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Investigating the Impact of Environmentally Relevant Imidazole Concentrations on the Antifungal Susceptibility and Community Composition of Soil Fungi

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Abstract

Miconazole and clotrimazole are environmentally-persistent drugs that are entrained into crop soils through the application of biosolids. There is concern that environmental exposure to such azole antifungals, which inhibit fungal growth by disrupting the production of the fungal cell membrane component ergosterol, promotes resistance in clinically or agriculturally relevant fungi. Thus, either environmentally-relevant or excessive levels of these drugs were applied to microplots over ten years and compared with drug-free plots. Overall, ergosterol quantification, plates counts, and identification of >250 fungal isolates showed lower fungal counts and species richness in plots receiving excessive drug amounts. In addition, fungi from treated plots did not show increased resistance to a panel of medical and agricultural azole drugs in disk diffusion assays. Altogether, while the highly contaminated soils showed lower fungal counts, lower species richness, and fewer isolates highly-susceptible to miconazole, increased resistance to azoles was not evident at environmentally relevant concentrations.

Keywords: Antifungal resistance, mycology, fungal community diversity, fungal infections, biosolids, wastewater contaminants

Summary for Lay Audience

Products sold to treat fungal infections –such as those of the hair, skin, nails, and urogenital tract– include drugs that specifically kill or slow the growth of fungi. These products tend to be washed down the shower, sink, or toilet and make their way into the sewage system. Following sewage treatment, some traces of drugs remain, and two medical antifungal drugs that particularly accumulate and persist in the environment, possibly due to their use in over-the-counter products, are clotrimazole and miconazole. Although not used to treat systemic infections, these drugs, which are chemically defined as azoles, are chemically related to azoles used to treat life-threatening, systemic fungal infections and agricultural azoles used to control crop diseases in agriculture. This reliance on azole products has raised concerns over azole contamination of the environment, resulting from the application of biosolid fertilizer derived from sewage treatment products onto soils. More specifically, there is concern that azole contamination of soils can shift the soil fungal community's population structure and lead to the selection of azole-resistant phenotypes.

To address these concerns, from 2010 to 2020, amounts of clotrimazole and miconazole typical to those found in biosolid fertilizer were annually added to 2 m^2 plots of soil grown with soybeans. Soil from these microplots, in addition to identical microplots receiving no drugs, were sampled, starting in 2018, for fungal isolation. The resulting fungal libraries were analyzed for changes in composition between the control and treated microplots. In addition, fungal libraries from the treated plots were compared to libraries from the control plots for their susceptibility to clotrimazole, miconazole, medical azoles used for systemic infections, and agricultural azoles used for crop protection.

Overall, it was found that the azole-treated soils showed lower fungal counts compared to the untreated soils and the soils treated with excess clotrimazole and miconazole showed lower species richness. On the other hand, there was no clear evidence that the fungal community recovered from the azole-contaminated soils were less susceptible to azoles than fungi recovered from the control plots. Nonetheless, several species with medical and agricultural significance were recovered from

the contaminated soils, highlighting the importance of monitoring soil fungi for increased azole resistance.

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List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
CLSI	Clinical and Laboratory Standards Institute
C-TAB	Cetrimonium bromide
CTZ	Clotrimazole
CZ	Czapek-Dox medium
DBDM	Dekkera/Brettanomyces differential medium
DG	Dichloran glycerol medium
DIF	Difenoconazole
DMSO	Dimethyl sulphoxide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FCZ	Fluconazole
GYP	Glucose-yeast extract-peptone medium
ITR	Itraconazole
LB	Luria-Bertani medium
MCZ	Miconazole
ME	Malt extract medium
MH	Mueller-Hinton medium
MIC	Minimum inhibitory concentration
PD	Potato dextrose medium
PRO	Propiconazole
RAPD	Random amplified polymorphic DNA
RB	Rose bengal medium
RPMI-1640	Roswell Park Memorial Institute 1640 medium
SD	Sabouraud dextrose medium
TEB	Tebuconazole
VRZ	Voriconazole
YEPD	Yeast extract-peptone-dextrose medium
YM-11	Yeast-mold 11 medium
ZOI	Zone of inhibition

Table of Contents

List of Figures

List of Tables

1   Introduction and Literature review

1.1 History and use of antifungal agents

Although the practice of treating microbial infections with biological preparations dates back to ancient cultures, German physician Paul Ehrlich kickstarted research into the synthesis of antimicrobial compounds derived from dyes in the early 1900s. However, possibly due to the relative rarity of fungal infections prior to the HIV/AIDS epidemic and lack of fungal targets that are not shared with humans, the development of antifungals lagged behind the development of antibacterials (Larkin *et al.*, 2019). Nonetheless, ergosterol, an essential fungal cell wall component not found in human cells, was found to be a suitable molecular target for the polyene and azole classes of antifungal drugs (Dixon & Walsh, 1996). Thus, during the 1940's to 1950's, treatment for fungal infections transitioned from relying on physical interventions, such as radiation therapy, and repurposed medications, such as potassium iodide and sulfonamide antibiotics, to pharmaceuticals designed for a fungal-specific mode of action (Larkin *et al*., 2019).

The polyene drugs are defined by macrocyclic rings containing polyene structures, which consist of multiple alternating single carbon-carbon and double carbon-carbon bonds, and are amphiphilic, meaning that they have hydrophilic and hydrophobic regions. These drugs primarily work by binding to ergosterol on the fungal cell surface, which results in the formation of pores through which cytoplasmic contents can leak through (Dixon & Walsh, 1996). Nystatin and amphotericin B, both isolated from *Streptomyces* species in 1950 and 1955, respectively, entered clinical use in the late 1950's (Larkin *et al*., 2019).

Subsequently, the azole drugs clotrimazole, miconazole, and econazole were developed and entered the market from the late 1960's to the early 1970's (Larkin *et al*., 2019). Azole drugs bind to and inhibit the enzyme lanosterol 14 α-demethylase (CYP51A1), which catalyzes the demethylation of lanosterol to ergosterol (Dixon & Walsh, 1996). Effects of this disruption include reduced membrane fluidity due to the absence of ergosterol, the build-up of toxic 14α-methylsterol by-products, and, possibly, an increase in saturated fatty acid content in the membrane leading to

further reduced membrane fluidity (Dixon & Walsh, 1996; Larkin *et al.*, 2019). In addition, at higher concentrations than those that produce effects via CYP51A disruption, azole drugs have an immediately lethal effect through direct membrane damage to both eukaryotic and prokaryotic cells (Van den Bossche *et al.*, 1983). Chemically, azoles are compounds defined by a five-membered ring containing two nitrogen atoms for imidazoles, or three nitrogen atoms for triazoles (Dixon & Walsh, 1996).

It has been estimated that while infectious diseases were the cause of one death per thousand deaths in the pre-antibiotic era of the 1940's, the age-standardized rate dropped to 0.1 per thousand in the 1970's due to widespread adoption of antibiotics (Griffiths & Brock, 2003). While the societal benefit of antifungal drugs has been less quantified than those of antibacterial drugs, the development of antifungal drugs has undoubtedly reduced the mortality rate associated with immunodeficiency and reduced the burden of dermatological fungal infections. For example, in a model of 40-year old patients with acute myeloid leukemia, it was estimated that prophylactic fluconazole administration approximately halved the probability of developing a fungal infection, from 0.157 to 0.076, and also slightly reduced the probability of dying from the infection, from 0.285 to 0.238, for an overall increased life expectancy of 0.9 years (Nomura *et al*., 2006).

1.2   Clinical, community, and agricultural use of antifungals

As of 2019, the World Health Organization List of Essential Medicines includes the imidazoles clotrimazole and miconazole (Figure 1), which are suitable for oral and topical use, and the second-generation triazoles fluconazole, itraconazole, and voriconazole (Figure 2), which are suitable for intravenous use (World Health Organization, 2019). In addition, the polyenes, nystatin and amphotericin B, and squalene monooxygenase inhibitors, such as terbinafine, are included (World Health Organization, 2019). Other effective antimycotic agents on the list include inorganic compounds, such as selenium sulfide and sodium thiosulfate, and small molecules that inhibit intracellular fungal targets, such as griseofulvin and flucytosine, which inhibit tubulin and thymidylate synthase, respectively (World Health Organization, 2019).
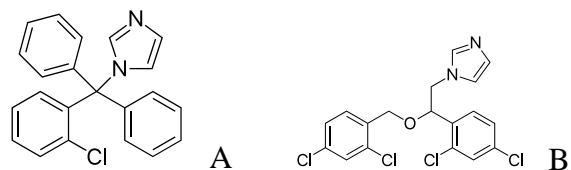
Figure 1 – Structures of select medical imidazoles

Clotrimazole (A) and miconazole (B) are medical imidazoles. Each is found as the active ingredient in around 300 over-the-counter products in Canada to treat athlete's foot (tinea pedis), jock itch (tinea cruris), ringworm (tinea corporis), superficial yeast growth on skin (tinea versicolor), a well as vaginal yeast infections and diaper rash (Wishart *et al*., 2018). Clotrimazole can be prescribed or found in over-the-counter medications in forms such as topical creams (typically 10 mg/g) and 10 mg lozenges. Miconazole is found in slightly higher concentrations for creams (20 mg/g) and tablets (50 mg) (Wishart *et al*., 2018). Neither medication is approved for systemic use.
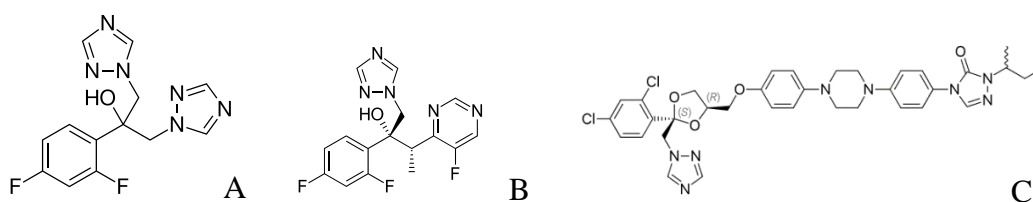
Figure 2 – Structures of select medical triazoles

Fluconazole (A) is a medical triazole used for the treatment of cryptococcal meningitis, *Candida* infections of various types, such as vaginal, systemic, and esophageal, as well as prevention of fungal lung disease in susceptible populations. It is available by prescription for intravenous use (2 mg/mL) and by prescription or over-the-counter in the form of 50-200 mg tablets. Voriconazole (B) is prescribed for intravenous use (10 mg/mL), and in tablet form (50-200 mg); however, it is not available over the counter (Wishart *et al*., 2018). It is approved for use for esophageal candidiasis in addition to invasive aspergillosis, which is intrinsically resistant to fluconazole (Leonardelli *et al*., 2016), and *Fusarium* infections, which are resistant to fluconazole and itraconazole (Stanzani *et al.*, 2007). Itraconazole (C) is not available over-the-counter or for intravenous use but can be prescribed for tablet/capsules (65-200 mg) and as an oral solution (10 mg/mL). Itraconazole is approved for a wider variety of fungal infections, including blastomycosis, histoplasmosis, aspergillosis, and onychomycosis, as well as preventing fungal infections in cystic fibrosis patients (Wishart *et al*., 2018).

According to the Medical Expenditure Panel Survey compiled by the U.S. Department of Health and Human Services, Agency for Healthcare Research and Quality, fluconazole was the most widely prescribed antifungal drug with 3.5 million annual prescriptions in the U.S. in 2017, followed by nystatin and ketoconazole, another medical imidazole, with 2.5 million prescriptions each (Agency for Healthcare Research and Quality, 2018). Less commonly prescribed antifungal drugs include clotrimazole, alone or in a mixture with the anti-inflammatory drug metamethasone, at 1 million and 850,000 annual prescriptions respectively, terbinafine at 1 million prescriptions, and miconazole with 225,000 prescriptions (Agency for Healthcare Research and Quality, 2018).

1.3 Fate of antifungals in the environment

Due to previous studies implying that soil environments can act as a reservoir of azole-resistant fungi that can cause mycosis in humans, it is essential to determine what concentration of azole drugs in the soil can promote antifungal resistance. Pathways leading to azole contamination of soils include applying agricultural antifungals to product crops against plant-pathogenic fungi, such as difenoconazole, tebuconazole, and propiconazole (Figure 3), as well as the application of antifungal-contaminated biosolids to agricultural lands.
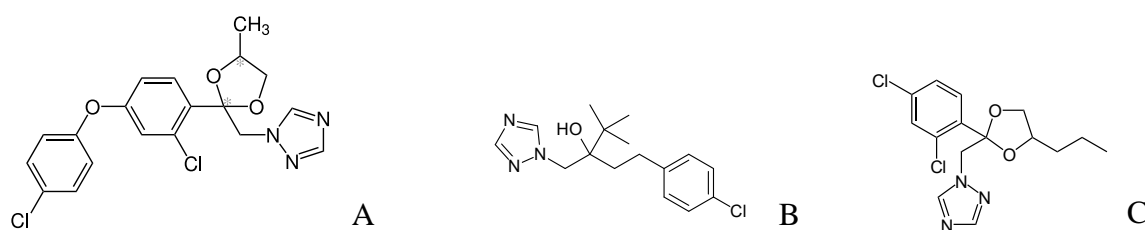
Figure 3 – Structures of select agricultural triazoles

Difenoconazole (A), tebuconazole (B), and propiconazole (C) are broad-spectrum fungicides approved for use in Canada to control fungal growth on turfgrass, wood, and crops such as cereals. To control plant pathogens, these agricultural triazoles can be applied via foliar application or seed treatment (Pest Management Regulatory Agency, 2016c, 2016b, 2016a).

During primary treatment of wastewater, solids are allowed to settle, and these solids are then biologically digested to create biosolids. Biosolids are then applied to agricultural fields as sources

of nutrients, such as nitrogen and phosphate, and micronutrients, such as copper, iron, molybdenum, and zinc. In Ontario, the application of biosolids to agricultural fields is governed by stringent regulations that consider parameters such as field slope, distance to residential areas, and amounts of plant-available nitrogen and phosphate (Government of Ontario, 2002).

A 2007 survey of Swedish sewage treatment plants estimated that 41% of purchased miconazole and all purchased clotrimazole could be accounted for in sewage sludge (Lindberg *et al.*, 2010). Furthermore, miconazole has previously been detected at concentrations of 477 μg/kg and 341 μg/kg in biosolids originating from Niagara Region sewage treatment facilities (Sabourin *et al.*, 2012) and the Robert O. Pickard Environmental Centre in Ottawa (Gottschall *et al.*, 2012), respectively. Clotrimazole was detected at a concentration of 492 μg/kg in biosolids originating from a Beijing sewage treatment plant (Chen *et al.*, 2013).

Following the application of biosolids, miconazole and clotrimazole persist in soils. A concentration of 127 μg miconazole/kg soil was detected 400 days following application of the Ottawa biosolids to a field at a rate of 22 Mg dry weight per ha (Gottschall *et al.*, 2012). Likewise, plots receiving three annual applications of the Beijing biosolids at a rate of 60 Mg/ha contained up to 65 and 41 μg miconazole and clotrimazole, respectively, per kg of soil (Chen *et al.*, 2013). Microcosm studies have shown that the half-life of clotrimazole decreases with temperature, with a half-life in loam soil of 275 days at 4°C and 68 days at 30°C (Sabourin *et al.*, 2011). In addition, in sludge-amended sandy-loam soil, the half-life of clotrimazole decreased from 126 to 29 days, as moisture content increased from 4.5 to 11% (García-Valcárcel & Tadeo, 2012)

1.4   Emergence of resistance to antimicrobial agents

The Centers for Disease Control and Prevention published in its *Antibiotic Resistance Threats in the United States, 2019* report that over 2.8 million cases of antibiotic-resistant infections—mostly caused by bacteria—in the U.S. were causing over 35,000 deaths per year (CDC, 2019). Multiple factors lead to the evolution, selection, and spread of antibiotic and antifungal resistance. These include the ability of the microorganisms to develop resistance due to over-use or inappropriate use of antibiotic or antifungal drugs, the spread of drug-resistant strains in environments such as

hospitals, and, in bacteria, the spread of genes conferring drug-resistance to drug-susceptible bacteria via plasmids, bacteriophages, or uptake of naked DNA (Levy, 2002). More specifically, fungi can develop resistance to azole drugs via upregulation of drug and toxin-removing efflux pumps, upregulation of CYP51A1-encoding genes, and modifications of the CYP51A1-azole binding sites (Fisher *et al.*, 2018).

The development of resistance to fluconazole, the most commonly prescribed azole drug, is exacerbated by the fact that fluconazole is fungistatic and not fungicidal, thus allowing for directional selection of the resistant phenotype among exposed isolates, and the fact that fluconazole resistance can occur by multiple mechanisms (Anderson, 2005). It follows that the international ARTEMIS Antifungal Surveillance Program detected that fluconazole resistance in the yeast *Candida glabrata* increased from 9% to 14% from 1992–2001 to 2001–2007 (Pfaller, Messer *et al.*, 2009). In addition, from 2005 to 2007, increases in voriconazole resistance in multiple clinical species of *Candida* have been found, including in *C. famata* (from 1% to 6%), *C. norvegensis* (0% to 7%), *C. lipolytica* (0% to 11%), and *C. pelliculosa* (14% to 17%) (Pfaller *et al.*, 2010). In addition, *Candida auris* is a multi-azole resistant pathogen first isolated in 2009; however, it has been argued that the recent spread of the species may be due to climate change as opposed to azole overuse (Casadevall, 2019).

1.5   Evidence of cross-resistance between azoles

Several *in vitro* studies have shown that the evolution of resistance to one azole, whether used for agricultural or clinical purposes, leads to increased resistance to other azoles. For example, following gradual adaptation of up to 30 μg/mL tebuconazole over eight weeks, the maize filamentous fungal pathogen *Colletotrichum graminicola* showed increased resistance to clotrimazole and miconazole in a skin infection assay (Serfling *et al.*, 2007). Likewise, isolates of the plant pathogen *Ustilago avenae* selected for resistance to the agricultural azole tetraconazole were also more resistant to clotrimazole, miconazole, and itraconazole than unadapted isolates (Köller & Wubben, 1989). Another study showed that a *Candida parapsilosis* strain selected for lower sensitivity to tetraconazole was also less sensitive to fluconazole, voriconazole, and itraconazole (Rocha *et al.*, 2016). Simultaneous incubation with promethazine, an efflux pump

inhibitor, did not lower the minimum inhibitory concentration (MIC) values to fluconazole, leading to the suggestion that increases in efflux pump activity could not solely be responsible for the increased resistance to fluconazole after tetraconazole adaptation (Rocha *et al*., 2016).

A recent study applied spores of triazole-susceptible *A. fumigatus* containing an antibiotic-resistance marker and tebuconazole to open fields and greenhouse soil planted with tomatoes (Cao *et al*., 2020). After four fungicide applications ov40
r fourteen days, the open fields contained up to 0.72 mg tebuconazole/kg soil, and the greenhouse soil contained up to 1.24 mg/kg soil. When *A. fumigatus* strains containing the antibiotic-resistance marker were isolated and tested for sensitivity against azoles, a higher number of triazole-resistant isolates were recovered from the greenhouse soils, and this was hypothesized to be due to the higher tebuconazole concentration. It was concluded that the tebuconazole exposure could induce resistance to the medical triazoles, itraconazole and voriconazole (Cao *et al*., 2020).

It follows that isolates that show reduced sensitivities to clotrimazole and miconazole following long-term exposure will likely show reduced sensitivities to other azole drugs regardless of whether they are meant for clinical or agricultural use.

1.6   Antifungal resistance between the environment and the clinic

Recent studies have noted genetic similarities between environmental and clinical fungal isolates and, thus, have implicated transmission of opportunistic pathogens residing in the environment to humans. For example, *Candida tropicalis* with identical identifying genomic sequences have been isolated from soil, fruits, and the bloodstream of azole-naïve candidiasis patients without correlation to time and treatment location within the hospital (Chen *et al*., 2019; Lo *et al*., 2017). In addition, because genetically similar isolates of *C. krusei* have been observed both in clinical settings and various environmental settings, these isolates were likely transmitted from the environment to humans (Douglass *et al*., 2018). These findings have important implications for clinical azole resistance as the mentioned *C. tropicalis* strain identified in environmental and

clinical samples had reduced sensitivity to clinical azoles, and *C. krusei* is intrinsically resistant to fluconazole.

Together, these findings beg the question: Is azole-resistance being selected for in the environment and then causing azole-resistant mycotic infections in humans? Evidence for such a phenomenon comes from clinical isolates of the filamentous fungi species *Aspergillus fumigatus* containing a TR34/L98H or TR46/Y121F/T289A mutation in the Cyp51A-encoding gene. The TR34/L98H mutation consists of a 34-bp tandem repeat in the promoter region and a leucine to histidine substitution in codon position 98 in the coding region. Meanwhile the TR46/Y121F/T289A mutation includes a 46-bp tandem repeat in the promoter region and a tyrosine to phenylalanine in position 121 and threonine to alanine in position 289 of the coding region. Computer modelling has suggested that the resistant phenotype of the TR34/L98H mutant is due to altered binding conformations between azole drugs and the modified Cyp51A enzymes (Bowyer & Denning, 2014; Snelders *et al*., 2012). Strains of *A. fumigatus* with this mutation can outcross with azole-sensitive strains and, thus, spread geographically (Camps *et al*., 2012). In addition, microsatellite genotyping studies have dated the origin of the TR34/L98H mutation to 1997, shortly after the approval of agricultural azoles such as propiconazole and tebuconazole in the early to mid 1990's (Snelders *et al*., 2012). As well, the tandem repeats of both the TR34/L98H and TR46/Y121F/T289A mutations are typical of azole-resistance mutations found in fungal plant pathogens (Chowdhary *et al*., 2013; Snelders *et al*., 2009). Given these points, it is possible that the two mutations originated in the environment before showing up in clinical settings.

It has also been shown that due to high levels of fungicides, such as tebuconazole, epoxiconazole, and prothioconazole, waste from flower bulbs, compost waste, and wood chippings were identified as being particularly conducive to the development of azole-resistant *Aspergillus fumigatus* (Schoustra *et al.*, 2019). In addition, a case study of an azole-naïve patient who developed fatal aspergillosis found genetically identical *A. fumigatus* strains containing the TR46/Y121F/T289A mutation throughout the patient's house months after death (Lavergne *et al.*, 2017). Altogether, these studies are evidence that *A. fumigatus* can develop azole resistance

in the environment and fungi residing in the environment can cause mycotic infections in humans.

1.7 Fungal ecology

The largest and best-studied subdivisions of the Dikarya kingdom are the Ascomycota and Basidiomycota. Common fungal pathogens include the candidiasis-causing Ascomycete yeasts *Candida albicans* and *Candida glabrata*, the cryptococcosis-causing Basidiomycete yeasts *Cryptococcus neoformans* and *Cryptococcus gattii*, and the aspergillosis-causing filamentous fungus *Aspergillus fumigatus*. Collectively these five species accounted for ~80% of fungal infections following organ transplants monitored by the American-based Transplant-Associated Infection Surveillance Network (Pappas *et al*., 2010).

With the exception of *Candida krusei*, detection of other common clinically relevant *Candida* species in environmental samples has been rare and, in the case of *C. albicans*, not uncommonly interpreted as sample contamination (Opulente *et al*., 2019). Likewise, clinically relevant yeasts have been described as 'not common or abundant' in soils (Yurkov, 2018). However, Opulente *et al*. succeeded in obtaining 28 isolates, including major *Candida* pathogens –*C. albicans, C. krusei, C. parapsilosis,* and *C. tropicalis*—from 931 samples of soil, plant matter, and fruits. Although the study could not determine if the *Candida* isolates could actively grow in soil, their isolation from soils proved that they persist outside of endothermic environmefnts and could possibly be transferred between endothermic organisms via soil (Opulente *et al*., 2019). Another study from 2019 questioned the long-held assumption that views *C. albicans* as an obligate commensal of endothermic animals (Bensasson *et al*., 2019). In this work, the authors isolated three strains of *C. albicans* from oak trees and suggested that the organisms were long-term inhabitants of the trees based on their high genetic diversity (Bensasson *et al*., 2019). On the other hand, the filamentous fungus *A. fumigatus* is readily isolated from environmental samples (for example, see Snelders *et al*., 2009).

Soil bacterial communities are typically more diverse and contain 2-3× the species richness compared to soil fungal communities (Peay *et al*., 2016). In addition, yeasts tend to be less

abundant than filamentous fungi in soils with counts of yeasts typically being in the range of hundreds to thousands of cells per gram (Yurkov, 2018). Nonetheless, soil fungi play important ecological roles, such as organic matter decomposition and nutrient mineralization. Globally, proximity to the equator and available nitrogen correlates with pathogen richness, moisture-related parameters correlate with saprotroph richness, and soil alkalinity and the presence of host plants correlates with ectomycorrhizal richness (Tedersoo et al., 2014). In addition, fungal biomass and diversity were shown to be associated with increases in nitrogen and nitrate concentration in grassland and forest soils in Germany (Birkhofer *et al*., 2012). Below topsoil, species richness broadly decreases with decreasing carbon availability, leaving mycorrhizal fungi and species able to break down lignin and tannin predominating lower depths of soils (Peay *et al*., 2016). Fungal communities in agricultural soil samples can also be influenced by factors such as tillage practice, fertilization intensity, and crop growth. For example, *Fusarium* spp. was found to be positively associated with conservation tillage while *Rhodotorula* spp. was positively associated with conventional tillage (Sommermann *et al*., 2018).

1.8   Review of Methods

1.8.1   Culture-based surveys of soil fungi

Culture-based surveys of soil fungal communities using a small number of isolation methods tend to produce collections dominated by a few species. For example, Aljohani *et al.* isolated 110 yeasts from 443 Cameroonian agricultural soil samples by inoculating 1 mL of chloramphenicol containing SD (Sabouraud dextrose) broth with 200 mg soil for 2-7 days at 30°C and then streaking the liquid enrichments onto yeast extract-peptone-dextrose agar for 48 hours of incubation (Aljohani *et al*., 2018). The resulting collection was dominated by *Cyberlindnera subsufficiens* (25% of isolates), *Cyberlindnera saturnus* (14%), *Torulaspora globose* (19%), and *Candida tropicalis* (18%) (Aljohani *et al*., 2018). *Cyberlindnera* and *Rhodotorula* species were also found to dominate in a study of endophytic yeast of the elm and oak trees, *Ulmus parvifolia* and *Quercus salicina* (Kim & Kim, 2017). Yeasts from the genera *Cyberlindnera* were also the dominant endophytic yeast isolated from the roots of the critically endangered fern *Mankyua chejuense* (Kim

et al., 2017). Enrichment-free protocols can also produce collections dominated by a few species. For example, of over 100 yeasts isolated by Slavikova *&* Vadkertiová from direct plating of a 1% soil slurry onto two agars at 7°C and 25°C, 80% belonged to four species (Sláviková & Vadkertiová, 2003).

1.8.2   Ergosterol quantification for estimating biomass

Quantification of ergosterol is a useful biomarker of fungal load in environmental samples, including soils (reviewed in Djajakirana *et al*., 1996; Montgomery *et al*., 2000). As caveats, however, ergosterol quantification underestimates counts of yeasts relative to filamentous fungi with a single yeast cell showing ergosterol amounts on the magnitude of 1×10^{-4} pg and a single filamentous fungi spore containing 1×10 levels (Pasanen *et al*., 1999). Also, ergosterol shows relatively slow degradation over two months in soils unexposed to sunlight (Mille-Lindblom, Wachenfeldt, & Tranvik, 2004) and soils briefly exposed to fungicides (Zhao, Lin, & Brookes, 2005), complicating the utility of ergosterol for quantifying living biomass.

1.8.3   Antifungal susceptibility testing

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI, previously NCCLS) developed recommendations for testing the sensitivity of isolates to clinical antifungal drugs. Both institutions have developed methods for antifungal susceptibility testing using broth dilutions. In this method, isolates are grown in broths containing various concentrations of a drug and the lowest drug concentration that leads to growth inhibition is noted as the minimum inhibitory concentration (MIC). For reference, some broths are left uninoculated and some isolates are grown in broths containing no drug.

The EUCAST method for testing yeasts isolates was optimized for testing *Candida* and *Cryptococcus* species and tests fluconazole from a range of 0.125 to 64 mg/L and itraconazole and voriconazole from a range of 0.008 to 4 mg/L. (EUCAST, 2020). In addition, a protocol for filamentous fungi was optimized for testing *Aspergillus* spp. and tests itraconazole from 0.0156 to

8 mg/L and voriconazole from 0.0312 to 16 mg/L (EUCAST, 2015). Both protocols are based on a 200 μL microbroth dilution assay using RPMI-glucose broth containing 2% glucose. Likewise, CLSI recommends similar microbroth dilution tests in its M27-A3 (CLSI, 2008a) and M38-A2 (CLSI, 2008b) protocols for testing yeasts and filamentous fungi, respectively.

A second method for testing the susceptibility of isolates to drugs involves streaking an agar plate with a suspension of an isolate and immediately placing a material containing the drug, such as a paper disk, onto the agar. The drug then diffuses outward from the material onto the surrounding agar such that the concentration of the drug steadily decreases in the agar with increasing distance from the drug-containing material. Following incubation, the distance from the disk at which the isolate can no longer grow is used as a measure of the isolates' sensitivity. Agar-based susceptibility tests can be quicker to perform than broth-based tests while still providing reliable results. Intra- and interlaboratory variation using an E-test strip, which is a plastic strip containing a gradient of a drug, on RPMI-glucose agar is relatively low making testing relatively reliable. One review where four laboratories performed 20 replicates of E-tests with *C. krusei* and *C. parapsilosis* isolates tested against ketoconazole and fluconazole showed agreement within a 3 $\log_2$dilution range 98-100% of the time (Pfaller *et al*., 1996).

Based on disk diffusion methods, CLSI produced the M44-A2 protocol (CLSI, 2009) for testing yeasts, which was optimized for testing *Candida* species against fluconazole and voriconazole, and the M51-A method (CLSI, 2010) for testing filamentous fungi. For both protocols, CLSI chose to use Mueller-Hinton agar—a complex medium—over RPMI (Roswell Park Memorial Institute) agar—a synthetic medium— due to its greater availability and lower cost at the expense of more significant batch-to-batch variability (CLSI, 2009). However, the activity of the imidazoles clotrimazole and miconazole have been shown to be antagonized by complex media such as SD agar and brain-heart infusion agar, but not present in synthetic media (Ester & Hoeprich, 1976).

To ensure reproducibility, the microbroth-based EUCAST 7.3.1 protocol for testing of yeasts (EUCAST, 2020) stipulates that the inoculum used for testing must be standardized to 0.5×10^5 to 2.5×10^5 CFU/mL of distilled water while the microbroth-based EUCAST 9.3.1 protocol for testing filamentous fungi (EUCAST, 2015) stipulates an inoculum size of 2×10^5 to 5×10^5

conidia/mL. Yeasts are to be grown between 34-37°C on SD or PD agar for 18-48 hours before adjusted to a set turbidity using a spectrophotometer. Meanwhile, filamentous fungi can be grown for 2-5 days at 35°C, or longer if sporulation does not occur, on any media that encourages sporulation. Then, a damp cotton swab is rubbed against filamentous fungi colonies and deposited into 5 mL distilled water with Tween 20, a surfactant. Next, to standardize the inoculum size, the suspension is either directly adjusted with distilled water after counting conidia using a haemocytometer, or the suspension is filtered through an 11 μM filter to filter out hyphae before using a spectrophotometer to adjust the density of conidia.

1.9 Objectives and hypothesis

Although numerous studies have raised concerns over residual antibiotics in the environment promoting the development of antibiotic resistance in soil bacteria, less is known about the effects of residual antifungal drugs on soil fungi. Given that miconazole and clotrimazole persist in the environment, microplots at Agriculture and Agrifood Canada London Research and Development Centre (AAFC) have been annually amended with environmentally relevant and excessive concentrations of miconazole and clotrimazole since 2010. This study aimed to elucidate the effects that the miconazole and clotrimazole in these plots have had on fungal community composition and antifungal resistance. My predictions were:

1) Fungal isolates belonging to the same species would show decreased sensitivities to clotrimazole and miconazole when isolated from the treated versus the control plots
2) The fungal community in the soils containing clotrimazole and miconazole would be enriched in wildtype strains with intrinsically low sensitivities to the drugs as, over the years, the drugs inhibited the growth of sensitive strains
3) Long-term exposure to clinical azoles would select for isolates resistant to agricultural azoles

2   Methods

2.1 Field experiment

In 2010, a field experiment was initiated at the AAFC-London to evaluate the long-term impact of biosolid-typical levels of clotrimazole and miconazole exposure on soil fungi. Annually, in June, solutions of 365 mg/mL miconazole nitrate in dimethyl sulfoxide (DMSO) and 500 mg/mL clotrimazole in methanol were mixed into four 1 kg portions of soil. These 1-kg portions of soil were then mixed into 2 m^2 microplots to a depth of 15 cm using a rototiller to produce four replicate microplots containing 1 mg/kg soil and four replicate plots containing 10 mg/kg soil of both miconazole and clotrimazole. Four replicate plots were left untreated. The plots were randomly distributed by treatment type across the field and were physically separated by fibreglass frames. The gray-brown Luvisol soil had previously been classified as having the following properties: a sand/silt/clay ratio of 18/67/15%, pH of 7.5, cation exchange capacity of 7.5, and organic matter content of 3.4%. The soil had no known prior treatment with manure or biosolids, and no known treatment with pesticides or fertilizers since 1986. The microplots were cropped annually with soybeans (*Glycine max* var. Harrosoy) and manually weeded. Each spring, eight 20 cm-long soil cores totalling 1 kg were collected from each plot. These soil samples were sieved and stored at -20°C for long-term storage. On the other hand, soil samples were sampled in August and October 2018, April 2019, and March 2020 and stored at 4°C or 15°C for up to six months prior to being used for fungal isolation (Figures 4-6).

2.2 Isolation of yeasts and filamentous fungi

To isolate yeasts and filamentous fungi colonies from the soils, first, soil slurries were prepared. To create soil slurries, five grams of each soil sample were measured out using a metal spatula that was sterilized with 70% ethanol between samples. The five gram soil aliquots were suspended in 45 mL of 0.002% sodium metaphosphate buffer (Fisher Scientific, Ottawa, ON) in 50 mL conical tubes (Corning Life Sciences, Corning, NY; or Sarstedt AG, Numbrecht, Germany). Soil slurries were stored for up to a week at 4°C before being used for fungal isolation. Soil slurries were mixed using a wrist action shaker (Burrell, Pittsburgh, PA) before being spiked into various broths or

plated onto multiple types of solid agar (Table 1; Figures 4-6). Soil slurries spiked into broths in 50 mL conical tubes were either rotated on a Roto-Torque (Cole-Palmer, Chicago, IL) or shaken at ~250 rpm on a shaker (New Brunswick Scientific, Edison, NJ) to encourage yeast-like growth in the supernatant and filamentous fungi to sink down into the pellet. After a brief incubation, 100 μL of the supernatant was then plated onto solid agar to selectively isolate yeasts. Alternatively, 100 μL of a 1:10 dilution of slurry or 50 or 100 μL of undiluted slurry was directly plated onto various antibiotic-containing agar plates to give 1, 5, or 10 mg of soil plated per agar plate. To plate broth supernatants or soil slurries, a metal or glass plate spreader was sterilized in 97% ethanol and passed over a flame between spreads. For each plating event, soils from all twelve plots were used, and a negative control of either sodium metaphosphate or MilliQ water was used. To increase the diversity of isolates and to minimize clonality in the fungal collection, new soil slurries were routinely prepared between plating events and in general, only one morphologically identical colony per plate was retained for the collection.

Commercially prepared agars used for isolation of yeasts and filamentous fungi from soils included dichloran-glycerol 18 agar (DG18) (Fluka, Steinheim, Germany), rose bengal (RB) and yeast-mold-11 (YM-11) agar (both from Hardy Diagnostics, Santa Maria, CA), and Sabouraud dextrose (SD), Mueller-Hinton (MH), malt extract (ME), Czapek-Dox (CZ), and potato-dextrose (PD) agars (all from Becton, Dickinson and Company, Sparks, MD). Agars made in-house included Dekkera/Brettanomyces differential medium (DBDM) and glucose-yeast-peptone (GYP) agars. The PD, MH, and GYP agars were used to support the growth of a wide range of organisms due to a nutrient-rich composition and a neutral pH. As for selective media, CZ uses nitrate as a sole nitrogen source, DBDM uses ethanol as sole carbon source, ME and SD both have an acidic pH, DG selects for osmophilic and xerophilic organisms due to low water activity, and RB agar contains rose bengal to inhibit the radial growth of certain filamentous fungi.

All agars and broths were amended with antibiotics to inhibit the growth of bacteria and select agars and broth were amended with selective fungicides to increase the diversity of fungi isolated overall. Antibiotic stocks of 100 mg/mL chloramphenicol in 97% EtOH, 500 mg/mL streptomycin in ddH$_2$O, 5 mg/mL chlortetracycline HCl in 0.5 N NaOH and a stock of 100 mg/mL cycloheximide in ddH$_2$O, were prepared in 15 mL or 50 mL conical tubes, filtered using a 0.22

μm filter, and stored in conical tubes at 4°C prior to use. All three antibiotics and cycloheximide were sourced from Sigma-Aldrich (St. Louis, MO). Antibiotics were added to the agar as noted Following initial isolation, in most cases, yeasts and filamentous fungi were sub-cultured to purity on the agar used for initial isolation after which axenic yeasts cultures were maintained on Sabouraud dextrose (SD) agar and axenic fungal cultures were maintained on Mueller-Hinton (MH) agar at room temperature. Yeasts isolated at 4°C were maintained at 4°C, 15°C or room temperature, depending on the observed maximum growth temperature. Likewise, filamentous fungi that were isolated at temperatures over 37°C were either maintained at 37°C or room temperature, depending on the observed growth rate. Preparation of glycerol stocks for yeasts varied by the period of isolation (Figures 4-6). On the other hand, filamentous fungal cultures were swabbed with sterile cotton swabs from growth on MH agar at room temperature and the resulting fungal fragments were suspended in Milli-Q water. The fungal suspensions were then used to create 25% v/v glycerol stocks to be frozen at -80°C.

Table 1 – Composition of Agars and Broths used for Fungal Isolation

With the exception of commercially-prepared RB and YM-11 agars, agars were either prepared in-house or made with the addition of agar base (Biobasic, Toronto, ON) to commercially-prepared broths. All agars contained 15 g/L of agar with the exception of commercially-prepared YM-11 agar, which used 13.5 g/L. For enrichment of yeasts, broths identical to YM-11 and RB agar were prepared in-house with the exception that trypan blue was not added to the YM-11 broth. An additional medium, termed yeast-extract peptone dextrose (YEPD), was prepared identically to GYP media with double the amounts of peptone and L-glucose. pH values of media were brought within the ranges shown using 1N NaOH or HCl.

	CZ	DBDM	DG18	GYP	ME	MH	PD	RB	SD	YM-11
pH at 25°C	7.3 ± 0.2	5.4 ± 0.2	5.6 ± 0.2	7.0 ± 0.2	4.7 ± 0.2	7.3 ± 0.1	7.2 ± 0.2	7.2 ± 0.2	5.7 ± 0.2	7.5 ± 0.5
Extracts (g/L)										
Beef extract						2				
Malt extract					6					
Potato extract							4			
Yeast extract				5	1.2					
Nitrogen Source (g/L)										
Caesin, acid digested						17.5				
Peptone, animal sources			5	10					10	20
Peptone, soy								5		20
Sodium nitrate	3									
Yeast nitrogenous base		6.7								

	1	2	3	4	5	6	7	8	9	10
Carbon Source										
Ethanol (mL/L)		60								
L-Glucose (g/L)			10	20	6		20	10	20	5
Maltose (g/L)					1.8					
Soluble starch (g/L)						1.5				
Sucrose (g/L)	30									
Inorganic Constituents (g/L)										
Dipotassium phosphate	1									5
Ferrous sulfate	0.01									
Magnesium sulfate	0.5		0.5					0.5		
Monopotassium phosphate			1					1		
Potassium chloride	0.5									
Sodium chloride										5
Additives (mg/L)										
Chloramphenicol	80	80	100	80	80	80	80	80	80	
Chlortetracycline HCl										200
Cycloheximide		100								
Dichloran			2							
Rose bengal								5		
Streptomycin	400	400		400	400	400	400	400	400	
Trypan blue		30								

Figure 4 – Isolation Procedure of Yeasts from August 2018 Soils

Sixteen yeasts were obtained by AAFC technician Andrew Scott (not shown on flowchart) where all but one isolate was obtained by direct plating of 10 mg of soil onto 0.2-fold strength (0.2×) SD agar with miconazole and clotrimazole, 0.2× SD agar containing cetrimonium bromide (noted by the '-C' affix to media names), 0.2× PD, and 0.2× YEPD agars. An additional isolate was obtained by performing an enrichment for yeasts with 0.2× SD-C broth and streaking the supernatant onto 0.2× SD-C agar using a 10 μL loop. For long-term storage, single colonies were amplified in 5 mL test tubes at 30°C with shaking at 250 RPM for 3-7 days using 2 mL of yeast extract-peptone-dextrose (YEPD) broth. Glycerol stocks were created using 800 μL of the liquid cultures and 800 μL of 50% glycerol. RT = room temperature.
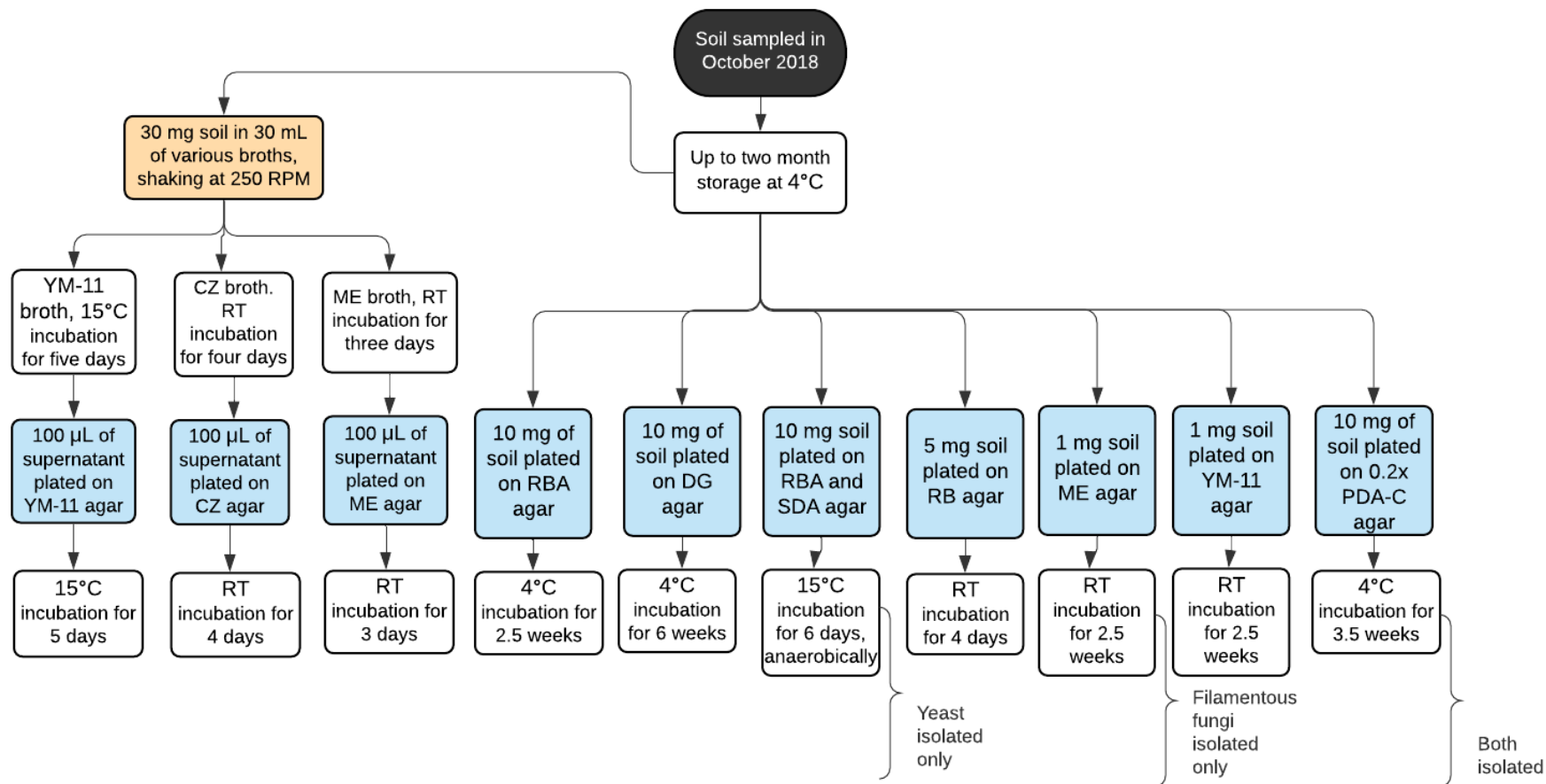
Figure 5 – Isolation of Yeasts and Filamentous Fungi from October 2018 Soils

For long-term storage of yeasts, stocks were created by sub-culturing isolates onto solid agar at least twice using single colonies, and then dispensing colonies from the agar into a 50:50 mix of broth and 50% glycerol using a 10 µL plastic loop.
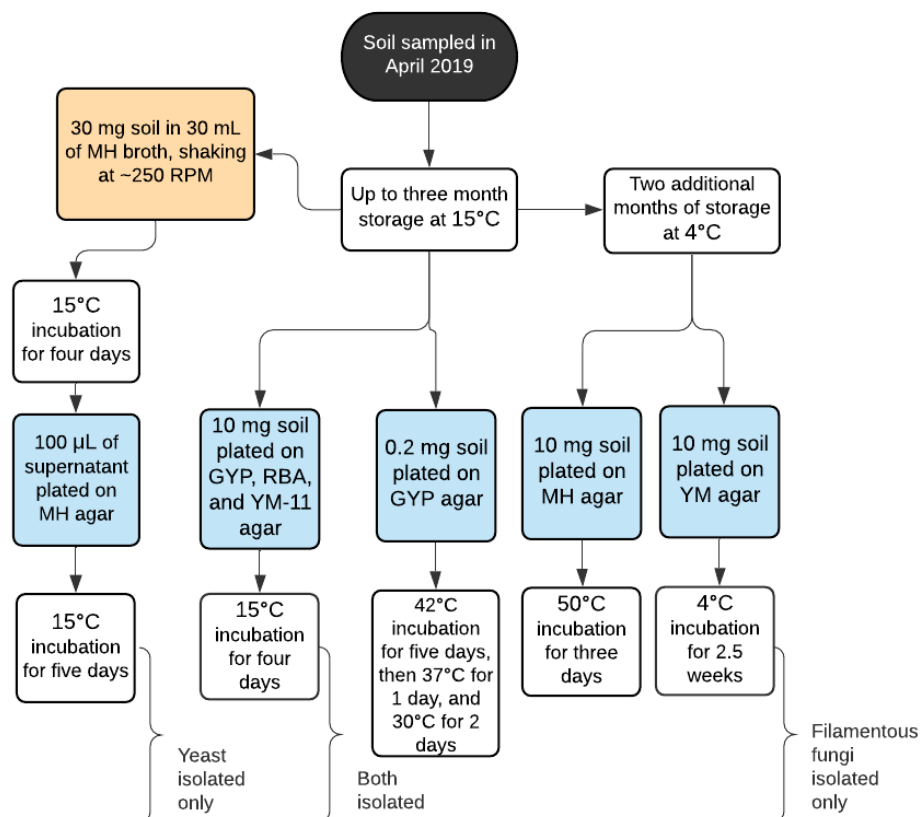
Figure 6 – Isolation of Yeasts and Filamentous Fungi from April 2019 Soils

To create long-term stocks, yeasts were sub-cultured on MH agar at the initial isolation temperature, and colonies were scrapped using a 10 μL loop and deposited in 800 μL of 0.9% NaCl and 800 μL of 50% glycerol. In addition to the fungi isolated from the above procedures, in February 2020, soils stored for three months at 15°C and then 8 additional months at 4°C were used to isolate 26 yeasts using a two-day enrichment of 30 mg soil in 0.2× Luria-Bertani broth and subsequent two-week incubation on 0.2× SD-C agar at room temperature. Seven of these yeasts were used for antifungal susceptibility testing. However, these yeasts were were not used for species richness calculations as these yeasts were specifically isolated to increase sample size for susceptibility testing of yeasts from the treated plots. Six sequenced filamentous fungi were also isolated by plating 10 mg of soil on 0.2× SD-C agar at 50°C for one week).

2.3   DNA extraction from fungal isolates

For isolating genomic DNA from yeasts, a protocol modified from Lõoke *et al.* (2011) was used. Briefly, yeast cells were grown for up to one week on SD agar at room temperature or 15°C and a sterile 10 µL plastic loop was used to deposit cells into a lysis solution containing 180 µL of 0.22 µm filter-sterilized 200 mM lithium acetate (Sigma, St. Louis, MO) and 20 µL of 10% sodium dodecyl sulfate in a 1.5 mL microcentrifuge tube. The mixtures were briefly vortexed, incubated at 70°C for up to 10 minutes and then centrifuged at $15,000 \times g$ for three minutes. The resulting supernatant was then used as a starting material for use with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The *Isolating Genomic DNA from Yeast* protocol included in the kit was followed starting from the protein purification step except that all centrifugation steps resulting in nucleic acid pellets were performed at $15,000 \times g$ for 10 minutes.

For isolating DNA from filamentous fungi, bead beating tubes from PowerSoil kits (Qiagen, Hilden, Germany) were amended with lysis buffer from the kits and half-filled with 50 x 10 mm blocks of fungal mass growing on MH agar for at least one week. The tube contents were then processed as per manufacturer's instructions for soil samples. Alternatively, following homogenization, bead beating tubes were centrifuged at $15,000 \times g$ for 5 minutes and the supernatant was used directly as a template for PCR amplification.

2.4   Identification of isolates

Initially, yeasts were grown on SD agar for up to a week and filamentous fungi were grown on MH agar for up to three weeks, both at room temperature, to group isolates by morphology. To further group the isolates, DNA was extracted from 88 yeasts representing 15 species and Random Amplified Polymorphic DNA (RAPD) PCR was performed based on the methods of Aljohani *et al*. (2018). In this method, PCR is performed using a single primer that amplifies repetitive sequences in the genomic DNA and the resulting amplicons are run on an agarose gel to group closely related strains. Briefly, in 25 µL PCR strip tubes, 10 µL reactions were composed of 0.4×buffer, 0.02 U/mL Taq polymerase, 2.4 mM $MgCl_2$ (all from Promega, Madison, WI), 320

µM dNTP mix (BBI Biotech, Berlin, Germany), and 1 µM of either the $(GACA)_4$ (5'-GACAGACAGACAGACA-3') or M13 primer (5'- GTAAAACGACGGCCAG-3') (Sigma Aldrich, St. Louis, MO), 2 ng/mL DNA, and autoclaved MilliQ water. The reactions were cycled using a protocol of denaturation at 98°C for two minutes, amplification for 45 cycles of 93°C for 20 sec, 50°C for 45 sec, and 72°C for 20 sec, followed by a hold at 72°C for six minutes and a cool down to 4°C. The $(GACA)_4$ and M13 PCR products were combined and run on a 1% agarose gel at 80V for 1.5-2.5 hours and bands were manually scored. PCR fingerprinting failed for filamentous fungi, possibly due to the DNA extraction technique.

Representatives of each fingerprint, as well as each morphologically unique isolate, were identified by sequencing ~250 bp of the ITS1 region. The ITS1-F forward (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS2 reverse primer (5'-GCTGCGTTCTTCATCGATGC-3') (White *et al.*, 1990) were used. The template used for the PCR was either yeast colonies grown for less than one-week and suspended in MilliQ water (ABS_{600} ~0.2), yeast DNA diluted to 10 ng/mL, or undiluted filamentous fungi DNA. The PCR mix for one reaction included 1x GoTaq Flexi buffer, 0.006 units/mL Taq polymerase, and 1.5 mM $MgCl_2$ (all from Promega Corporation, Madison, WI), 200 µM dNTP mix (BBI Biotech, Berlin, Germany) and 0.2 µM of the forward and reverse primers (MilliporeSigma, St. Louis, MO), 10 µL of template, with MilliQ water up to 25 µL. The PCR protocol was denaturation at 95°C for 5 min, amplification for 35 cycles of 94°C for 30 sec, 52°C for 30 sec, and 68°C for 30 sec, followed by a cooling down to 4°C. Four reactions per sample were performed. After reactions were pooled, the reactions were checked for purity on a 1% agarose gels for an amplicon size of 250-600 bp and cleaned up using the Wizard SV Gel and PCR clean-up kit (Promega, Madison, WI). Purified DNA template was adjusted to 3-10 ng/µL using a NanoDrop ND1000 (NanoDrop Technologies, Wilmington, DE) for quantification and MilliQ water for dilutions. Reactions of 10 µL DNA and 5 µL of 2 µM forward or primer were submitted to Robarts Research Institute (London, ON) for Sanger sequencing.

Sequences were trimmed on both ends to exclude regions with significant no call bases, queried against the GenBank non-redundant nucleotide database using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (NCBI BLAST) (Altschul, Gish,

Miller, Myers, & Lipman, 1990), and then species given the highest scoring matches were recorded. Partial ITS sequences returning the same highest-scoring species match were aligned using MUSCLE to look for sub-groups within the species. Higher-order taxonomic classification corresponding to subkingdom, phylum, subphylum, class, order, family were resolved using RStudio 3.6.2 and the taxize 0.9.92 package (Chamberlain & Szöcs, 2013) using genus names as queries and the taxonomy database from NCBI as a reference.

2.5   Viable plate counts

To estimate changes in viable soil fungal counts due to clotrimazole and miconazole exposure, soils were cultured on agar-solidified RB and DG18 media and the resulting number of colonies were counted (Table 1). The soil used for the plate counts was stored at 4℃ for less than 2.5 weeks after being sampled in November 2018 (Figure 5). For each plate, 100 µL of a 100 mg/mL soil slurry, corresponding to 10 mg of soil, was spread using a sterile plate spreader and incubated at 4℃. Colonies were counted 1.5 weeks and 6 weeks post-incubation for the RB and DG18 plates, respectively. For a second plating set, soil slurries were prepared in duplicate for each plot from soils stored at 15℃ for three months after being sampled in April 2019 (Figure 6). As before, 10 mg of each soil slurry was plated onto RB agar, but this time, incubated at 15℃ for four days. Plates that were overgrown or had large filamentous fungal colonies taking over half of a plate, which totalled two out of 24 plates, were excluded from plate counts. For plates containing large filamentous fungal colonies on one end of the plate, or for plates containing too many colonies overall, half-sections of the plates were counted.

2.6   Ergosterol quantification for estimating biomass

The soil samples used for ergosterol quantification were sampled in August 2018 and stored at 4℃ in the dark in tied, clear plastic bags. The samples were briefly removed from storage twice for fungal cultivation before being used for ergosterol quantification in March 2020.

Ergosterol quantification was performed by the AAFC technologist Lou Ann Verellen. Briefly, 5 grams of soil, 3.5 grams of Ottawa Sand (Fisher Chemical, Ottawa ON), and 6 mL of methanol were combined in a 20 mL polyethylene scintillation vial, vortexed for 10 seconds and mixed using a wrist action shaker at room temperature for an hour. The mixture was then centrifuged for 10 minutes at 3000 rpm in the Labofuge 6000 (Heraeus Christ, Osterode, Germany) and 1 mL of supernatant was filtered through a 0.2 mm polytetrafluoroethylene syringe filter and stored in a 1.5 mL microcentrifuge tube.

Following filtration, 20 μL of the supernatant was used for injection and ergosterol was measured using an Agilent 1260 Infinity high performance liquid chromatography system with a C-18 reverse-phase column (Agilent Eclipse XDB-C18, 4.6mm x 100mm, 3.5μ). Parameters for the chromatography included a mobile phase of Acetonitrile:Methanol (70:30 v/v), a flow rate of 1.2 mL/min, and temperature of 25 °C. Ergosterol was detected using an Agilent Diode Array detector at a wavelength of 282 nm.

The retention time was 8.6 min as determined with an ergosterol standard (Sigma-Aldrich, St. Louis, MO). To produce the ergosterol standard curve, the ergosterol standard (10 mg/mL in chloroform) was measured at concentrations of 0.125, 0.25, 0.50, 0.75, 1.0, and 2.5 μg/mL.

2.7   Assessing species richness within treatment groups

To assess differences in species richness between control, low treatment, and high treatment plots, fungal isolates were identified and tallied by treatment type. For yeasts, isolates were clustered at the species level by sequencing while RAPD PCR and morphology were used to identify isolates without sequencing data. Seventeen out of 173 yeasts in the collection could not be classified at the time of writing. For filamentous fungi, only ITS sequencing was used to enumerate species as sequenced isolates with identical morphologies were shown to belong to different species and RAPD fingerprinting assays failed to produce discernible banding patterns.

2.8 Antifungal susceptibility testing

Drug stocks were prepared in 15 mL polypropylene conical tubes (Corning Inc., Corning, NY), by preparing stock solutions of 12.8 mg/mL clotrimazole (Sigma, St. Louis, MO), miconazole nitrate, fluconazole, itraconazole, difenoconazole, propiconazole and tebuconazole (all from AK Scientific, Union City, CA), and 6.4 mg/mL voriconazole (AK Scientific, Union City, CA). DMSO (Sigma, St. Louis, MO) was used a solvent for all drugs except for clotrimazole and tebuconazole, which were prepared in 95% ethanol (Commercial Alcohols, Toronto, ON). After allowing the drugs to dissolve in solution for at least two hours, the solutions were aliquoted into autoclaved 1.5 mL Eppendorf tubes and either used immediately or stored at -80°C. Disks of Qualitative Type 2 Whatman filter paper (Whatman, Maidstone, UK) were cut into disks 7 mm in diameter using a hole puncher and laid out onto sterile glass Pyrex petri dishes using sterile forceps. The Petri plates were then wrapped in aluminum foil, autoclaved on a dry setting of 121°C, 15 p.s.i. for 15 minutes and cooled to room temperature in a laminar flow hood before use.

To prepare azole-containing filter paper disks, fluconazole stocks were diluted to 5 µg/µL, voriconazole stocks were diluted to 0.2 µg/µL, and all other drug stocks were diluted to 2 µg/µL in their appropriate solvent. Then, 5 µL of each drug were aliquoted onto the paper disks, while on the glass Pyrex Petri dishes, in a laminar flow hood, to produce 10 µg disks of clotrimazole, miconazole, itraconazole, tebuconazole, difenoconazole, and propiconazole, 20 µg disks of fluconazole, and 1 µg disks of voriconazole. The concentration of each medical azole disk was equal to the concentration of commercially prepared disks from Rosco Diagnostica (Rosco, Taastrup, Denmark), with the exception that the commercially prepared fluconazole disks are 25 µg/disk. Disks were dried in a laminar flow hood for a minimum of two hours after drug application to a maximum of overnight prior to use or storage. For storage, the Pyrex petri dishes containing the disks were placed in a metal canister with a desiccator, both previously dried and sterilized at 120°C in an oven but brought to room temperature in the flow hood, and then the canister was stored at -80°C.

RPMI-glucose agar was prepared by combining 10.4 g RPMI powder (formulation 31800-089; Thermo Fisher Scientific, Waltham, MA), 18 g dextrose (Sigma, St. Louis, MO), and 15 g agarose

(Biobasic Canada, Toronto, ON) with 800 mL ddH$_2$O, microwaving until constituents were fully dissolved, and then autoclaving at 121°C, 15 p.s.i. for 15 minutes. Separately, 34.53 g of MOPS (Sigma, St. Louis, MO) and 0.15 g L-glutamine (Thermo Fisher Scientific, Waltham, MA) were combined in 200 mL ddH$_2$O, sterilized through a 0.22 μm filter, and then added to the agarose mixture. After adjusting the pH of the agar to 7.0 using 6N NaOH, the agar was poured into plates 5 mm thick. Mueller-Hinton agar (Becton, Dickinson and Company, Franklin Lakes, NJ), formulated to contain 20–25 mg/L calcium and 10–12.5 mg/L magnesium, was prepared per manufacturer's direction and poured into agar plates with 5 mm of thickness.

For testing yeasts, single colonies growing on SD agar at room temperature were suspended in Milli-Q water and adjusted to a turbidity of 0.07-0.11 absorbance at 590 nm in UVette® spectrophotometry tubes (Eppendorf AG, Hamburg, Germany). In most cases, colonies were grown for 48-60 hours, however, slow-growing colonies of *Mrakia* spp., *Solicoccozyma terrea*, and *Filobasidium oeirense* were grown on SD agar for up to one week. Susceptibility testing was performed by streaking the suspensions on RPMI–glucose agar using a sterile cotton swab and briefly drying the suspensions prior to disk application. For most isolates, clotrimazole, miconazole, itraconazole, voriconazole, and fluconazole disks were applied to one agar plate and difenoconazole, propiconazole, and tebuconazole were applied to a second agar plate. Plates were incubated at 30°C, or at 15°C if the maximum growth temperature was determined to be below 30°C, typically, for 48 hours, and the zones of inhibition were measured from edge to edge using a ruler. Sensitivity assays of the slow-growing colonies of *Solicoccozyma terrea* and *Filobasidium oeirense* were incubated for 120 hours. Assays using *Mrakia* spp. were incubated for 96 hours, and assays including *Vishniacozyma victoriae*, *Solicoccozyma aeria*, *Hannaella coprosmae* and select isolates of *Bullera alba* and *Sampaiozyma ingeniosa* were incubated for 72 hours. Plates were also imaged on a flat surface using a levelled gel imager camera alongside an adjacent ruler for documentation purposes.

For filamentous fungi, 10-100 μL of cryopreserved stock were pipetted onto three spots of a 0.2× PD agar plate and plates were incubated upright at room temperature for one to three weeks. Fungal biomass was obtained by gently swabbing colonies with a sterile cotton swab and suspending the biomass into approximately 500 μL of Milli-Q water in a 1.5 mL microcentrifuge tube. While the

biomass was not filtered to exclude all but conidia, large hyphal fragments were prevented from being introduced to the suspension by discarding fragments stuck to the end of the pipette tip. When possible, suspensions were adjusted to a turbidity of $ABS_{590} = 0.07$-0.11. Otherwise, initial fungal suspensions showing a turbidity below $ABS_{590} = 0.07$ were used as-is. Suspensions were spread with sterile cotton swabs onto MH agar, and Rosco Neo-Sensitabs™ (Rosco, Taastrup, Denmark) containing 10 µg clotrimazole or miconazole were placed onto the agar, and the plates were incubated at 25°C for 48-60 hours. Again, plates were imaged using the gel imager; however, unlike with the yeasts, measurements for zones of inhibition were measured from the images using the ruler as a guide.

2.9 Quality control for antifungal susceptibility testing

Selected yeasts were tested in duplicate to assess the impact of variation between sets of tests caused by variation in disk and plate properties, such as composition and dryness, inoculum preparation, such as density and colony freshness, and testing parameters, such as zone of inhibition measurements and incubation time. The largest variation was noted for an isolate of *Schwanniomyces occidentalis* showing a zone of inhibition to clotrimazole of 36 mm in one test and 44 mm in another test. Reproducibility also suffered in tests where isolates produced small zones of inhibition with extensive trailing growth towards a disk. For example, *Rhodotorula* isolates initially retested due to showing no zone of inhibitions when tested against miconazole and itraconazole, later showed zones of inhibition around 10 mm (Table 2). When multiple measurements of isolates were taken, the average zone of inhibition between trials was used in subsequent calculations. Filamentous fungi were tested using commercially prepared azole disks and yeasts tested against agricultural azoles were tested using limited batches of media, limiting batch variation.

To test for reproducibility using different agars and disk preparation methods, two isolates of *Rhodotorula mucilaginosa* were tested in duplicate using paper disks on Mueller-Hinton agar and two isolates each of *Cyberlindnera saturnus* and *R. mucilaginosa* were tested on the standard RPMI-glucose agar with commercially-prepared 10 µg clotrimazole or miconazole Rosco Neosensitab disks as opposed to in-house filter paper disks. Mueller-Hinton agar provided less

definition around the miconazole and itraconazole disks than RPMI-glucose agar, possibly due to the lack of a dye in the former. Still, the zones of inhibition to clotrimazole were within error range to those on RPMI-glucose agar. In addition, although the Neosensitab disks were significantly larger at 10 mm in diameter versus 7 mm in diameter for the filter paper disks, the susceptibility and resistance phenotypes were identical using the different disks for the *C. saturnus* and *R. mucilaginosa* isolates.

To confirm that zones of inhibition were caused by the drug impregnated into the disk and not the solvent carrier, preliminary experiments were undertaken with disks containing only the ethanol or DMSO solvents. In no instances did either solvent inhibit the growth of 25 yeasts (18 *C. saturnus*, three *R. mucilaginosa*, two *Debaryomyces hansenii*, and two *Bullera alba*) or 25 filamentous fungi of various species tested.

Table 2 – Average ZOI (mm ± Standard Deviation) of Yeasts Tested using Different Batches of Media

To check for variation in zones of inhibition measurements with sensitivity testing of yeasts, select yeasts were tested using different batches of media. Isolate A was identified as *Schwanniomyces occidentalis* and tested against three batches of media while other isolates were tested against two different batches and isolates B and C were identified as *Rhodotorula mucilaginosa*, isolates D and E were *Cyberlindnera saturnus*, isolate F was *Bullera alba*, and isolate G was *Cutaneotrichosporon terricola*. R = resistant phenotype, n.d. = not tested in duplicate.

	Clotrimazole	Miconazole	Fluconazole	Voriconazole	Itraconazole
Isolate A	40.0 ± 3.3	26.7 ± 2.1	29.0 ± 1.0	35.3 ± 3.3	25.3 ± 0.9
Isolate B	27.5 ± 0.5	10.0/R	R/R	R/R	11.0/R
Isolate C	29.0 ± 0	R/R	R/R	R/R	11.1/R
Isolate D	24.0 ± 0	19.5 ± 0.5	R/R	18.5 ± 1.5	14.0 ± 0
Isolate E	22.5 ± 1.5	20.0 ± 0	R/R	19.0 ± 1.0	14.0 ± 0
Isolate F	35.5 ± 0.5	R/R	R/R	R/R	23.0 ± 2
Isolate G	35.0 ± 1.0	32.0 ± 2.0	n.d.	n.d.	n.d.

2.10 Determining levels of susceptibility or resistance to drugs

For tested yeasts, the majority of susceptibility tests to the eight azole drugs either produced large, clearly-defined zones of inhibition, indicating that an isolate was susceptible to the drug, or no zone of inhibition surrounding the drug disks, indicating resistance. However, several isolates tested against particular drug disks showed extensive 'trailing growth' indicative of a fungistatic drug effect. These isolates produced microcolonies directly surrounding the drug disk with colony size increasing in size with increasing distance to the disk. In these cases, zones of inhibition were measured from edge-to-edge using normal-sized colonies, as determined by colonies growing in the absence of drug, as endpoints. Examples of tests returning results and their classification can be found in the Appendix (Supplementary Figure 1).

2.11 Statistical analysis

To determine if resistance to one azole drug was associated with resistance to another azole drug, pair-wise comparisons were performed. Contingency tables comparing the number of isolates determined to be resistant or susceptible to each drug were constructed as shown in the Appendix (Tables 3 and 4). Fisher Exact tests were performed using an on-line calculator (Stangroom, 2020) with a significance level of 0.05.

In addition, to estimate the relationship between susceptibility to one drug versus another drug, linear regression analysis across all isolates was performed using the zones of inhibition to the two drugs to the same isolate as variables.

Any potential treatment effect on the sensitivity of recovered fungi to the azole drugs by soil treatment origin was determined using a two-tailed T-test for two independent means and calculated using an online calculator and a significance level of 0.05 (Stangroom, 2020b). Data was compiled such that tests that produced no zone of inhibition in the disk diffusion assay, which

indicated that the tested isolate was resistant to the tested drug, were represented in the dataset with a zone of inhibition value of zero millimeters.

3   Results

3.1   Impact of azole exposure on culturable fungal propagules

The azole concentrations in the treated soils steadily increased with annual azole application to an average, with noted standard deviation between the four treatment plots, of 0.22 ± 0.09 mg/kg of miconazole and 0.13 ± 0.04 mg/kg of clotrimazole in the low treatment soils and 19.58 ± 3.77 mg/kg of miconazole and 24.95 ± 6.52 mg/kg of clotrimazole in the high treatment soils (Figure 7).
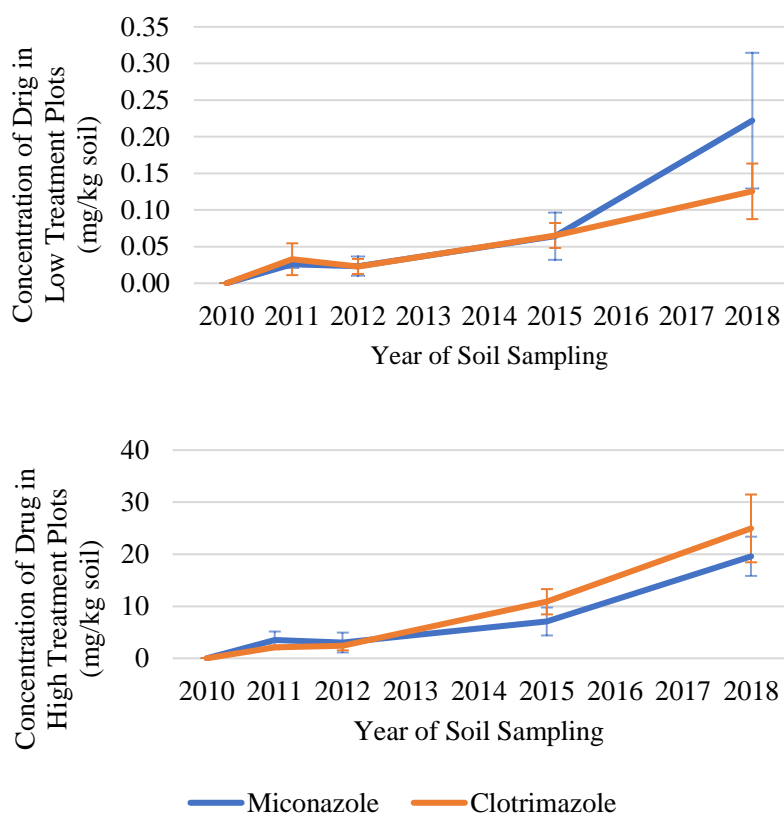

Figure 7 – Concentration of Azoles in the Low and High Treatment Plots Over Time

Average concentration of the drugs in the four low and four high treatment plots collected prior to annual drug application is shown with the error bars showing standard deviation in azole concentrations between the plots.

The impact of long term fungicide exposure on viable populations of filamentous fungi and yeasts was evaluated by viable plate counts on RB and DG agar using soils sampled in 2018 following nine annual drug applications. Statistical tests performed using a two-tailed T-test for two independent means (Stangroom, 2020b) using the plate count data found higher colony numbers on plates plotted with control soil than plates plotted with high treatment soil on RB agar ($p \leq 0.01$ for incubations at both 4°C and 15°C) but this difference was not seen on the DG agar incubated at 4°C ($p = 0.32$) (Figure 8).
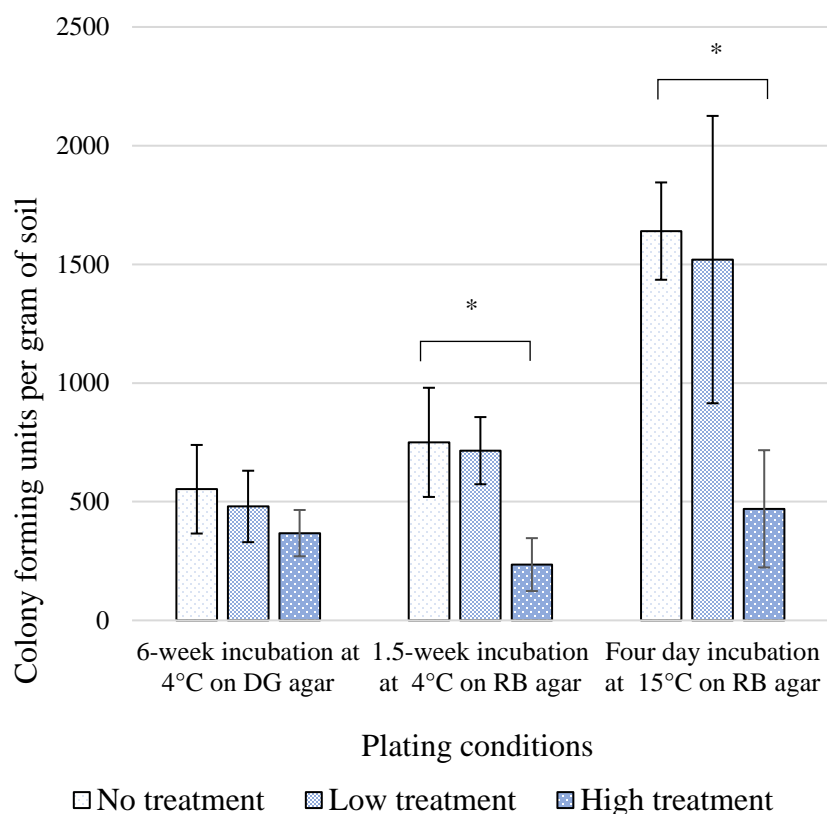
Figure 8 – Plate Counts from Soils Receiving No, Low, or High Azole Treatments

Plate counts show reduced fungal load in high treatment plots compared to control plots on RB agar bur not DG agar. An outlier plate from the control plot #26 soil with 260 CFU/g soil and an outlier plate from the high treatment soil with 1,800 CFU/g soil were excluded from the RB agar incubated at 15°C bar chart; however, even with these outliers $p = 0.01$ between the control and high treatment plots. Significant differences between treated plots versus controls are marked with an asterisk.

In addition, to estimate fungal biomass, ergosterol in extracts of soil in August of 2018 was quantified by high performance liquid chromatography (Figure 9). The ergosterol concentration in the high treatment versus control soils was significantly lower in the soils.
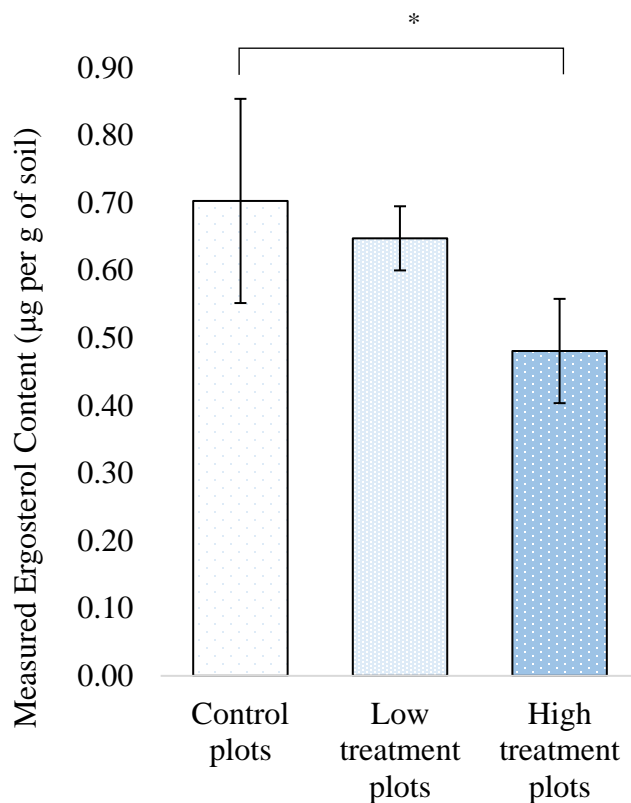
Figure 9 – Ergosterol Content in Control versus Azole-treated Soils

Ergosterol decreased in the high treatment plots receiving annual applications of azoles versus untreated control plots after eight years of annual azole application.

3.2   Fungal cultivation and isolation

Soil from each of the 12 plots were plated onto 0.2× strength SD and 0.2× PD agar amended with 1 mg/L miconazole and 1 mg/L clotrimazole in DMSO (Figure 4). For the 0.2× SD agar plates, one of four plates inoculated with control soil yielded bacterial growth, and of the four plates inoculated with high treatment soil, one showed bacterial growth, and another showed filamentous fungi growth. No growth was observed on any other plate after eight days. On 0.2× PD agar, microbial growth was noted on all four plates containing high treatment soil, two of four plates

containing low treatment soils, and none of the four plates containing control soils (Figure 10). Organisms from these plates were not retained in the collection.

A   B
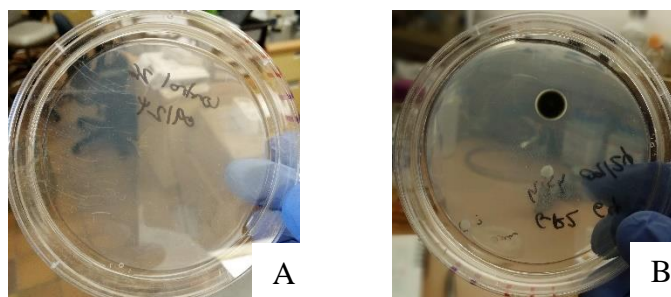
Figure 10 – Observations from Azole-treated Soils Plated on Antifungal-containing Media
Growth was observed on 0.2-strength potato dextrose plates containing clotrimazole and miconazole when plated with the high treatment soils but not the control soils. None of the plates plated with the four control soils produced growth (A), but all of the plates plotted with high treatment soil produced growth (B).

Overall, 42 yeasts were obtained from soils sampled in August 2018. All obtained isolates from the plating on DBDM agar matched to *Cyberlindnera saturnus* and all but two of thirty isolates obtained on the 0.2× strength media containing C-TAB corresponded to *C. saturnus*, *Rhodotorula mucilaginosa*, or *Bullera alba*.

Seventy-three yeasts were isolated from the October 2018 soils. *C. saturnus* was the only species isolated on the CZ agar, and was the predominant species isolated from the YM-11 agar, along with *R. mucilaginosa*, and the ME agar, along with *R. mucilaginosa* and *Debaryomyces* spp. The yeasts from the 4°C incubations were more diverse with, among 15 isolates from each set, 11 species from the RB agar and more than eight species from the DG agar. Anaerobic incubation yielded few yeasts, five among two sets of SD and RB agar plates, with isolates belonging to *Sampaiozyma ingeniosa* (*n*=2), *Hannaella coprosmae*, *D. hansenii*, and *R. mucilaginosa*. In addition, fifty-five filamentous fungi that were used for sensitivity testing were isolated from the October 2018 soils. *Mucor* and *Clonostachys* species were the predominant isolates from the RB agar and *Cladosporium* and *Mortierella* species were the predominant isolates from the 0.2× PD-C agar.

From the soils sampled in April 2019, forty-two yeasts from more than nine species were obtained from the enrichment in MH broth and incubation on MH agar. The high efficiency of this isolation may be attributed to the fact that the soils were put into enrichment within days of being sampled from the field with a 15°C storage in the interim. In addition, forty-five filamentous fungi used for sensitivity testing were obtained from the soils. Of note, the isolates from the incubation on GYP at 15°C were dominated by *Mucor* and *Mortierella* species while the MH incubation at 50°C showed growth of the pathogen *Aspergillus fumigatus*.

3.3   Identification of fungal isolates

Of the 173 yeasts isolated prior to February 2020, 81 were identified by ITS sequencing, 43 were identified by a combination of RAPD PCR and morphology and 46 were preliminarily identified by morphology only. Paired-end sequencing of 26 amplicons produced an identical overlapping sequence that could be used for identification so single-end sequencing was used thereafter. Sequenced isolates from the yeast collection yielded 31 unique ITS sequences representing 28 species (Table 3). Four isolates gave less than 99% ID to any isolate given by NCBI BLAST and one isolate did not have any close matches to any other classified organism in the database.

For initial identification, all yeasts were streaked onto $1\times$ SDA and checked after at least one week of growth at room temperature. *C. saturnus* was distinguished by white colonies showing a raised centre surrounded by web-like growth while *D. hansenii* was distinguished by flat, white colonies and *B. alba* showed small, beige colonies. Meanwhile, *Rhodotorula* showed pink-red, smooth, sometimes mucoid, colonies and Wang *et al.* (2015) was used as a guide to distinguish *Rhodotorula* from similarly pigmented yeasts.

Following grouping by morphology, RAPD PCR using genomic DNA from 88 yeasts was used to further group together isolates with identical morphologies (Figure 11).
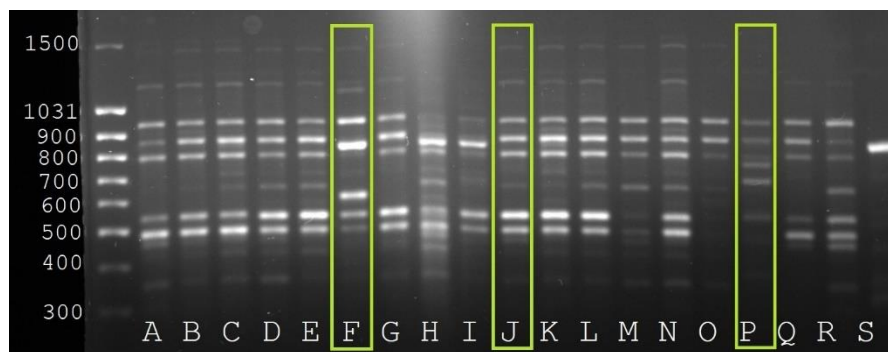
Figure 11 – Representative RAPD PCR Gel for Identification of *Cyberlindnera saturnus*
PCR fingerprints highlighted in green were identified by ITS sequencing as *Cyberlindnera saturnus* and all other isolates were assumed to be *Cyberlindnera saturnus* due to similar fingerprints and morphology.

Yeasts showing unique morphologies or RAPD PCR fingerprints in addition to representative isolates from yeasts with identical morphologies and RAPD PCR fingerprints were sequenced. Overall, by morphology only, 11 isolates were assigned to *Cyberlindnera saturnus*, 12 isolates to *Rhodotorula*, three isolates to *Debaryomyces*, and one isolate to *Bullera alba*. An additional 30 isolates were identified as *C. saturnus*, 10 isolates were identified as *R. mucilaginosa*, and three isolates were identified as *D. hansenii* using a combination of morphology and by sequencing a representative of a PCR fingerprint.

For filamentous fungi, suspensions of filamentous fungal colonies were aliquoted onto 0.2× PDA and 1× MH agar in triplicate. Colonies were imaged following adequate growth. By morphology, four isolates were identified as *Mucor* spp., three isolates each as *Cladosporium* spp., *Clonostachys* spp., and *Mortierella* spp., and two isolates as *Aspergillus* species.

In addition, partial ITS sequencing was performed on 73 filamentous fungal isolates from the collection, yielding 27 unique ITS sequences representing 22 species (Table 4).

While species-level resolution was obtained for most isolates, the sequencing could not distinguish between the members of certain species-complexes, such as the *Fusarium incarnatum-equiseti* and *Cladosporium cladosporioides* species complexes, as well as *Aspergillus wentii* and closely related species and *D. hansenii* and closely related species (Supplementary Tables 1 and 2).

Table 3 – **Community Composition of Yeasts Isolated from the Soils.**

Isolate counts were primarily identified by ITS sequencing, with sequences provided in Supplementary Table 1.0., with counts of isolates preliminarily identified by morphology or RAPD PCR noted by parentheses. A single yeast isolate from the high treatment plot, noted as 'Isolate FK01_169', showed no significant match in the NCBI database even when using pair-ended sequencing.

Phylogenetic Data of Isolate						Count by Plot Origin		
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Control**	**Low**	**High**
Basidiomycota	Tremellomycetes	Tremellales	Bulleraceae	*Bullera*	*alba*	2 (1)	3	3
Basidiomycota	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Cutaneotrichosporon*	*terricola*	1		
Ascomycota	Saccharomycetes	Saccharomycetales	Phaffomycetaceae	*Cyberlindnera*	*saturnus*	2 (12)	4 (22)	5 (7)
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Cystofilobasidium*	*infirmominiatum*	2	2	
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Cystofilobasidium*	*capitatum*		1	
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*	*nepalensis, hansenii, vietnamensis* (A)	1 (2)	1 (1)	2 (3)
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*	*nepalensis, hansenii, vietnamensis* (B)	1		
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales	*Diutina*	*catenulata*	1		
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*	*oeirense*		1	
Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	*Goffeauzyma*	*gastrica*	1		
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Hannaella*	*coprosmae*	1		
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Hannaella*	*oryzae* (A)	1		
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Hannaella*	*oryzae* (B)	1		
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae	*Leucosporidium*	*creatinivorum, yakuticum, scottii*	1	1	
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Mrakia*	*arctica, gelida*		3	
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Mrakia*	*blollopis*	2		
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Mrakia*	*arctica*	1		

NA	NA	NA	NA	NA	FK01_169			1
Basidiomycota	Tremellomycetes	Tremellales	Rhynchogastremataceae	*Papiliotrema*	*flavescens*		1	
Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*mucilaginosa*	8 (5)	4 (7)	8 (11)
Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*graminis*	3	1	
Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*babjevae*	1		
Basidiomycota	Microbotryomycetes	Microbotryomycetes	Chrysozymaceae	*Sampaiozyma*	*ingeniosa*	2	2	3
Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Schwanniomyces*	*occidentalis*		1	
Basidiomycota	Tremellomycetes	Filobasidiales	Piskurozymaceae	*Solicoccozyma*	*terrea*			1
Basidiomycota	Tremellomycetes	Filobasidiales	Piskurozymaceae	*Solicoccozyma*	*aeria*		1	
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Tausonia*	*pullulans* (A)	1	1	1
Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Tausonia*	*pullulans* (B)			1
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	sp. isolate C220			1
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*heimaeyensis, foliicola*			1
Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*victoriae*	1		

Table 4 – Community Composition of Filamentous Fungi Isolated from Soils. Sequences are provided in Supplementary Table 2.

Phylogenetic Data of Isolate						Count by Plot Origin		
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Control**	**Low**	**High**
Ascomycota	Leotiomycetes	Leotiomycetes	Leotiomycetes	*Acremonium*	sp. MJ35			1
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Acrophialophora*	*levis*	1	2	
	Mucoromycetes	Mucorales	Mucoraceae	*Actinomucor*	*elegans* (A)	3	1	
	Mucoromycetes	Mucorales	Mucoraceae	*Actinomucor*	*elegans* (B)	1		
Ascomycota	Sordariomycetes	Hypocreales	Stachybotryaceae	*Albifimbria*	*verrucaria, viridis*	1		
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*fumigatus, fumigatiaffinis*	1	1	1
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	[multiple]	1		
Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	[multiple]		2	
Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	*Clonostachys*	*rosea* (A)	4	8	5
Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	*Clonostachys*	*rosea* (B)		1	1
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*oxysporium*	3	1	1
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*incarnatum-equiseti*	1	1	
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Metarhizium*	*robertsii*	1	1	
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Metarhizium*	*marquandii*	1	1	
	Mucoromycetes	Mucorales	Mucoraceae	*Mortierella*	*alpina* (A)		2	1
	Mucoromycetes	Mucorales	Mucoraceae	*Mortierella*	*alpina* (B)			1
	Mucoromycetes	Mucorales	Mucoraceae	*Mortierella*	*alpina* (C)		1	
	Mucoromycetes	Mucorales	Mucoraceae	*Mortierella*	*gamsii*		1	
	Mucoromycetes	Mucorales	Mucoraceae	*Mortierella*	*hyalina*		1	

41

	Mucoromycetes	Mucorales	Mucoraceae	*Mucor*	*circinelloides*	1		1
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Neosartorya*	*udagawae*	1		
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*oxalicum* (A)	1		2
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*oxalicum* (B)	1		
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*novae-zeelandiae*	1		
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Plectosphaerella*	*cucumerina*		1	
Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	*Purpureocillium*	*lilacinum*		2	
	Mucoromycetes	Mucorales	Lichtheimiaceae	*Rhizomucor*	*variabilis*	3	4	3

3.4   Fungal population structure analysis

When looking at yeasts, the collection from the control plots have notably more species than those from the high treatment plots. Of 34 sequenced yeasts isolates from the control plots and 27 isolates each from the low treatment and high treatment plots, 18 (52% of identified isolates), 15 (55%), and 10 unique species (37%) were observed (Table 3), respectively. On the other hand, for filamentous fungi, 17 species of 26 identified isolates (65%), 17 species of 31 isolates (55%), and 10 species of 17 (59%) isolates were observed from the control, low, and high treatment plots, respectively (Table 4). Although the yeast collection from the high treatment plots has a notably lower species richness than the yeast collection from the control plots, a similar effect was not seen with filamentous fungi, possibly due to under-sampling of the filamentous fungi community.

3.5   Sensitivity of isolates to drugs

With respect to the medical imidazoles, clotrimazole and miconazole, of 138 tested yeasts to each drug, 93.5% and 86.2% of isolates were determined to be sensitive to clotrimazole and miconazole, respectively. Of the sensitive isolates, 3.9%, were determined to be only weakly susceptible to clotrimazole but notably more, 16.8%, were weakly susceptible to miconazole (Figure 12).

In contrast to the medical imidazoles and agricultural triazoles, the percentage of sensitive isolates to the medical triazoles fluconazole and voriconazole was significantly lower. Of 119 tested isolates, only 14.3% and 58.9% of isolates showed sensitivity to fluconazole and voriconazole, respectively. On the other hand, most isolates, 94.1% of 119 isolates, were sensitive to itraconazole. The percent of weakly susceptible isolates was relatively high with 17.6%, 11.4%, and 12.5% of susceptible isolates to fluconazole, voriconazole, and itraconazole, respectively, being defined as weakly susceptible (Figure 12).

With respect to the agricultural triazoles, 100% of 104 and 98 isolates tested against difenoconazole and tebuconazole, respectively, showed a sensitive phenotype by the end of the

standard assay. Meanwhile, 93.3% of 104 isolates tested against propiconazole showed a sensitive phenotype (Figure 12).

Distribution of susceptibilities of isolates tested against various drugs
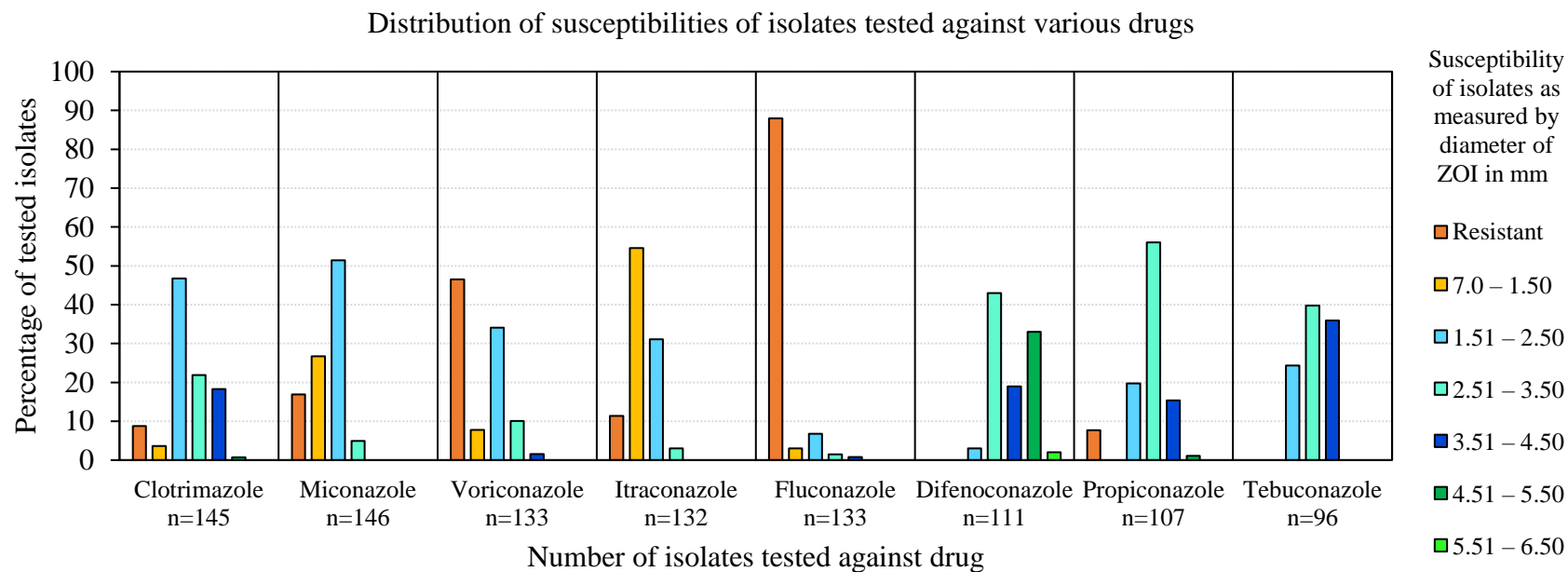
Figure 12 – Distribution of Susceptibilities of Yeasts Tested against Various Azoles

Sensitivity of yeasts isolated from all soils tested against eight azole drugs show that the tested yeasts were most susceptible to the tested agricultural triazoles difenoconazole, propiconazole, and tebuconazole, followed by the medical imidazoles clotrimazole, miconazole, and then the medical triazoles voriconazole, itraconazole, and fluconazole. Susceptibility of isolates to the drugs were ranked by diameter of the zones of inhibition that the drug disks produced by a disk diffusion assay.

3.6 Cross-resistance between drugs against yeasts

Overall, resistance to voriconazole was associated with resistance to all other medical drugs and propiconazole (p<0.001 for each pair-wise comparison) (Supplementary Table 3.1-3.5). Also, resistance to itraconazole was correlated with resistance to fluconazole (p=0.002; Supplementary Table 3.6) and resistance to either was significantly correlated with resistance to miconazole (Supplementary Table 3.7- 3.8). However, resistance to clotrimazole was not associated with resistance to miconazole (p=0.107; Supplementary Table 3.9), fluconazole (p=0.050; Supplementary Table 3.10), or itraconazole (p=0.123; Supplementary Table 3.11). As for the agricultural triazoles, resistance to propiconazole was associated with resistance to the medical imidazoles, clotrimazole (p<0.001; Supplementary Table 3.12) and miconazole (p<0.001; Supplementary Table 3.13), in addition to voriconazole, but not to itraconazole (p=0.479; Supplementary Table 3.14) or fluconazole (p=1; Supplementary Table 3.15). Analyses of cross-resistance could not be made for difenoconazole and tebuconazole toward the other drugs as all isolates tested against these two drugs were susceptible.

Cross-resistance to multiple drugs was often associated with particular species. Strains of *Bullera alba* were frequently multi-drug resistant with two out of nine tested strains being resistant to clotrimazole, miconazole, voriconazole, fluconazole, and propiconazole and an additional strain being sensitive to all of the above drugs except clotrimazole. In addition, all tested *Rhodotorula mucilaginosa* isolates were resistant to fluconazole, with the majority of isolates also being resistant to voriconazole, and four out of 41 strains showing complete resistance to miconazole and itraconazole as well. In addition, five out of six *Sampaiozyma ingeniosa* strains were resistant to miconazole, voriconazole and fluconazole.

Regression analysis did not indicate that the extent of sensitivity, as measured by the size of the zone of inhibition, for a given isolate to any azole was directly correlated with the sensitivity to any other azole (Table 5).

Table 5 – Regression Analysis using ZOI (mm) of the Same Isolate Tested Against Two Drugs as Variables. R^2 values shown.

	Clotrimazole	Miconazole	Voriconazole	Itraconazole	Fluconazole	Difenoconazole	Propiconazole
Miconazole	0.00						
Voriconazole	0.01	0.46					
Itraconazole	0.01	0.37	0.35				
Fluconazole	0.01	0.19	0.51	0.16			
Difenoconazole	0.41	0.06	0.22	0.03	0.38		
Propiconazole	0.05	0.45	0.35	0.12	0.21	0.01	
Tebuconazole	0.22	0.10	0.15	0.02	0.20	0.50	0.09

Table 6 – Mean ZOI (mm ± standard deviation) of Isolates Tested Against Azoles by Plot Type Origin.

	Clotrimazole	Miconazole	Voriconazole	Itraconazole	Fluconazole	Difenoconazole	Propiconazole	Tebuconazole
Yeasts								
Control	23.5 ± 10.7	14.3 ± 7.3	8.5 ± 12.4	14.8 ± 7.1	66.0 ± 10.8	42.4 ± 9.9	27.3 ± 10.9	31.6 ± 6.3
Low treatment	22.8 ± 7.8	14.4 ± 6.3	12.7 ± 10.2	14.4 ± 5.5	81.0 ± 8.3	34.3 ± 9.5	28.5 ± 9.1	30.2 ± 7.4
High treatment	21.9 ± 11.8	11.7 ± 8.0	90.0 ± 11.3	12.0 ± 6.3	44.0 ± 7.1	38.7 ± 9.1	25.9 ± 11.5	32.6 ± 2.6
Filamentous fungi								
Control	22.2 ± 8.8	17.5 ± 11.6						
Low treatment	25.2 ± 11.6	17.2 ± 11.9						
High treatment	21.9 ± 2.2	13.1 ± 11.8						

3.7   Susceptibility patterns of isolates to medical imidazoles

The susceptibility of isolates obtained from control and treated soils toward clotrimazole and miconazole were not found to be different. The average zone of inhibition (ZOI), with sample-based standard deviation to clotrimazole was 23.5 ± 10.7 mm, 22.8 ± 7.8 mm, and 21.9 ± 11.8 mm when testing yeasts from the control ($n=50$), low ($n=53$), and high treatment ($n=43$) plots, respectively (Table 6). The average ZOI when testing 33 filamentous fungi from the control plots, 31 isolates from the low treatment plots, and 24 isolates from the high treatment plots was 22.2 ± 8.8 mm, 25.2 ± 11.6 mm, and 21.9 ± 2.9 mm, respectively (Table 6). In both cases, As shown by a two-tailed T-test for two independent means, there was no significant difference in average zone of inhibition between tests using isolates from the control versus low treatment plots ($p=0.68$ for yeasts, $p=0.23$ for filamentous fungi) or between the control versus high treatment plots ($p=0.43$ for yeasts, $p=0.88$ for filamentous fungi). Likewise, the average ZOI using miconazole disks and yeasts from the control, low, and high treatment plots showed no difference in susceptibilities between yeasts from the control plot versus the low treatment plots ($p=0.92$), or versus the high treatment plots ($p=0.12$) (Table 6). Similarly, with respect to all tested filamentous fungi, the average ZOI using control versus low treatment-origin isolates was not statistically significant ($p=0.19$), nor was the difference when testing control versus high treatment-origin yeasts ($p=0.17$) (Table 6).

The sensitivities of isolates from within a given species were tested to clotrimazole and miconazole to compare the degree of variation from isolates originating from the control plots with isolates originating from the treated plots. On a species-level, zones of inhibition were very similar when testing 53 *Cyberlindnera saturnus* isolates against clotrimazole and miconazole, with average ZOI not being statistically significantly different when testing isolates from the control versus low treatments ($p=0.90$) and control and high treatments ($p=0.63$) (Figure 13). A similar lack of treatment effect was seen with 17 *Clonostachys rosea* isolates and 10 *Rhizomucor variabilis* isolates tested against the medical imidazoles (Figure 13).

Although *Rhodotorula* isolates from the control treatment appeared to be more resistant to clotrimazole than the isolates from the high treatment, with an average zone of inhibition of 34.2

± 2.7 mm when testing the control-origin isolates versus 37.2 ± 2.6 mm when testing the high treatment-origin isolates (p=0.006), there was no statistically significant difference in average ZOI when testing control versus low-treatment origin isolates (p=0.11) at 34.2 ± 2.7 mm versus 36.1 ± 3.1 mm (Figure 13).

To further test *Rhodotorula* isolates against clotrimazole, two additional tests were performed. Isolates were grown on SD agar plates at room temperature and then sub-cultured on MH agar and incubated for approximately 12 hours at 30°C. Following growth, a 10 µL loop of cells was used for susceptibility testing using RPMI-glucose agar and Neosensitab disks. Susceptibility tests were then performed except seven isolates each from the control and high treatment plots were incubated with reduced oxygen by incubation in a large BD GasPak EZ container (Becton, Dickinson and Company, Franklin Lakes, NJ) containing one Mitsubishi AnaeroPouch-MicroAero pouch (Mitsubishi Chemical Holdings, Tokyo, Japan), and 17 isolates from the control plots and 11 isolate from the high treatment plots were incubated under standard conditions for seven days. Although two MicroAero pouches are needed to create the standard microaerophilic environment in a large GasPak EZ container, a Mitsubishi Anaero-Indicator indicated reduced oxygen concentration within the container throughout the incubation period of 48 hours. In both cases, *Rhodotorula* isolates from the high treatment plots were not any more or less susceptible to clotrimazole then those from the control plots (p=0.18 for the reduced oxygen incubation, and p=0.37 for the seven-day incubation). Interestingly, in both cases this time, isolates from the control plots appeared to be more susceptible to the clotrimazole than those from the high treatment plots with average zones of inhibition of 37.0 ± 2.1 mm versus 34.7 ± 3.6 for the reduced oxygen trial and 34.2 ± 2.4 versus 33.1 ± 4.6 mm for the seven-day incubation. Overall, given the increased testing, it appears that the initial results of *Rhodotorula* isolates from the high treatment being more susceptible to clotrimazole than isolates from the control was due to a sampling error.

3.8 Isolates highly susceptible to medical imidazoles

Eleven yeasts, representing 7.6% of the collection, were considered to be highly susceptible to miconazole due to ZOI measurements being greater than or equal to 25.0 mm (Figure 12). These isolates were also susceptible to clotrimazole and were largely isolated from the plots receiving no

azole fungicides. The isolates represented 17% of tested isolates from the control plots (n=8 of 47) and were identified as *Bullera alba*, *Cutaneotrichosporon terricola*, *Diutina catenulata*, *Hannaella oryzae* (n=2), *Mrakia arctica, Sampaiozyma ingeniosa*, and *Vishniacozyma victoriae*, 3.8% of isolates from the low treatment plots (n=2 of 53) and included *Schwanniomyces occidentalis* and *Solicoccozyma aeria*, and 4.5% of tested isolates from the high treatment plots (n=2 of 44) which included a *Bullera alba* isolate and an unidentified psychrophilic isolate. As calculated by a Fisher exact T-test, the proportion of isolates highly susceptible to miconazole was statistically significant between the control and low treatment plots (p=0.04; Supplementary Table 4.1) but not between the control and high treatment plots (p=0.09; Supplementary Table 4.2).

On the other hand, when defining yeasts highly sensitive to clotrimazole as having a ZOI $\geq$ 300 mm in diameter (n=51) in the disk diffusion assays, 46%, 26%, and 32% of control, low treatment, and high treatment-origin isolates were defined as highly susceptible. Increasing the threshold for high susceptibility to a ZOI $\geq$ 350 mm included 30 isolates from all plots and led to 24%, 17%, and 20% of the control, low treatment, and high treatment collections falling under the definition. Given the lack of a treatment effect, isolates highly sensitive to clotrimazole were not analyzed further.
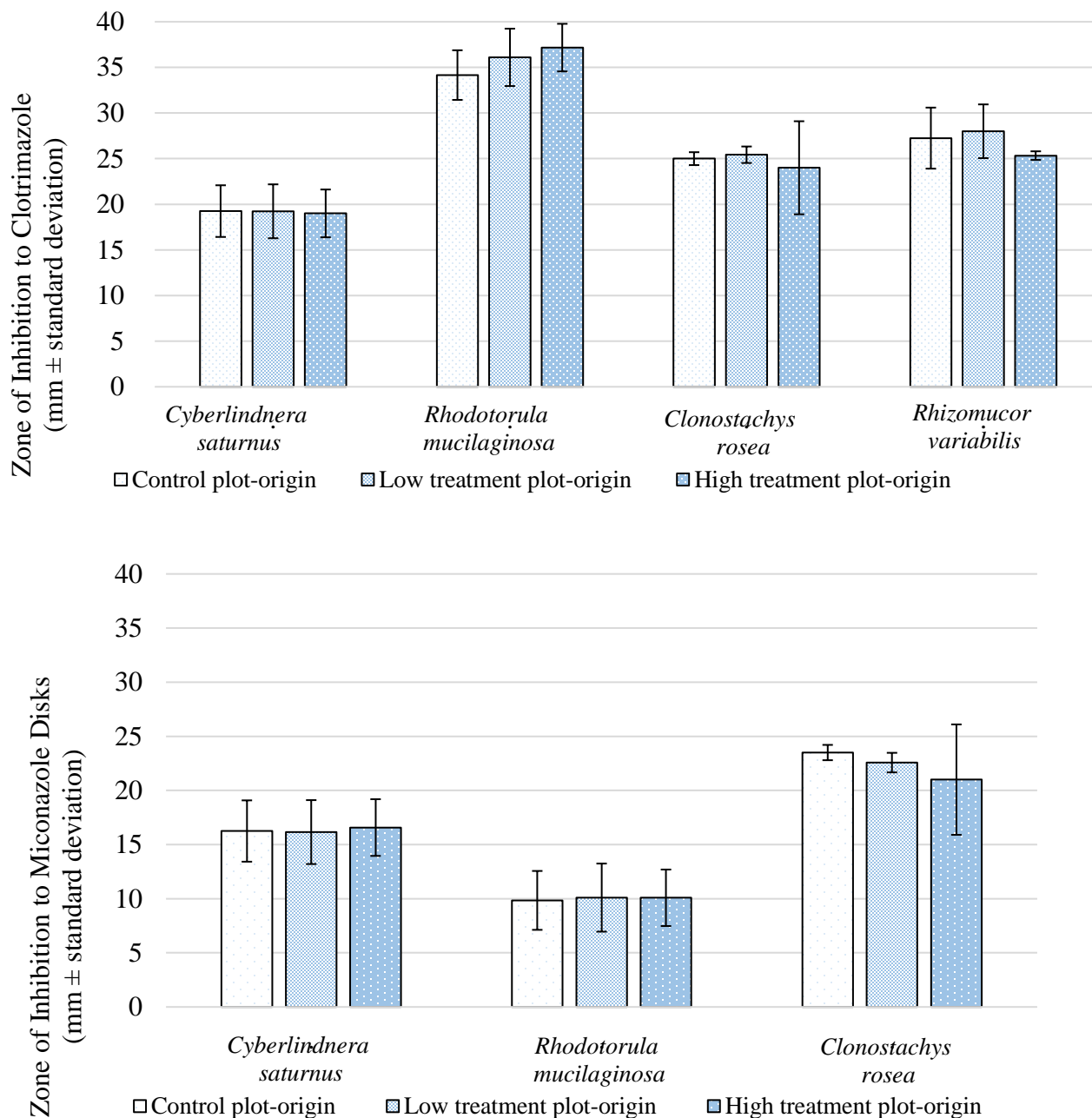
Figure 13 – Sensitivities of Selected Species to Clotrimazole and Miconazole

Sensitivity assays with select yeasts and filamentous fungi species against 10 μg clotrimazole disks shows a significant difference in zones of inhibition when testing *R. mucilaginosa* isolates from the control versus high treatment plots. No other statistically significant pair-wise difference was detected. Likewise, sensitivity assays with selected yeasts and filamentous fungi species against 10 μg miconazole disks showed no differences in average zones of inhibition based on soil type origin of the isolates. Isolates of *Rhizo. variabilis* were resistant to miconazole.

3.9 Susceptibility patterns of isolates to medical triazoles

Of 146 yeasts tested against itraconazole, the average ZOI using isolates from the control plots versus the high treatment plots was slightly higher at 14.8 ± 7.1 versus 12.0 ± 6.3 mm ($p=0.06$). Nonetheless, *C. saturnus*, *R. mucilaginosa*, and *D. hansenii* isolates from the control versus high treatment plots were not significantly more or less sensitive to itraconazole ($p=0.35$, $p=0.46$, and $p=0.44$, respectively) (Figure 14). Similarly, among 96 tested yeasts against voriconazole, there was no significant difference in average ZOI when testing isolates from the control versus low treatment plots ($p=0.37$) or high treatment plots ($p=0.13$) to the disks. Likewise, no difference in average ZOI to voriconazole was found when testing *C. saturnus* isolates from the control versus the low treatment plots ($p=0.88$) or versus the high treatment plot ($p=0.17$) (Figure 14). Only 25 of 158 tested yeasts were susceptible to fluconazole but, overall, no significant difference was found in average ZOI when testing isolates from the control versus treated plots.

3.10 Susceptibility patterns of isolates to agricultural triazoles

Similar to the medical imidazoles and medical triazoles, for the agricultural triazoles, in general, no statistically significant differences were found between the average zones of inhibition for isolates from the control plots ($n=38$ to 39 for each drug) versus the low treatment plots ($n=38$ for difenoconazole and propiconazole, $n=30$ for tebuconazole), or for the control versus the high treatment plots ($n=34$ for difenoconazole and propiconazole, $n=30$ for tebuconazole). For isolates tested against the propiconazole disks, the average zones of inhibition was 27.3 ± 109 mm, 28.5 ± 91 mm, 25.9 ± 11.5 mm for control, low treatment, and high treatment-origin isolates, respectively and for isolates tested against tebuconazole, the averages were 31.6 ± 6.3 mm, 30.2 ± 7.4 mm, and 32.6 ± 6.2 mm, respectively (Table 6). However, isolates from the low treatment plot appeared to be significantly more resistant to difenoconazole than those from the control treatment plots ($p=0.0004$) with an average zone of inhibition of 34.3 ± 9.5 mm for isolates from the low treatment plot versus 42.4 ± 9.9 mm for the isolates from the control plots (Table 6). When the average zone of inhibition from the control plot isolates was compared to that of the high treatment isolates, 38.7 ± 9.1 mm, the difference was not significant ($p=0.11$) (Table 6). While the average zones of

inhibition toward the three agricultural azoles for the yeast *Cyberlindnera saturnus*, *Rhodotorula mucilaginosa*, and *Debaryomyces* spp. showed little variation per species per drug, as typical for the clinical azoles, large variation in particular was seen within *Bullera alba* isolates toward difenoconazole. Isolates of *B. alba* from the control (*n=3*), low treatment (*n=3*), and high treatment (*n=3*) plots produced average zones of inhibition to difenoconazole at 43.0 ± 4.6 mm, 33.7 ± 15.5 mm, and 29.3 ± 6.0 mm, respectively.
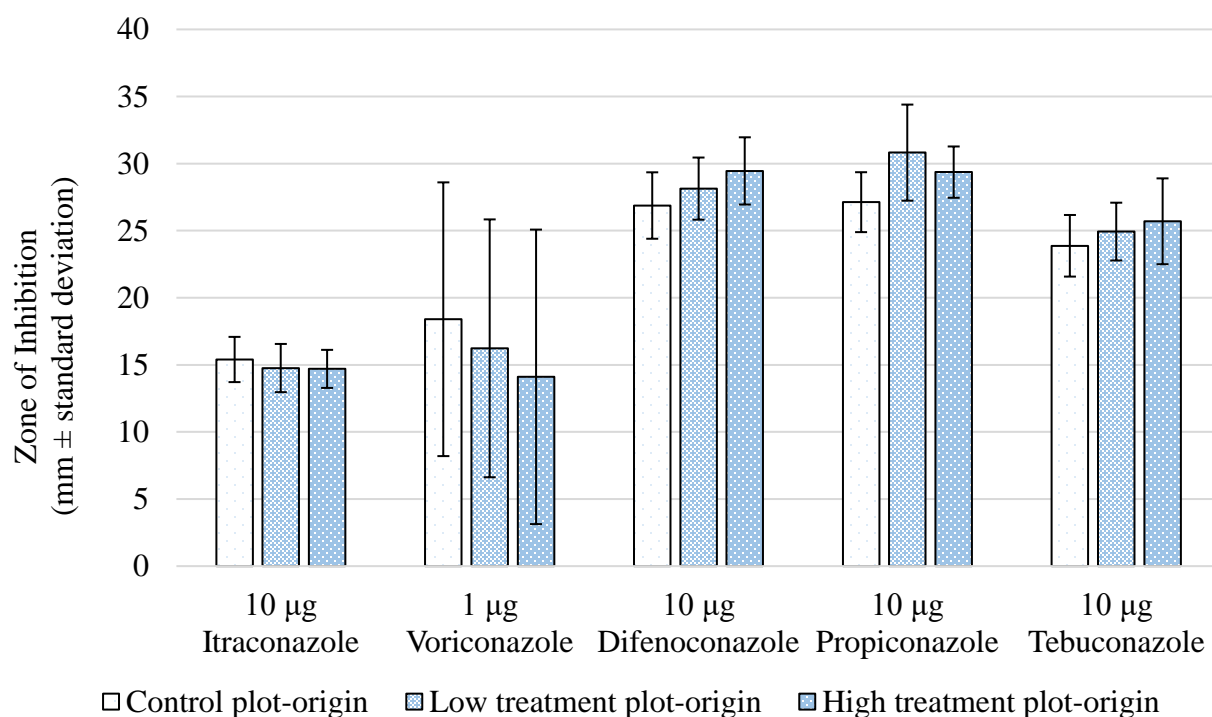

Figure 14 – Sensitivity of *Cyberlindnera saturnus* Isolates to Triazoles

No significant difference in average ZOI was noted in tests using yeasts from the control versus treated plots.

4 Discussion

4.1 Impact of fungicide exposure on population dynamics

As shown by the plate counts on RB agar, the lower number of recovered colonies per gram of soil plated between the soils from the control versus the high treatment suggests that the clotrimazole and miconazole in the high treatment plots led to lower viable fungal propagules (Figure 8). The fact that the high treatment soils sampled in 2018 had lower ergosterol content per gram of soil than the samples from the control plots further shows that the high treatment soils have reduced fungal load due to the long-term effects of the antifungal drugs in the soil (Figure 9). The increase in ergosterol content seen in the control soils versus the high treatment soils sampled in 2018 may have been due to increased fungal growth in the rhizosphere of the cropped soybeans over eight years (Figure 9).

The RB and DG agars contain rose bengal and dichloran, respectively, which inhibit the radial growth of fungi that can spread over large portions of the plate in a species-dependent manner (King *et al*., 1979). This leads to higher colony counts and the effective isolation of slower-growing species (Henson, 1981; Hocking & Pitt, 1980; King *et al*., 1979). In addition, incubation at 4°C selects for yeasts, especially psychrophilic yeasts, over filamentous fungi, and the addition of 220 g/L glycerol in the DG agar selects for xerophilic species. One possible reason why the colony counts on DG agar did not show a significant difference between treated and untreated soils is due to the sporulation of filamentous fungi after the six week incubation period. Nonetheless, the RB plates incubated at 15°C for four days were almost exclusively dominated by filamentous fungi growth while still showing significant differences in colony counts between the control versus high treatment soils.

The observation that both the yeasts and filamentous fungi collections from the control plots showed lower diversity in the control plots versus the treated plots can be attributed to the fact that several species were isolated only from the control plots but not the treated plots. Several yeasts, amounting to 13.7% of the collection, were identified as belonging to species isolated only once

(Table 2). Half of these unique isolates were isolated from the control plots while the remaining half were isolated evenly between the low treatment and high treatment plots. In addition, five *Mrakia* spp., and four isolates each of *Cystofilobasidium infirmominiatum* and *Rhodotorula graminis* were isolated from the control and low treatment plots, but not the high treatment plots. Likewise, for the filamentous fungi collection, 16.4% of identified isolates (12 of 73 isolates) belonged to species represented only once in the dataset with half of these species being isolated from the control plots (Table 3).

For the yeast collections from the control plots and high treatment plots, respectively, *Cyberlindnera saturnus* comprised 20% and 27% of the collections while *Rhodotorula mucilaginosa* comprised 32% and 25%. However, it should be noted that the fungal collections were constructed with the goals of maximizing diversity in the collections and minimizing clonality. In particular, when broths enriched for yeasts were plated onto agar, if colonies with identical morphologies were observed on the same agar plate, only one isolate was retained for the collection. In addition, if more than one identical colony of a filamentous fungi were growing on the same agar plate plated with soil, only one colony was retained for the collection. Thus, it is not possible to easily determine from the fungal libraries if species that were readily culturable are more or less represented in the control or treated soils as the enrichment process would distort counts of yeasts. Nonetheless, although yeasts were not tallied by morphology noted per agar plate, in certain instances, when each soil was directly plated onto agar, it was clear that colonies of certain morphologies were less prevalent on plates containing high treatment soils compared to control soils. For example, red pigmented colonies were observed on all four DG agar plates plated with control soil on but were not observed on any of the four plates plated with high treatment soil. A sampling of these red pigmented colonies identified two as *Rhodotorula*.

4.2 No evidence of increased azole resistance in isolates from azole-containing soil

As shown by the disk diffusion assays, in general, yeasts and filamentous fungi isolated from the soils receiving clotrimazole and miconazole were not detected to be any more or less sensitive to the tested azoles when compared to isolates from the control plots. This pattern held when looking at all isolated yeasts or filamentous fungi from a soil treatment type and when looking at specific species, such as the yeast *Cyberlindnera saturnus* and *Rhodotorula mucilaginosa* and the filamentous fungi *Clonostachys rosea* and *Rhizomucor variabilis*. However, some statistical tests did show significant differences in sensitivities between sensitive isolates from different treatments, as determined by smaller average zones of inhibition. For example, in the case of difenoconazole, isolates from the low treatment appear to be more resistant to those from the control with an average zone of inhibition nearly 10 mm smaller (p=0.0004) while the isolates from the high treatment were not any more resistant (*p*=0.11). It could be argued that these p-values are affected by low statistical power of the tests, as the average zone of inhibition from the low treatment isolates was 34.3 ± 9.5 mm versus 42.4 ± 9.9 mm for the isolates from the control, which puts the two averages within the 20% error range for ZOI measurements (Table 4.0; Table 6).

4.3 Susceptibilities of tested species versus literature values

Similar to the results from this study showing all *R. mucilaginosa* isolates being sensitive to clotrimazole, an analysis of 72 strains of *Rhodotorula*–including five strains of *R. mucilaginosa*–from hospitals in Iran (Seifi *et al*., 2013) in addition to five *R. mucilaginosa* strains from pigeon feces in Saudi Arabia (Abulreesh *et al*., 2019) showed all *R. mucilaginosa* strains being sensitive to clotrimazole. In this study, 80.5% of *Rhodotorula* isolates were considered to be weakly susceptible to the miconazole disks, with an additional 17% being considered resistant and a single isolate being classified as sensitive. Likewise, a study of 30 clinical *Rhodotorula* strains–21 of them being *R. mucilaginosa*—from French hospitals found that miconazole was the only drug of eight tested drugs showing a range of sensitivities, with most isolates being marked as

'intermediate' (Preney *et al*., 2003). The clinical strains from Iran also showed a range of sensitivities to miconazole, especially with *R. mucilaginosa* (Seifi *et al*., 2013).

In comparison to the relatively few *Rhodotorula* isolates tested to the medical imidazoles in the literature, much more information exists on the sensitivity of clinical *Rhodotorula* isolates to the medical triazoles. A review of 131 *Rhodotorula* isolates tested using the established NCCLS and EUCAST methods, in addition to the commercially-available agar-based Etest and the microbroth dilution-based Sensititre YeastOne tests, concluded that fluconazole, voriconazole, and itraconazole were inactive against the majority of tested isolates (Gomez-Lopez *et al*., 2005). The MIC_{90} values for 29 isolates tested by the reviewers were found to be >64, 8, and 8 mg/L to fluconazole, itraconazole, and voriconazole, respectively. This is in accordance with this study, which ultimately classified all *Rhodotorula* isolates as resistant to fluconazole, 85.4% of isolates being resistant to voriconazole with the remaining being weakly susceptible, and 78% of isolates being resistant to itraconazole with 14.6% being weakly susceptible and 7.3% being susceptible.

Nonetheless, previous studies have noted several differences between clinical and non-clinical *Rhodotorula* strains. For example, while clinical *Rhodotorula* strains are frequently noted as fluconazole-resistant (Tuon & Costa, 2008), the *Rhodotorula* strains from pigeon feces in Saudi Arabia were noted for their susceptibility to fluconazole (Abulreesh *et al*., 2019), a result shared with five *Rhodotorula* strains isolated from bird feces in Malaysia (Lord *et al*., 2010). In addition, a sample of clinical isolates was shown to have greater biofilm capacity compared to environmental isolates (Nunes *et al*., 2013). As for sensitivity to agricultural azoles, a metagenomic study of pear fruit fields treated with various fungicides, including azoles such as difenoconazole and tebuconazole, found that the proportion of *Rhodotorula* isolates, primarily represented by *R. glutinis*, increased in the fungicide-treated fields compared to control fields, which suggests relative resistance to these drugs (Zambounis *et al*., 2020).

As for isolated filamentous fungi, *Actinomucor elegans, Mortierella alpina*, *Mucor circinelloides, and Rhizomucor variabilis* were isolated from the soils and were expected to be resistant to the azole drugs. These species belong to the Mucoromycotina order, which causes the highly-lethal disease Mucormycosis (Jeong *et al*., 2019). A single amino acid change in residue 129 of CYP51

F5 confers resistance to certain azoles, such as voriconazole and fluconazole, but not others, such as itraconazole (Caramalho *et al*., 2017). The mechanism of resistance to the former drugs and sensitivity to itraconazole has been elucidated to involve a tertiary hydroxyl group that interferes with hydrogen bonding between the azole drug and the lanosterol 14a-demethylase enzyme (Sagatova *et al*., 2016). Sun *et al*. reported that MIC values, as determined by CLSI microbroth dilutions, for seven clinical *Mucor* sp. were all above 64 μg/mL for fluconazole and ranged between 32-64 μg/mL for voriconazole (Sun *et al*., 2002). Meanwhile, the MIC values toward itraconazole ranged from 0.25-8 μg/mL (Sun *et al*., 2002).

As expected, given the data from the literature, isolated *Mucor circinelloides* (*n*=2) and *Rhizomucor variabilis* (*n*=9) were resistant to fluconazole and voriconazole and weakly susceptible to miconazole and itraconazole. Interestingly, however, the isolates were clearly susceptible to clotrimazole. In a preliminary test, a single tested *M. circinelloides* isolate from the high treatment plots showed sensitivity to difenoconazole and tebuconazole and resistance to propiconazole. This finding could be useful as Mucor rot has been described as an emerging postharvest disease caused by *Mucor* spp. affecting multiple crops, such as mandarin oranges in California (Saito *et al*., 2014). Resistance of the species *Mucor rouxii* to propiconazole has previously been described (Weete & Wise, 1987).

4.4 Spectrums of activity and co-resistance between azoles

A previous study analyzed the antifungal susceptibility of 1,698 yeasts from various sources (Desnos-Ollivier *et al*., 2012). The study defined isolates with a high MIC as being able to grow in ≥8 ug/mL fluconazole, ≥0.25 ug/mL voriconazole, and ≥0.5 ug/mL of itraconazole. Fluconazole was effective against the smallest proportion of environmental isolates, with 45% having a high MIC toward the drug, while only 18-21% of environmental isolates had a high MIC against voriconazole or itraconazole. The study also made the surprise finding that, when considering isolates from all origins, which included isolation from human, food, and industrial sources, Basidiomycetes were more likely to yield a high MIC to the three drugs than Ascomycetes with high MIC rates of 75-76% compared to 16-34% (Desnos-Ollivier *et al*., 2012).

In agreement with the study, as shown in Figure 12.0, a higher proportion of isolates were resistant to fluconazole than the other two tested medical triazoles. In addition, of the tested medical imidazoles, clotrimazole was effective against a larger proportion of isolates compared to miconazole, which agrees with a previous study of yeasts with medical significance (Yamaguchi *et al*., 1983). When broken down by phylogeny, 82 Basidiomycete yeasts strains were tested against the medical azoles with 8.5% and 26.8% being resistant to the imidazoles clotrimazole and miconazole, respectively, and 15.8%, 69.5%, and 95.1% being resistant to the triazoles itraconazole, voriconazole, and fluconazole, respectively. On the other hand, of 63 Ascomycete yeasts, all were susceptible to the medical azoles with the exception of two isolates of *Debaryomyces* to fluconazole. All isolated filamentous fungi, except for the Mucormycetes, belonged to the Ascomycota.

One unexpected finding from the azole sensitivity tests was that co-resistance to clotrimazole and miconazole was not widely noted either in isolates obtained from the control plots or from the plots receiving annual applications of clotrimazole and miconazole (Table 5.0; Supplementary Table 3.9). Of the seven clotrimazole-resistant yeasts, one isolate identified as *Filobasidium oeirense* and four isolates identified as *Tausonia pullulans* were sensitive to miconazole while two isolates identified as *Bullera alba* were resistant to both drugs. On the other hand, most isolates of *Sampaiozyma ingeniosa* were miconazole-resistant and only weakly susceptible to clotrimazole while *Rhodotorula mucilaginosa* isolates were commonly miconazole-resistant while being clearly susceptible to clotrimazole. With respect to filamentous fungi, the fact that the tested Mucormycetes were sensitive to clotrimazole but not the other tested azole drugs lends further evidence that the spectrum of activity of clotrimazole is significantly different from that of the other clinical azoles. Overall, it was clear that, miconazole-resistant isolates were not likely to be clotrimazole-resistant and clotrimazole-resistant isolates were not likely to be miconazole-resistant.

4.5   Isolates with little prior azole documentation

As for the identified isolates determined to be very susceptible to miconazole, the azole-susceptibilities of the corresponding species have not been well-described in the literature. There were no previous reports of the susceptibility of *Sampaiozyma ingeniosa* to the tested azoles and *Cutaneotrichosporon terricola*, *Schwanniomyces occidentalis*, *Solicoccozyma aeria*, and *Mrakia* spp. to any azole in the literature. Of note, the disk diffusion assays were performed at either 15°C or 30°C, while previous studies of environmental antifungal resistance tend to focus on yeasts with medical or agricultural significance or those that can grow readily during testing. For example, Maciel *et al.* did not test the azole susceptibility of *Cutaneotrichosporon terricola* due to its inability to grow at 37°C (Maciel *et al.*, 2019), and the disk diffusion assay performed by Perini *et al.* where *Mrakia spp.* isolates were grown on SD agar for four days and then tested on RPMI-glucose agar at 15°C for 2-7 days failed due to a lack of growth on test plates (Perini *et al.*, 2019). However, in this study, growing *Mrakia* isolates for seven days on SD agar and then performing a four-day disk diffusion assay on RPMI-glucose agar plates, both at 15°C, successfully allowed for azole susceptibility testing of 4 out of 6 *Mrakia* isolates.

On the other hand, a previous mycobiome sequencing study of the wheat phyllosphere mycobiome after application of Viverda, an agricultural fungicide containing epoxiconazole in addition to fungicides from other classes, identified *V. victoriae* as being the most susceptible species to the effects of the fungicide (Knorr *et al.*, 2019). Disk diffusion assays using the difenoconazole, propiconazole, and tebuconazole disks on the same plate failed twice with indications of large zones of inhibition only appearing after six-days of incubation. The findings from this study, as well as the findings from the disk diffusion assay, indicate that this species may be hyper susceptible to azoles.

A literature review yielded few results on the antifungal susceptibility of several other species isolated and tested in this study. For example, the sole reference for the antifungal susceptibility of *C. saturnus* comes from a single isolate obtained from Piracicaba River, Brazil that was tested against a panel of drugs, including fluconazole and itraconazole, using a broth microdilution test at 37°C for 48 hours. The test yielded MIC_{80} values for fluconazole (>64 µg/mL) and itraconazole

(0.25 µg/mL) marking relative resistance and susceptibility to the drugs, respectively, similar to the results obtained in this study (Medeiros *et al.*, 2008). However, when tested by disk diffusion assays, *C. saturnus* isolates showed trailing endpoints toward all tested azole drugs and continued growth within the zones of inhibition following the standard disk diffusion assay. Thus, *C. saturnus* may be more resistant to azole drugs than suggested by both studies. The azole susceptibility of *C. saturnus* may be of future interest due to its previously described abundance in soils and ability to promote plant growth.

4.6   Isolates with human pathogenic potential

A previous analysis of 5,000 yeasts from 1,000 environmental samples yielded 54 strains of budding yeasts with human pathogenic potential and found that pathogenic yeasts were associated with soils compared to other sampling locations such as fruit, leaves, and wood (Opulente *et al.*, 2019). This study, on the other hand, yielded relatively more isolates classified as opportunistic human pathogens including yeasts, such as *Rhodotorula mucilaginosa*, that are not classified as 'budding yeast'.

Debaryomyces hansenii and *Rhodotorula mucilaginosa* are two opportunistic human pathogens that are commonly isolated from environmental sources. *D. hansenii* (syn. *Candida famata*) is particularly associated with dairy products as well as being a cause of infections of the bloodstream, peritoneum, retina, and tissue of the mid-chest (reviewed in Beyda *et al.*, 2013). The ARTEMIS global antifungal surveillance program found that of over 250,000 clinical *Candida* isolates collected globally between 1997 and 2007, 0.3% were identified as *Candida famata* (Pfaller *et al.*, 2010). In addition, *C. famata* was isolated from 2.1% of 642 blood samples producing fungal isolates at Teikyo University Hospital, Japan between 1979 and 1995 (Kawakami *et al.* 1980). Although being a rare cause of infections, at least one case of invasive infection has been detected in a patient with no underlying conditions or risk factors (Wong *et al.*, 1982). On the other hand, clinical infection of *D. hansenii* may be subject to overestimation due to misidentification of *Pichia guilliermondii* (syn. *Candida guilliermondii*) as *D. hansenii* (Desnos-Ollivier *et al.*, 2008).

Another candidiasis-causing yeast, *Diutina catenulata* (syn. *Candida catenulata*) was isolated from a control plot. The species is commonly isolated from environmental samples, particularly dairy products, and is a rare cause of invasive infections in immunocompromised hosts (Radosavljevic *et al*., 1999).

Rhodoturla mucilaginosa has been described as an emerging opportunistic pathogen (Pfaller & Diekema, 2004; Wirth & Goldani, 2012). As reviewed by Wirth & Goldani (2012), *Rhodotorula* species are ubiquitous in the environment, having been isolated from environmental samples, such as air, soil, and food products, as well as being detected as the most common microorganism from the hands of hospital workers. *R. mucilaginosa* is also a common contaminant of catheters due to its strong affinity for plastic. It follows that most cases of infections involve central venous catheter (CVC) use and, most likely due to the increase in CVC use, reports of infections have increased in haematological patients since 1985. Infections can also, especially in HIV-positive patients, lead to serious complications such as meningitis and endophthalmitis (Wirth & Goldani, 2012). Overall, *Rhodotorula* was found in 4.2% of clinical isolates (*n*=8821) in the ARTEMIS project, being the 4th-most common non-*Candida* yeast (Pfaller, Diekema, *et al*., 2009).

No other yeasts of clinical relevance was identified in the collection. Although some case studies note *Tausonia pullulans* (syn. *Trichosporon pullulans*) as a cause of trichosporonosis in immunocompromised hosts, reports of *T. pullulans* infections may be due to misidentification (Holland *et al*., 2004).

For filamentous fungi, the most clinically relevant group isolated from the soils was *Aspergillus* spp., which cause a group of diseases called aspergillosis. Aspergillosis can present as respiratory infections as spores of the fungi are introduced into the lungs. Patients with asthma or cystic fibrosis can suffer from allergic bronchopulmonary aspergillosis, which is an allergic reaction to the fungi that can damage lung tissue (Latgé, 1999). In addition, those with pre-existing lung conditions can suffer from aspergilloma, balls of the fungi in the lungs, which are commonly asymptomatic but can also lead to fatal internal bleeding due to disrupted blood vessels (Latgé, 1999). In addition, aspergillosis can present as an invasive infection in

immunocompromised patients with severity and symptoms depending on the organ infected and the underlying condition (Latgé, 1999).

In addition, *Purpureocillium lilacinum* has been noted for causing invasive infections in immunocompromised patients. The fungus can spread quite readily, for example, in contaminated lotion in a hospital (Orth, 1996). Spores of the species are used in the agricultural setting as a biocontrol agent where it has been suggested that they pose a risk to immunocompromised people (Luangsa-Ard *et al*., 2011). The species can cause serious eye infections, such as keratitis and endophthalmitis, in those who wear soft contact lenses or have trauma to the eyes (Todokoro *et al*. 2014) and can also cause dermatological infections without predisposing conditions (Saghrouni *et al*., 2013).

Fusarium species are found in environmental samples, such as soil and air, and are opportunistic human pathogens. For immunocompetent people, *Fusarium* infections can cause onychomycosis in those that walk outdoors with exposed toenails, cutaneous infections for burn victims, and keratitis for contact lens users. On the other hand, in cancer patients, those with severe burns, or organ transplant patients, skin, lung, and sinus infections can develop (Dignani & Anaissie, 2004). In institutions monitoring severely immunocompromised patients, such as cancer patients and marrow transplants, *Fusarium* infections were documented at incidences of around 0.2-1.2% of patients (Dignani & Anaissie, 2004).

Other isolated filamentous fungi have been identified as the causative agent of an infection only recently. For the first time, in 2017, Chowdhary *et al.* reported *Penicillium oxalicum* as the cause of invasive infections in three cases involving immunocompromised patients (Chowdhary *et al*., 2017). In addition, for the first time, in 2020, Masetti *et al.* reported *Albifimbria verrucaria* as the causative agent of an invasive infection in human, a child with neuroblastoma (Masetti *et al*., 2020).

4.7 Isolates with notable agricultural relevance

The two most commonly isolated yeasts from the soils, *Cyberlindnera saturnus* and *Rhodotorula mucilaginosa*, have previously been studied for their association with crops, especially with respect to their ability to promote plant growth. The first report of an auxin-producing endophytic yeasts was for an isolate of *Cyberlindnera saturnus* (syn. *Williopsis saturnus*). This endophyte of maize roots was found to produce the plant growth promoting hormones indole-3-acetic acid and indole-3-pyruvic acid *in vitro* and increase root and shoot growth of maize following seedling inoculation (Nassar *et al*., 2005). Isolates of *R. mucilaginosa* have been found to be endophytes of cottonwood and produce indole-3-acetic acid (Xin *et al*., 2009).

Compared to the isolated yeasts, the isolated filamentous fungi strains have been documented more thoroughly for agricultural relevance, where they range from pathogenic to growth-promoting. For example, species in the *Fusarium solani* complex are a cause of sudden death syndrome of soybeans in North America (Aoki *et al*., 2003), while those in the *F. oxysporum* complex can cause root rot and seedling blight (Lanubile *et al*., 2015). On the other hand, it has been shown that inoculation of seeds and subsequent colonization of maize plants with *Metarhizium robertsii* leads to higher above-ground biomass, suppression of growth of black cutworm larvae on leaves, and alteration expression of plant defense genes (Ahmad *et al*., 2020). Strains of *Clonostachys rosea* have been reported to cause root rot in soybeans (Bienapfl *et al*., 2012) while strains have also been positively associated with suppression of *Sclerotinia sclerotiorum,* the fungal pathogen causing white mould, and increased shoot length upon inoculation (Rodríguez *et al*., 2015).

4.8 Future experiments and closing remarks

Given the number of filamentous fungi recovered with medical and agricultural significance, future experiments can test the recovered filamentous fungi to medical triazoles and agricultural triazoles. In addition, while initial colony counts were performed on agar containing 1 mg/L clotrimazole and 1 mg/L miconazole, little growth was observed on plates (Figure 10). Thus, future experiments can plate the treated and untreated soils on agars containing various lower

concentrations of the drugs to obtain suitable plate counts. These plate counts can be used to see if the treated soils contain more azole-resistant isolates than the untreated soils. In addition, replica plating from agar containing no azole drugs to agar containing miconazole can be used to further test the hypothesis that the control soils contain more isolates that are highly susceptible to miconazole. Finally, in order to see if any particular species shows differential abundance between the control and treated soils, targeted plate counts could be used. For example, *Debaryomyces hansenii* isolates can be selectively grown in conditions of 5% glucose and 10% NaCl (Breuer & Harms, 2006) and *Rhodotorula* species can be readily identified by pink-red colonies on SD agar. In addition, qPCR assays can also be used. Assays have already been published for quantifying *Cystofilobasidium infirmominiatum* (Spotts *et al*., 2009), a yeast isolated from the control and low treatment plots but not the high treatment plots, and *Vishniacozyma victoriae* (Rush *et al*., 2020), a yeast found to be highly susceptible to miconazole and the agricultural fungicide Vividera. In addition, assays for quantifying the medically and agriculturally important filamentous fungi *Mucor circinelloides* and *Clonostachys rosea* also exist (Bernal-Martinez *et al.;* Gimeno *et al*., 2019). Microbial community profiling using next-generation sequencing techniques can also further elucidate changes in community composition between the treated and untreated soils.

Overall, it was found that soils amended with environmentally-relevant concentrations of miconazole and clotrimazole showed reduced fungal load and diversity compared to control soils. While notable changes in azole susceptibility were not detected among the fungal isolates from the treated versus control soils, there was some indication that the treated soils contained fewer isolates highly susceptible to miconazole. Given the isolation of medically and agriculturally important fungi, future studies on the impact of azole contamination of soils is justified.

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6 Appendix A

Supplementary Table 1 – Partial ITS Sequences of Yeasts

Top match	Consensus Sequences	Query length, nt	% query cover	% ID
Bullera alba	CATTAGTGATTGACCTCCGGGTCTTAATAACTATAATCTTCTACCTCTGT GAACCGTTGGTCTTCGGACCGATTCTTATAACAAACATCTGTGTAATGAA CGTAACCTATATTAATCATATAAAACTTTTAACAACGGATCTCTTGGCTC TCGCATCGATGAAGAACGC	169	100	100
Cutaneotricho- sporon terricola	CATTAGTGAATTGCTCTCTGAGCGTTAACTATATCCATCTACACCTGTGA ACTGTTGATTGACTTCGGTCAGTTACTTTTACAAACATTGTGTAATGAAC GTCATGTTATTATAACAAAAATAACTTTCAACAACGGATCTCTTGGCTCT CGCATCGATGAAGAACGC	168	100	100
Cyberlindnera saturnus	CATTAAAGTATTCTTCGGTGCAGCCAGCGCTTCCACAGCGCGGCAGCCCA AACCTTACACACTGTGATTAGTTTTTTTACTATTTACTTTGGCTGCGCAA GTGGCCAAAGGTCTTAAACACAAAGATTTATATCTTTTTTTACAAAATTT AGTCAATGAAGTTTTAATACTATAATCTTCAAAACTTTCAACAACGGATC TCTTGGTTCTCGCATCGATGAAGAACGCAGC	231	100	100
Cystofilobasidium capitatum	CTGCGGAAGGATCACTAAAGAATTCGCCCTTCGGGGCTCTCTTTATTCAC ACACCCCTGTGCACTTTGGCCACCTCTTTGTTGAGGTGTGTCTTTTTAAT TACCATACCCTATAAACACAAGTTATTGAATGTAAAATCGTTATAAACTA ATATAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGC A	189	100	97.9
Cystofilobasidium infirmominiatum	CACTAGAGATTTCGCCCTCCGGGGCTCTCTTTCTTCACACACCCCTGTGC ACTTTGGCTGCCGCTTCATTGCGGTGGTCTTTTTAATAATTACCATACCC ATATACACAAGTCATTGAATGTAAAATCGTTATAAACTAATATAACTTTC AACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA	201	100	100
Debaryomyces nepalensis, D. hansenii, D. vietnamensis	CATTACAGTATTCTTTTTGCCAGCGCTTAATTGCGCGGCGAAAAAACCTT ACACACAGTGTTTTTTGTTATTACAAGAACTCTTGCTTTGGTCTGGACTA GAAATAGTTTGGGCCAGAGGTTTACTGAACTAAACTTCAATATTTATATT GAATTGTTATTTATTTTAATTGTCAATTTGTTGATTAAATTCAAAAAATC TTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAA	245	100	100

Debaryomyces nepalensis, D. hansenii, D. vietnamensis	CATTACAGTATTCTTTTTGCCAGCGCTTAATTGCGCGGCGAAAAAACCTT ACACACAGTGTTTTTTGTTATTACAAGAACTCTTGCTTTGGTCTGGACTA GAAATAGTTTGGGCCAGAGGTTTACTAAACTAAACTTCAATATTTATATT GAATTGTTATTTATTTTAATTGTCAATTTGTTGATTAAATTCAAAAAATC TTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAA	245	100	100
Diutina catenulata	CTGCGGAAGGATCATAAAAAACTAATTTACACGCGATTTAAAATTGCTTA CTTCAATAACCTATTAAACAATCAACCAACTAATACCAAAAACTTCCAAC AACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA	137	100	100
Filobasidium oeirense	CATTAATGAATATAAATCGTACTGTTCACGCAGTATGTGGGGTGGTGACT TCGGTCCCGCTCATTCATATCCATAACACCTGTGCACTGTTGGATGCTTG CATCCACTTTTAAACTAAACATTATTGTAACAAATGTAGTCTTATTATAA CATAATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGAT	195	100	100
Goffeauzyma gastrica	CATTAATGAATTCTGAGTAGGCTCTGCCTCTCACCCTTCATATCCATAAA CACCTGTGCACAGTCGGACCCTTGAGTCTTTCGGGACTCCCGGTGTCTTG ACGATCTTATCAAACAACAATGTAACCAATGTAATCATTATTATAACATA ATAAAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGC	200	100	100
Hannaella coprosmae	CATTATTAAATGCGAAGCCATCACTGGCCTAGCTTTATAATCCCTTATTA CACCTGTGCACCTTATTGTCTTTGGACAATTTTAACAAACATCAGTGTAA AGAATGTAAACTATTATAATAAATACAACTTTTAACAACGGATCTCTTGG CTCTCGCATCGATGAAGAACGC	172	100	100
Hannaella oryzae	CATTATTAAACGCGAAGCCATCACAGGCCTAGCTTTATAATCCTTATTAC ACCTGTGCACCTTATTGTCTTCGGACAATCTTTAAAAACAACAGTGTAAC GAATGTAAACTATTATAAAATAATACAACTTTTAACAACGGATCTCTTGG CTCTCGCATCGATGAAGAACGC	172	100	98.84
Hannaella oryzae	CTAGCTTTATAATCCTTATTACACCTGTGCACCTTATTGTCTTCGGACAA TCTTTACAACAACAGTGTAACGAATGTAAACTATTATAAAATAATACAA CTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCA	145	100	100
Leucosporidium scottii	CATTAGTGAATATTAGCGCATCTACTTGTAGAGCGTGACCTCCACTTTCT AACTCTGTGCATTTATTGGCGGAAGGACTTGAGCAATCGAGTTTCCTTCT GCGGCTCATTTTAAAACACTAGTTAAAGTATGTAACGAAATATCGAAACA AAAAAAACTTTCAACAACGGATCTCTTGGCTTGCGCATCGATGAAGAACG CAGCAA	202	100	99.5
Mrakia arctica, M. gelida	CACTAGTGATTAAATCGAGAGCGTCTTCATTGACCTCTCACCCTTCACAT CCACATACACCTGTGCACCGTTTGACTCTTTTAAAAGACGCAAGTCTGCA	212	100	99.06

	AAGAGAGTCATCAATTTTATACATACCCCAGTCTTATGAATGTAACAGTT TTAATAAACAAAATAAAACTTTTAACAACGGATCTCTTGGTTCTCGCATC GATGAAGAACGC			
Mrakia blollopis	CACTAGTGATTAAATCGAGAGCGTCTTCATTGACCTCTCACCCTTCACAT CCACATACACCTGTGCACCGTTTGGCTCTTATAAAAGACGCAAGTCTGCA ATGAGAGTCATCAATTTTATACATACCCCAGTCTTATGAATGTAACAGTT TTAATAAACATAATAAAACTTTTAACAACGGATCTCTTGGTTCTCGCATC GATGAAGAACGC	212	100	100
Mrakia arctica	CACTAGTGATTAAATCGAGAGCGTCTTCATTGACCTCTCACCCTTCACAT CCACATACACCTGTGCACCGTTTGGCTCTTTTAAAAGACGTAAGTCTGCA AAGAGAGTCATCAATTTTATACATACCCCAGTCTTATGAATGTAACAGTT TTAATAAACAAAATAAAACTTTTAACAACGGATCTCTTGGTTCTCGCATC GATGAAGAACGC	212	100	100
FK01_169	CATTAGTGAATCTAGCGTGTCTTGCCCTCGAGCAGAGCGCGACCTCTCAC TCTATACACTGTGCACTTAATAATCGTGGACGAAACTGAAGCCTCTTGGC CGACGTGACATCTACGTCTTATTTTATACATGAGTAAACGTATGTCATTA TATTTAAAAAAGAAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCG ATGAAGAACGC	211	NA	NA
Papiliotrema flavescens	CATTATTGATTGGTCGAAAGACCTTATCAGATTCTACCACCTCTGTGAAC CGTTGACCTCCGGGTTAATAATCAAACATCAGTGTAACGAACGTAAGAGT ATCTTAATTAAACAAAACTTTTAACAACGGATCTCTTGGCTCTCGCATCG ATGAAGAACGCA	162	100	100
Rhodotorula babjevae	CATTAGTGAATCTAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACT TTCTAACCCTGTGCATCTGTTAATTGGAATAGTAGCTCTTCGGAGTGAAC CACCATTCACTTATAAAACACAAAGTCTATGAATGTATACAAATTTATAA CAAAACAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGA ACGC	204	100	100
Rhodotorula graminis	CATTAGTGAATCTAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACT TTCTAACCCTGTGCATCTGTTAAATTGGACTAGTAGCTCTTCGGAGTGAA CCGCCATTCACTTATAAAACACAAAGTCTATGAATGTATACAAATTTATAA CAAAACAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGA ACG	203	100	100
Rhodotorula mucilaginosa	CATTAGTGAACATAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACT TTCTAACCCTGTGCACTTGTTTGGGATAGTAACTCTCGCAAGAGGGCGAA	204	100	100

	CTCCTATTCACTTATAAACACAAAGTCTATGAATGTATTAAATTTTATAA CAAAATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGA ACGC			
Sampaiozyma ingeniosa	CATTAGTGAATTTAGCGCATCTGCTTTGCAGAGCGTGACCTCCACTTTCT AACTCTGTGCACTTAATGGCGGAAGAGATGAAATATGCTCTTCTGCGGCT CATTTTATAACACTAGTTAAAGAATGTAACGAAATATCGAAACAAAAAAA AACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGC	196	100	100
Schwanniomyces occidentalis	CATTACAGTATTCTTTTTGCCAGCGCTTAATTGCGCGGCGAAAAAACCTT ACACACAGTGTTTTTTGTTATTACAAGAACTTTTGCTTTGGTCTGTCTCT AGAAATAGAGTTGGGCCAGAGGTTTAACTAAACTTCAATTTTATATTGAA TTGTTTTTTAATTAATTGTCAATTTGTTGATTAAATTCAAAAAATCTTCA AAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA	248	100	100
Solicoccozyma aeria	CATTAGTGAATTAAACATGCTTGGTGTCTTCGCTTCGGCAAGGCCCTTGC TTAACTCACATCCCAACACCTGTGAACTGTAAGGCGCATGACTAGGTTCG CCCAAGTCATCGTCTGCCCTTTTTAACAAACAATTAATGTAACAAACGTA GTCTTATTATAACCTAATAAAACTTTCAACAACGGATCTCTTGGCTCTCG CATCGATGAAGAACGC	220	100	100
Solicoccozyma terrea	CATTAGTGAATTAAACATGCTTGGTGTCTTCGCTTCGGCAAGGCCCTTGC TTAAATCACATCCTAACACCTGTGAACTGTAAGACGTATGATGAGGTCTT TGGCCAAGTCATCGTCTGCCCATTTTTAACAAACAATTAATGTAACAAAC GTAGTCTTATTATAACCTAATAAAACTTTCAACAACGGATCTCTTGGCTC TCGCATCGATGAAGAACGCA	220	100	100
Tausonia pullulans	CACTAGTGATTAAATCGAGCGTGTCTTCATTGACCGCTCACCCTTCTCAC CATCCACATACACCTGTGCACTGTTTAGCCTGAGCCGGTTTTCCGGTCCA GGTTATCATTTCATACAAACTCTAGTCTTATGAATGTAAACGTTTTAATA ACATAATAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAG AACGC	205	100	100
Tausonia pullulans	CACTAGTGATTAAATCGAGCGTGTCTTCATTGACCGCTCACCCTTCTCAC CATCCACATACACCTGTGCACTGTTTAGCCTGAGCCGTTTCGGTTCAGGT TATCATTTCATACAAACTCTAGTCTTATGAATGTAAACGTTTTAATAACA TAATAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAAC GCA	203	100	100
Vishniacozyma foliicola	GATCATTAATGATGCCCTCGAAGTCCTTGGACTGGTAGGGTTTGTGTCGG TCTCTTCGGAGTCGACCTTATCTCACACACCGTGAACTGTGGCTTCGGCC	189	100	100

	ATTTACACAAACTGTTAGTAATGAATGTAATATCATAACAAACATAAAAC TTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCA			
Vishniacozyma sp. isolate C220	CATTAATAATGCCCTCTGACTTCGGTCAGCTGGGTTCAAATGAGTGCCTT CTCTTCGGAGTTGGCCATCCTCACACACCGTGAACTGTGGCTTCGGCCAT CACAAACTGTTAGTAATGAATGTAATATCATAACAAAAACAAAACTTTTA ACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCA	194	100	99.48
Vishniacozyma victoriae	CATTAATAGTGCTCATTGACGCAAGTCAGTGAGTTAGATCTGCTCTCTTC GCAAGAAGAGGGTTTCCATACACACCGTGAACTGTGGCTTCGGCCATCAC AAACTGTTAGTAATGAATGTAATATCATAACAAAAACAAAACTTTTAACA ACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCA	186	100	97.31

**Supplementary Table 2 – Partial ITS Sequences of Filamentous Fungi**

Top match	Consensus sequence	Query length, nt	% query cover	% ID
Acremonium sp. MJ35	ATTACTGAGTGTAAAAACTCCCAACCCTTTGTGAACATACCTCTGTTGCTTCGGCGGATGCGCCCCGGGCGCGCCTTCTAGGAGGCGTGCCCCGGAACCAGGCGCCCGCCGGGGGACCTACAAACTCTTGTATTCCTAGCGGATCTCTGAGTGTGATTTAAACAAATCAAATCAAAACTTTCAACAACGGATCTCTTGGCTCTAGCATCGATGAAGAACGC	221	100	100
Acrophialophora levis	ATTACAGAGTTGCAAAACTCCCCAAACCATTGTGAACCTTACCTTCAACCGTTGCTTCGGCGGGCGGGCCACAGCGCCCCCCGGGCCCCCCCAGCGGGGCGCCCGCCGGAGGATACCCAAACTCTTGATACTTTATGGCCTCTCTGAGTCTTCTGTACTGAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA	217	100	100
Actinomucor elegan	CATTAAATAAAACTTGAGGGGAAACTGGGCTTACGGGCTTGGTTTTTCTCTTATTTTTTACCGTGAACTGTCTTATAGCATGGCGCTAGTAGAGATGCCTGAGCCGCCATACGGGGTAGGCGGCACAGGATGATTTTAATCGAAGCCATGGTCAAGCCGACTTTTTTTCAGCTTGGTACCCCAAAAATTAATTATTCTACCAAATGAATTCAGTATTAATATTGTAACATGGGCTCGCTGAAAGGTGGCCTATAAAACAACTTTTAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA	305	99	100
Actinomucor elegans	CATTAAATAAAACTTGAGGGGAAACTGGGCCTACGGGTTTGGTTTTTCCCTTATTTTTTACCGTGAACTGTCTTATAGCATGGCGCTAGTAGAGATGCCTGAGCCACCATACGGGGTAGGCGGCACAGGATGATTTTAATCGAAGCCATGGTCAAGCCGACTTTTTTTCAGCTTGGTACCCCAAAAATTAATTATTCTACCAAATGAATTCAGTATTAATATTGTAACATGGGCTCGCTGAAAGGTGGCCTATAAAACAACTTTTAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA	305	99	100
Albifimbria verrucaria, viridis	TACCGAGTTTACAAACTCCCAAACCCTTTGTGAACCTTACCATATTGTTGCTTCGGCGGGACCGCCCCGGCGCCTTCGGGCCCGGAACCAGGCGCCCGCCGGAGGCCCCAAACTCTTATGTCTTTAGTGGTTTTCTCCTCTGAGTGACACATAAACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA	214	100	100

Aspergillus fumigatus, fumigatiaffinis	CATTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTCTATCG TACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCC CTGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTC TGAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAAC AACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCA	237	100	100
Aspergillus [multiple]	TACCGAGTGAGGACCTAACCGGTCCAACCTCCCACCCGTGTCTATCGTAC CTTGTTGCTTCGGCGGGCCCGCCATTCGTGGCCGCCGGGGGGCATCTCGC CCCCGGGCCCGCGCCCGCCGGAGACACCAACACGAACACTGTCTGAAGGT TGCAGTCTGAGTCGATTTATTTAATCGTTAAAACTTTCAACAACGGATCT CTTGGTTCCGGCATCGATGAAGAACGCAGCA	231	99	100
Cladosporium [multiple]	CATTACAAGTGACCCCGGTCTAACCACCgggatgTTcATAACCCTTTGTT GTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCG GGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAAT TAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGA A	201	100	100
Clonostachys rosea (A)	CATTACCGAGTTTACAACTCCCAAACCCATGTGAACATACCTACTGTTGC TTCGGCGGGATTGCCCCGGGCGCCTCGTGTGCCCCGGATCAGGCGCCCGC CTAGGAAACTCAACTCTTGTTTTATTTTGGAATCTTCTGAGTAGTTTTTA CAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG AAGAACGCA	209	100	100
Clonostachys rosea (B)	CATTACCGAGTTTACAACTCCCAAACCCATGTGAACATACCTACTGTTGC TTCGGCGGGATTGCCCCGGGCGCCTCGTGTGCCCCGGATCAGGCGCCCGC CTAGGAAACTTAATTCTTGTTTTATTTTGGAATCTTCTGAGTAGTTTTTA CAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG AAGAACGCA	209	100	100
Fusarium oxysporum	CATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGC CTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGAC CCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAAT CAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA	200	100	100
Fusarium incarnatum-equiseti	CATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCTATACGTTG CCTCGGCGGATCAGCCCGCGCCCTGTAAAAGGGACGGCCCGCCCGAGGA CCCTAAACTCTGTTTTTAGTGGAACTTCTGANTAAAACAAACAAATAAAT CAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATC	187	100	100

Metarhizium robertsii	TACCGAGTTATCCAACTCCCAACCCCTGTGAATTATACCTTTAATTGTTG CTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTT AATAAGTATCTTCTGAGTGGTTAAAAAAATGAATCAAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGA	176	100	100
Metarhizium marquandii	TACCGAGTTTTCAACTCCCAAACCCACTGTGAACATATACCATTTGTTTA TTCGTTGCCTCGGCGGGGTTCTACTCCCTGGAGACAGGGGGCCAGCCCCCG CCGGTGTAACACCCAAAACCCTGAATGTGTACCCGTTACACGGCAGTATT ACTCTGAGTCACATCATTTTAAATGAATCAAAACTTTCAACAACGGATCT CTTGGTTCTGGCATCGATGAAGAACGCAGCA	231	100	100
Mortierella alpina (A)	CATTCATAATCAAGTGTTTTTATGGCACTTTCAAAAATCCATATCCCCTT GTGTGCAATGTCATCTCTCTGGGGGCTGCCGGCTGTCAAAAGCCGTGTGG TCACCTTTGGGATTTATATCTACTCAGAACTTTAGTGATTTTGTCTGAAA CATATTATGAATACTTAATTCAAAATACAACTTTCAACAACGGATCTCTT GGCTCTCGCATCGATGAAGAACGCA	225	100	100
Mortierella alpina (B)	CATTCATAATCAAGTGTTTTTATGGCACTTTCAAAAATCCATATCCACCT TGTGTGCAATGTCATCTCACTGGGGGCCACCGGCTGTCAAAAGCCGTCTG GTCACCTTTGGGATTTATATCTACTCAGAACTTTAGTGATTTTGTCTGAA ACATATTATGAATACTTAATTCAAAATACAACTTTCAACAACGGATCTCT TGGCTCTCGCATCGATGAAGAACGCA	226	100	100
Mortierella alpina (C)	CATTCATAATCAAGTGTTTTTATGGCACTTTCAAAAATCCATATCCACCT TGTGTGCAATGTCATCTCTCTGGGGGCTGCCGGCTGTCAAAAGCCGTGTG GTCACCTTTGGGATTTATATCTACTCAGAACTTTAGTGATTTTGTCTGAA ACATATTATGAATACTTAATTCAAAATACAACTTTCAACAACGGATCTCT TGGCTCTCGCATCGATGA	218	100	100
Mortierella gamsii	CATTCATAATAAGTGTTTTATGGCACTTTTTAAATCCATATCCACCTTGT GTGCAATGTCAGTTGTTCTCTTTTTTGAGAATGACCAAACATCAACTTAT TCTTTAACTCTTTGTCTGAAAAATATTATGAATAAAATAATTCAAAATAC AACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCA	197	100	100
Mortierella hyalina	CATTCATAATAAGTGTTTTATGGCACTTTTTTAAATCCATATCCACCTTG TGTGCAATGTCAGGGTTGGTTTCTCTCTTTTGAGAGATCAACCCCAAACA TCAACTCTATCTTAACTCTTTGTCTGAAAAATATTATGAATAAACAATT CAAAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGA ACGCA	205	100	100

Mucor circinelloides	CATTAAATAATCAATAATTTTGGCTTGTCCATTATTATCTATTTACTGTG AACTGTATTATTACTTGACGCTTGAGGGATGCTCCACTGCTATAAGGATA GGCGATGGAGATGCTAACCGAGTCATAATCAAGCTTAGGCTTGGTATCCT ATTATTATTTACCAAAAGAATTCAGAATTAATATTGTAACATAGACCTAA AAAATCTATAAACAACTTTTAACAACGGATCTCTTGGTTCTCGCATCGA TGAA	254	100	100
Neosartorya udagawae	CATTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTCTATCG TACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCC TCGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACTCTGTTC TGGAAGTATGCAGTCTGAGTTGATTATCATAATCAGTTAAAACTTTCAAC AACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCA	237	100	100
Penicillium oxalicum (A)	TTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATCGTA CCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGGGGGGCATCTGCCC CCGGGCCCGCGCCCGCCGAAGACACACAAACGAACTCTTGTCTGAAGATT GCAGTCTGAGTACTTGACTAAATCAGTTAAAACTTTCAACAACGGATCTC TTGGTTCCGGCATCGATGANGAACGCAGC	229	99	100
Penicillium oxalicum (B)	CATTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATCG TACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGGGGGGCATCCGC CCCCGGGCCCGCGCCCGCCGAAGACACACAAACGAACTCTTGTCTGAAGA TTGCAGTCTGAGTACTTGACTAAATCAGTTAAAACTTTCAACAACGGATC TCTTGGTTCCGGCATCGATGAAGAACGCA	229	100	100
Penicillium novae-zeelandiae	CATTACCGAGCGAGGATTCTCTGAATCCAACCTCCCACCCGTGTTTATTG TACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGGGGGGCATCTGC CCCCGGGCCCGCGCCCGCCGAAGACACCTTGAACTCTGTATGAAAATTGC AGTCTGAGTCTAAATATAAATTATTTAAAACTTTCAACAACGGATCTCTT GGTTCCGGCATCGATGAAGAACGCA	225	100	100
Plectosphaerella cucumerina	CATTACTGAGTACTACACTCTCTACCCTTTGTGAACTATTATACCTGTTG CTTCGGCGGCGCCCGCGAGGGTGCCCGCCGGTCTCATCAGAATCTCTGTT TTCGAACCCGACGATACTTCTGAGTGTTCTTAGCGAACTGTCAAAACTTT TAACAACGGATCTCTTGGCTCCAGCATCGATGAAGAACGCA	191	100	100
Purpureocillium lilacinum	CATTACCGAGTTATACAACTCCCAAACCCACTGTGAACCTTACCTCAGTT GCCTCGGCGGGAACGCCCCGGCCGCCTGCCCCCGCGCCGGCGCCGGACCC AGGCGCCCGCCGCAGGGACCCCAAACTCTCTTGCATTACGCCCAGCGGGC	241	100	100

	GGAATTTCTTCTCTGAGTTGCACAAGCAAAAACAAATGAATCAAAACTTT CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA			
Rhizomucor variabilis	CATTAAATAATCAATAATTTTGGCTTGTCCATTATTATCTATTTACTGTG AACTGTATTATTACTTGACGCTTGAGGGATGCTCCACTGCTATAAGGATA GGCGGTGGGGATGTTAACCGAGTCATAGTCAAGCTTAGGCTTGGTATCCT ATTATTATTTACCAAAAGAATTCAGAATTAATATTGTAACATAGACCTAA AAAATCTATAAAACAACTTTTAACAACGGATCTCTTGGTTCTCGCATCGA TGAAGAACGCA	261	100	100
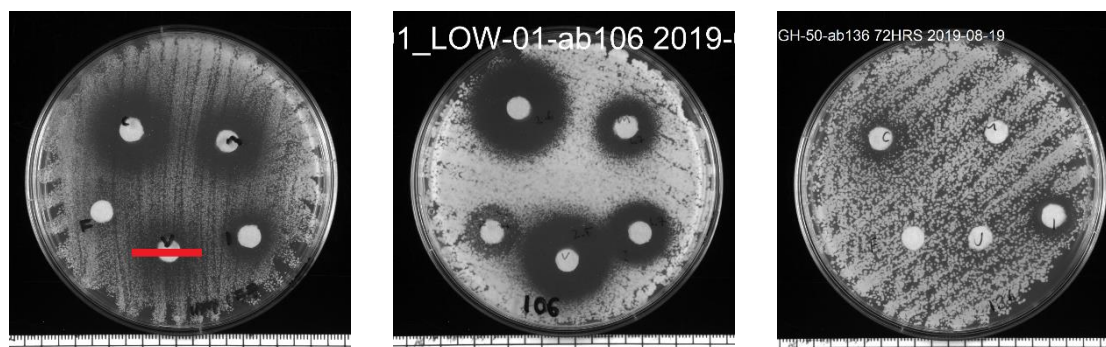
Supplementary Figure 1 – Examples of Disk Diffusion Assay Interpretations
The shown *Cyberlindnera saturnus* isolate (left) was determined to be sensitive to clotrimazole and miconazole (top rows), as well as itraconazole (bottom right), but resistant to fluconazole (bottom left), and weakly susceptible to voriconazole (bottom centre). The zone of inhibition measurement for voriconazole, which showed trailing growth, was determined by measuring from the edges of the apparent start of the trailing growth and is illustrated here by the red bar. Likewise, the shown *Debaryomyces hansenii* isolate (middle) was determined to be resistant to fluconazole (bottom left) due to the normal-sized colonies within a zone of 70 mm from the disk. Also, the ZOI against clotrimazole and itraconazole for the shown *Sampaiozyma ingeniosa* isolate (right) was determined to be between 70 and 150 mm due to small zones of inhibition reaching just below 150 mm to each disk.

Supplementary Table 3 – Contingency Tables used for Fisher Exact Tests, Analysis of Drug Cross-resistance

3.1 - Number of sensitive and resistant isolates to clotrimazole, voriconazole, or both drugs*

	VRC^S	VRC^R
CTZ^S	81	46
CTZ^R	1	7

3.2 - Number of sensitive and resistant isolates to miconazole, voriconazole, or both drugs*

	VRC^S	VRC^R
MCZ^S	81	30
MCZ^R	1	22

3.3 - Number of sensitive and resistant isolates to fluconazole, voriconazole, or both drugs*

	VRC^S	VRC^R
FCZ^S	46	0
FCZ^R	36	52

3.4 - Number of sensitive and resistant isolates to voriconazole, itraconazole, or both drugs*

	ITR^S	ITR^R
VRC^S	52	1
VRC^R	39	14

3.5 - Number of sensitive and resistant isolates to propiconazole, itraconazole, or both drugs*

	PRO^S	PRO^R
VRC^S	73	0
VRC^R	26	7

3.6 - Number of sensitive and resistant isolates to itraconazole, fluconazole, or both drugs

	ITR^S	ITR^R
FCZ^S	46	0
FCZ^R	74	14

3.7 - Number of sensitive and resistant isolates to miconazole, fluconazole, or both drugs

	FCZ^S	FCZ^R
MCZ^S	41	72
MCZ^R	0	47

3.8 Number of sensitive and resistant isolates to miconazole, itraconazole, or both drugs

	ITR^S	ITR^R
MCZ^S	110	5
MCZ^R	13	10

3.9 - Number of sensitive and resistant isolates to clotrimazole, miconazole, or both drugs

	MCZ^S	MCZ^R
CTZ^S	121	20
CTZ^R	5	3

3.10 - Number of sensitive and resistant isolates to clotrimazole, fluconazole, or both drugs*

	FCZ^S	FCZ^R
CTZ^S	46	80
CTZ^R	0	8

3.11 - Number of sensitive and resistant isolates to clotrimazole, itraconazole, or both drugs*

	ITR^S	ITR^R
CTZ^S	158	12
CTZ^R	6	2

3.12 - Number of sensitive and resistant isolates to propiconazole, clotrimazole, or both drugs*

	PRO^S	PRO^R
CTZ^S	97	3
CTZ^R	1	4

3.13 - Number of sensitive and resistant isolates to propiconazole, miconazole, or both drugs*

	PRO^S	PRO^R
MCZ^S	88	1
MCZ^R	8	6

3.14 - Number of sensitive and resistant isolates to propiconazole, voriconazole, or both drugs

	PRO^S	PRO^R
ITR^S	79	6
ITR^R	7	1

3.15 - Number of sensitive and resistant isolates to propiconazole, fluconazole, or both drugs

	PRO^S	PRO^R
FCZ^S	9	0
FCZ^R	77	7

Supplementary Table 4 – Contingency Tables used for Fisher Exact Tests, Analysis of Isolates Highly Susceptible to Miconazole

4.1 - Number of highly susceptible isolates to miconazole isolated from the control versus the low treatment plots

	ZOI < 250 mm	ZOI >= 250 mm
From control plots	39	8
From low plots	51	2

4.2 - Number of highly susceptible isolates to miconazole isolated from the control versus the high treatment plots

	ZOI < 250 mm	ZOI >= 250 mm
From control plots	39	8
From high plots	42	2

7 Curriculum Vitae

Farhaan Kanji

Education

University of Western Ontario, London, ON
Master of Science, Biology, 2018–2021
Demonstrated skills in written communication, technical writing aptitude, and data analysis to complete lab-based thesis studying antifungal resistance in agricultural soils

McMaster University, Hamilton, ON
Bachelor of Science, Molecular Biology and Genetics, 2012–2017 (GPA: 3.4)
Completed life sciences, mathematics, and statistics courses, and final-year thesis on next-generation sequencing marker selection for microbiome profiling.

Experience

Agriculture and Agri-Food Canada, London, ON
Summer research affiliate program, 05/2019 – 08/2020
Used problem solving, laboratory, and organizational skills to gather data for MSc thesis. Routinely performed media preparation, cell culturing, PCR, and disk diffusion assay tests.

Department of Biology, University of Western Ontario
Fall/Winter graduate teaching assistant, 09/2018 – 04/2020
Demonstrated oral communication and interpersonal skills while holding office hours for a second-year course in genetics and instructing first-year students in basic laboratory techniques

National Research Council Canada, Saskatoon, SK
Co-op student, 01/2016 – 08/2016
Independently performed RT-PCR and ddPCR assays, and prepared PCR amplicon libraries for next-generation sequencing. Prioritized tasks and finished assigned assays ahead of schedule.

Department of Biology, McMaster University
Research assistant, 05/2015 – 08/2015
Successfully worked in a team by assisting graduate students with microbiological techniques.

School of Geo. & Earth Sci., McMaster University
Work-study, 05/2013 – 08/2013
Demonstrated advanced use of Microsoft Office to track and share data and ability to quickly learn new computer software.

Volunteer Work

STEM Fellowship Science Fair, Hamilton ON, 01/2017 – 09/2017
Led first- and second-year undergraduates in science fair as project leader and won third place

iGEM Foundation, McMaster University, 01/2013 – 10/2016
Mentored on synthetic biology techniques in early years and provided mentorship in later years