoter. TR_{46}/Y121F/T289A indicates all isolates with a 46 base pair tandem repeat in the *cyp51A* promoter.



TR_{34}	TR_{34}/L98H
TR_{46}	TR_{46}/Y121F/T289A
Type 1	T248N/E255D
Type 2	Y46F/V172M/I242V/T248N/E255D/K427E
Type 3	Y46F/V172M/T248N/E255D/K427E
WT	WT
Other	A9T/Y46F/V172M/T248N/E255D/K427E V172M/T248N/E255D V172M/T248N/E255D/K427E W415G Y46F/H147Y/V172M/T248N/E255D/K427E Y46F/V172I/T248N/E255D/K427E

Figure 2.2. Azole-resistance phenotype of *Aspergillus fumigatus* isolates with each *cyp51A* genotype . Pan-azole-resistant denotes resistance to multiple medical azoles and TEB. Resistant to TEB denotes only resistance to tebuconazole. Azole-sensitive denotes no resistance to azoles. Azole-resistant denotes resistance to only 1 medical azole and possibly TEB. TR_{34} indicates the TR_{34}/L98H allele. TR_{46} indicates the TR_{46}/Y121F/T289A allele. Type 1 indicates the T248N/E255D allele. Type 2 indicates the Y46F/V172M/I242V/T248N/E255D/K427E. Type 3 indicates the Y46F/V172M/T248N/E255D/K427E allele. WT indicates a wild-type allele with no SNPs. Other indicates a complete unique allele.



TR_{34}	TR_{34}/L98H
TR_{46}	TR_{46}/Y121F/T289A
Type 1	T248N/E255D
Type 2	Y46F/V172M/I242V/T248N/E255D/K427E
Type 3	Y46F/V172M/T248N/E255D/K427E
WT	WT
Other	A9T/Y46F/V172M/T248N/E255D/K427E V172M/T248N/E255D V172M/T248N/E255D/K427E W415G Y46F/H147Y/V172M/T248N/E255D/K427E Y46F/V172I/T248N/E255D/K427E

Figure 2.3. Product of origin for isolates of *Aspergillus fumigatus* with each *cyp51A* genotype. Food product denotes almond, grape, peanut, and pecan. Lawn and garden product denotes compost, soil, and bulbs. TR_{34} indicates the TR_{34}/L98H allele. TR_{46} indicates the TR_{46}/Y121F/T289A allele. Type 1 indicates the T248N/E255D allele. Type 2 indicates the Y46F/V172M/I242V/T248N/E255D/K427E. Type 3 indicates the Y46F/V172M/T248N/E255D/K427E allele. WT indicates a wild-type allele with no SNPs. Other indicates a complete unique allele.

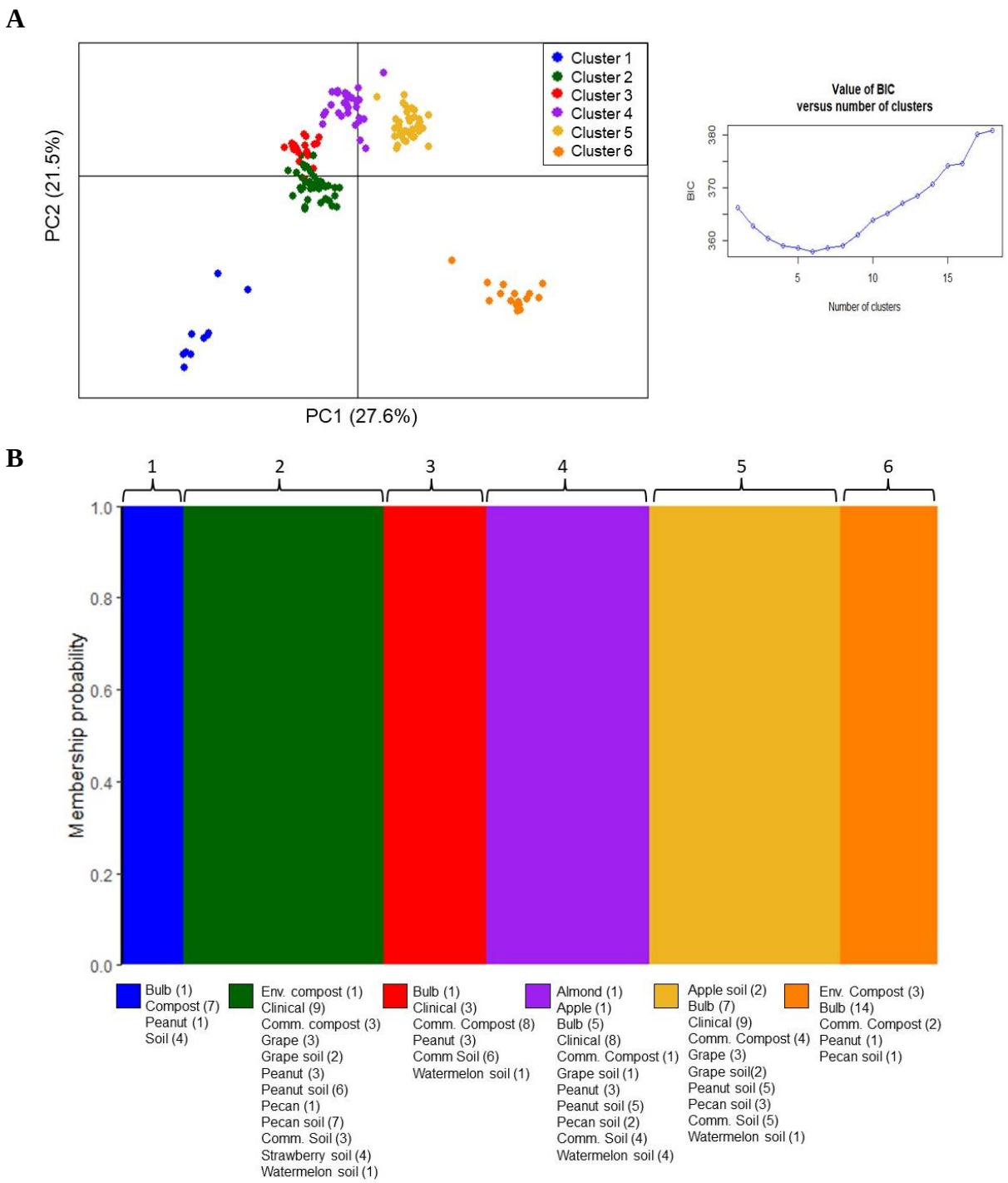
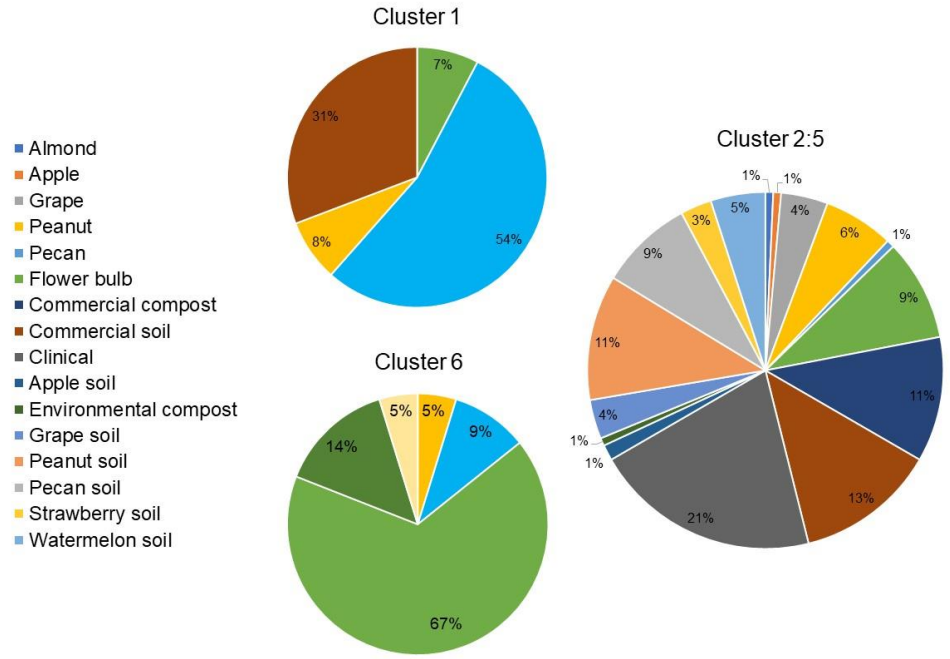
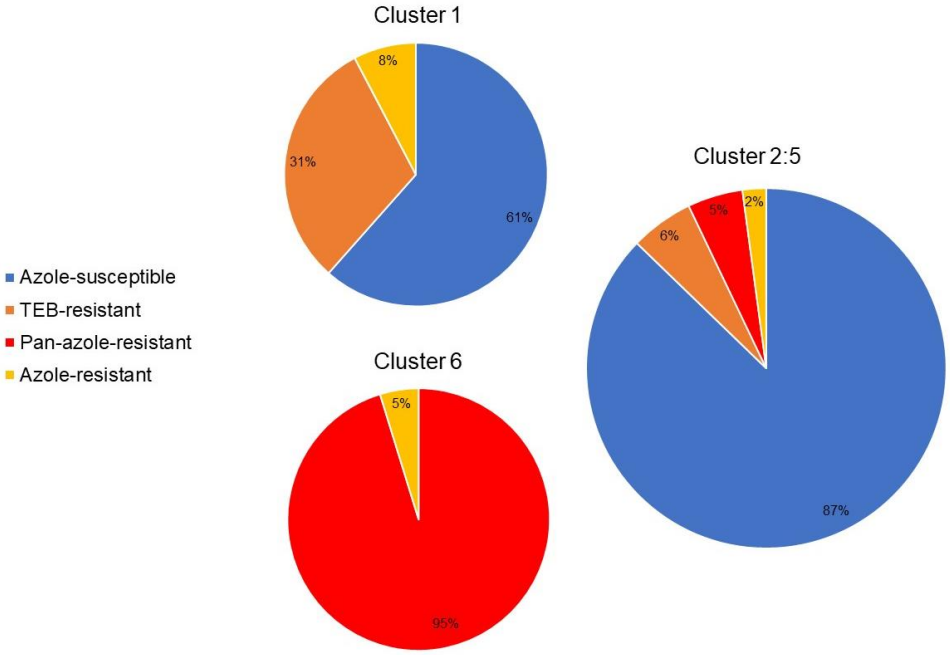


Figure 2.4. Clustering based on discriminate analysis of principal components (DAPC.) A. DAPC scatter plot of the first two principal components. Each dot represents an isolate and the color shown indicates the cluster composition. The Bayesian information criterion (BIC) for each K is shown in the upper right-hand corner. B. Histogram of membership probability of 185 *A. fumigatus* isolates based on DAPC. The color shown indicates the cluster composition.

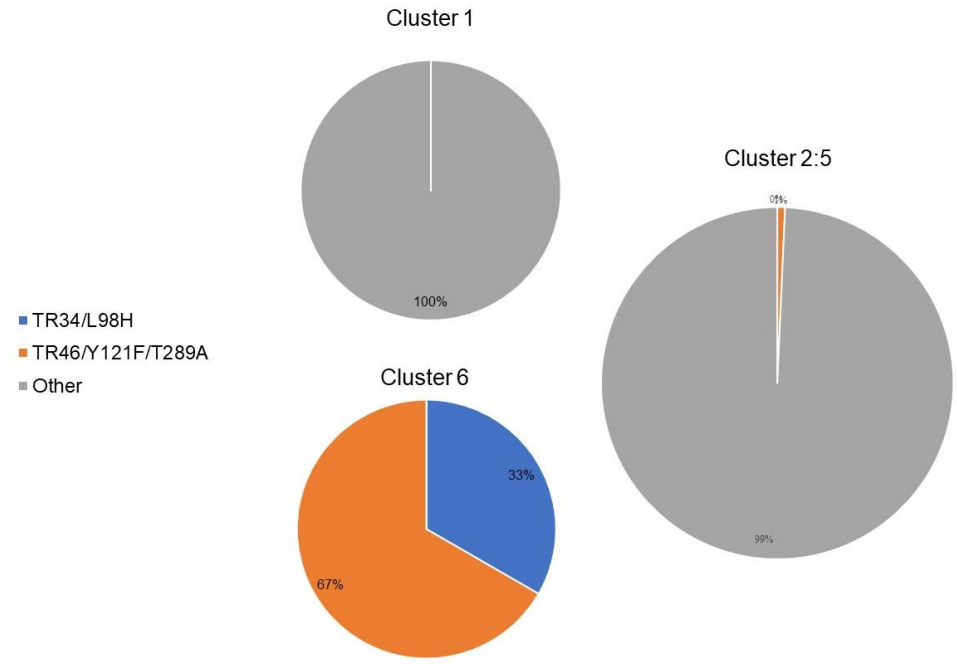
A


Cluster 1
7%
31%
8%
54%
Cluster 2:5
1%
1%
4%
6%
1%
9%
11%
4%
1%
1%
21%
13%
11%
9%
3%
5%
Cluster 6
5%
5%
9%
14%
67%
Almond
Apple
Grape
Peanut
Pecan
Flower bulb
Commercial compost
Commercial soil
Clinical
Apple soil
Environmental compost
Grape soil
Peanut soil
Pecan soil
Strawberry soil
Watermelon soil

B


Cluster 1
8%
31%
61%
Cluster 2:5
2%
5%
6%
87%
Cluster 6
5%
95%
Azole-susceptible
TEB-resistant
Pan-azole-resistant
Azole-resistant

C



D

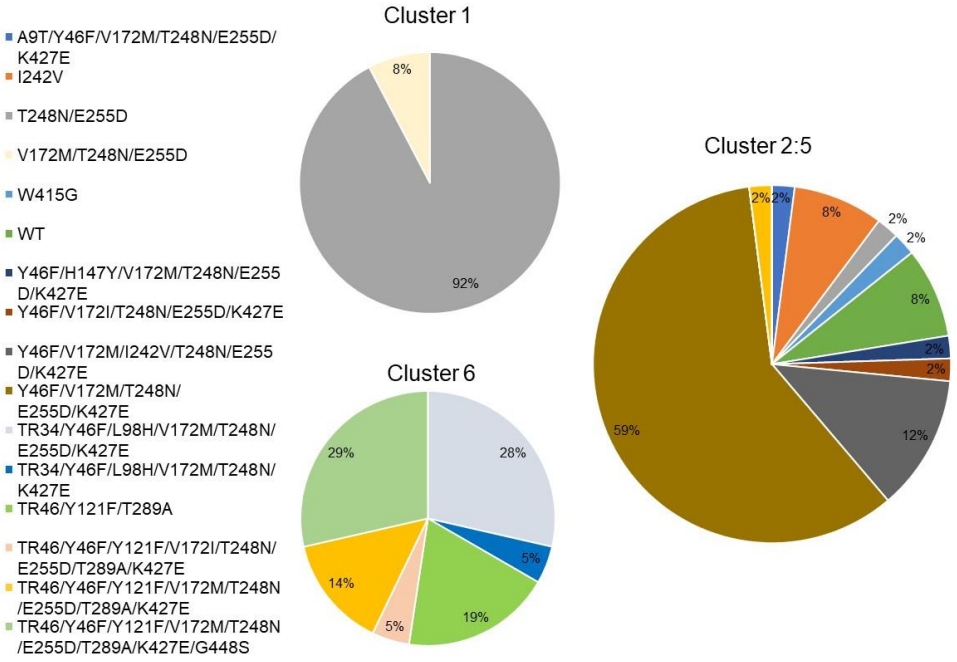
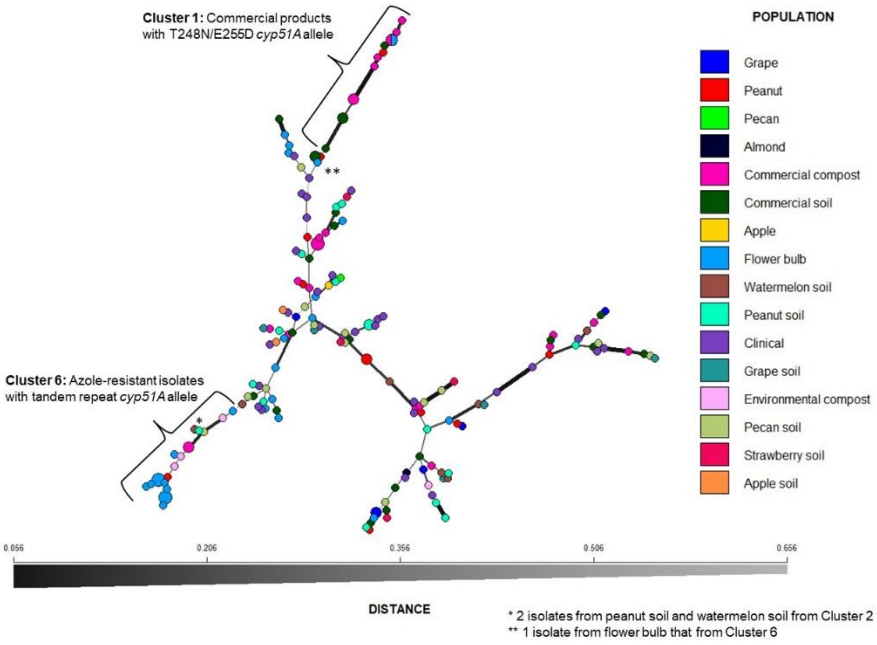


Figure 2.5. Pie charts showing the proportion of *Aspergillus fumigatus* isolates in each cluster by A. Setting or substrate of origin. B. Azole-resistance phenotype. C. Presence of a tandem repeat in the *cyp51A* promoter region. D. *cyp51A* genotype.

A



B

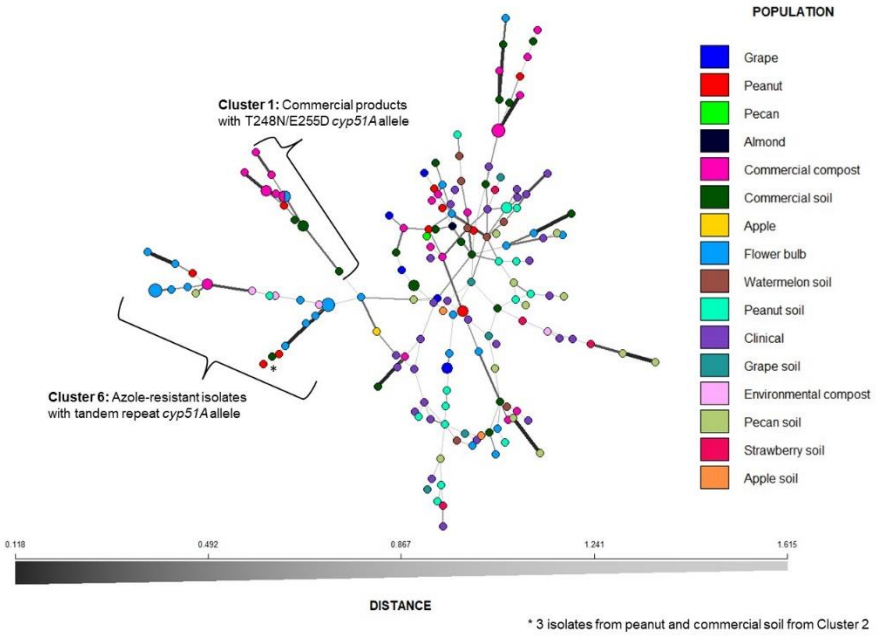


Figure 2.6. Minimum spanning networks based on A. Bruvo's genetic distance and B. Nei's genetic distance. Each circle represents a microsatellite genotype. The size of the circle indicates how many isolates have that genotype. The color of the circles indicates the original substrate of origin. The line thickness and color represent genetic distance between each isolate.

CHAPTER 4

CONCLUSIONS

Azole-resistant *A. fumigatus* has been found in many environments across the world. It is most commonly-documented in Europe, but has also been found in Asia, Africa, South America, North America, India, and the Middle East. Isolates of azole-resistant *A. fumigatus* were most commonly documented in soil, air, and plant debris across the world. Developed environments were the most common environments to find azole-resistant isolates, but agricultural environments were the most commonly sampled. This indicates that developed environments may be under-sampled and should be more thoroughly surveyed for resistance. Very few studies across the world viewed commercial products, and those that did found azole-resistance in flower bulbs, soil, and compost. We chose to survey commercial products in the United States in order to document the extent of azole-resistant *A. fumigatus* in these products since they could serve as reservoirs for these potentially deadly strains. We identified azole-resistant isolates in peanuts, pecans, almonds, soil, compost, and flower bulbs. Pan-azole-resistant isolates were found in compost, soil, flower bulbs, and peanuts. The peanuts were from the US, but the other products had mainly unknown origins, or origins outside of the United States, such as the Netherlands. The dominant mechanisms of pan-azole-resistance found were TR_{34}/L98H and TR_{46}/Y121F/T289A alleles, but we also found resistant isolates with the H147Y mechanism and non-*cyp51A* mechanisms of resistance as evident by no tandem repeats or SNPs directly tied to resistance in *cyp51A.* Population genetic analyses of these isolates along with others from a survey of agricultural and clinical environments in the southeastern United States found that

these isolates form 3 clusters loosely based on *cyp51A* genotype rather than product of origin. This is consistent with previous population genetics studies of azole-resistant *A. fumigatus.* Further surveys of commercial products throughout the United States and throughout the world is needed to document the spread of azole-resistance into unsuspected areas.