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Comparing development of drug resistance by *Cryptococcus neoformans* to chemically distinct azole anti-fungal compounds

Lindsey Burke

Comparing development of drug resistance by *Cryptococcus neoformans* to chemically distinct azole anti-fungal compounds

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By Lindsey Burke

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Abstract

Cryptococcus neoformans is a fungus that primarily infects humans who have weakened immune systems. An azole drug, Fluconazole, is commonly administered against *C. neoformans* in regions were cryptococcosis is most prevalent, most notably Sub-Saharan Africa. However, *C. neoformans* can gain resistance to Fluconazole through becoming an aneuploid. To better understand the basis of resistance, we employed a disk diffusion assay and investigated several chemically-distinct azole compounds with anticryptococcal properties for their effectiveness against *C. neoformans* and to identify potential differences in the capacity of the fungus to become resistant to each of the tested compounds. Different *C. neoformans* strains were tested, including both mating types. We found that Isavuconazole, Voriconazole, Difenoconazole, and Efinaconazole were superior to Fluconazole in preventing the occurrence of resistance, whereas Ketoconazole, and Myclobutanil demonstrated a relatively higher incidence of resistance. Our study has also demonstrated that the antifungal drugs differ significantly in their stability when added to the semi-solid rich growth media, which may partly explain differences in the occurrence of antifungal resistance.

Acknowledgements

I would like to first thank Dr. Lukasz Kozubowski for being a wonderful mentor throughout my time in his research lab. He gave me the guidance I needed and provided support with all my experiments. I am grateful for all the opportunities I have been given through the Kozubowski lab. In addition, I would like to thank Stephani Martinez Barrera and Rodney Colon-Reyes, two graduate students who also supported my learning and aided in my experiments. I would also like to thank David Kitch and Disha Qanungo, two fellow undergraduate students who helped me complete my experiments.

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Background

Cryptococcus neoformans

Cryptococcus neoformans is a basidiomycete fungus that can cause meningoencephalitis in immuno-compromised patients (Indnurm, 2005). This fungus can be found in the environment, namely avian excreta, soil, and trees. The number of cases per year of cryptococcal meningitis is almost one quarter of a million, with 181,000 deaths annually (Iyer, 2021). The primary population that it infects is those with HIV/AIDS, where, if treatment is limited, it can have a 30% mortality rate (Indnurm, 2005). The general pathogenic route is entering the lungs and then spreading to the central nervous system. *C. neoformans* is special in that it's able to cause a lethal disease in humans and grow at human body temperature. As fungi are eukaryotes, their cell biology is similar to that of humans. Therefore, treating *C. neoformans* is difficult as there are few drugs available, and many of those drugs exhibit considerable toxic side effects.

Current Treatments

There are three main classes of antifungal agents that are currently used for treatment in clinics: polyenes, azoles, and pyrimidine analogues (Iyer, 2021). Each of these drug classes are used to varying degrees depending on the severity of the infection and what medical resources are available. Azoles are a group of interest as they are both cheaper and less toxic than the other two main classes. Therefore, azoles are often used in resource-limited countries where people cannot afford expensive treatments. These treatments work, but the drug classes are not often used as a monotherapy unless absolutely necessary due to the variety of resistance mechanisms *C. neoformans* has developed (Hope et al., 2019).

Each drug class has its own mode of action. The mechanisms of action of polyenes and azoles relate to ergosterol, an essential component of the plasma membrane (Iyer, 2021). Polyenes directly bind to ergosterol, which results in making the plasma membrane "leaky" and leads to burst of cellular reactive oxygen species (ROS) whereas azoles inhibit biosynthesis of ergosterol by inhibiting an enzyme in the ergosterol biosynthesis pathway, Erg11. The main drug in the polyenes category is Amphotericin B and the main drug in the azole category is Fluconazole. Pyrimidine analogues block DNA synthesis through causing RNA miscoding by working as an antimetabolite (Vermes, 2000). The main drug in the pyrimidine analogues category is Flucytosine.

Amphotericin B is the usual go-to drug for treatment of cryptococcosis . While being very effective in killing *C. neoformans*, it has high toxicity and there are problems with getting it to developing countries that don't have sufficient resources (Iyer, 2021). Currently, researchers are working on a less toxic alternative, such as including a liposome bilayer-coated Amphotericin B and putting Amphotericin B in lipid-containing crystal nanoparticles (Lu et al., 2019; Stone et al., 2016). An additional approach that can be used is a combination therapy. Amphotericin B treatment followed by Flucytosine and then Fluconazole has been shown to have decreased toxicity and be more effective (WHO, 2018). A final treatment option that is used is Fluconazole by itself. This treatment method is only used when Amphotericin B and Flucytosine are not available, which is the case in many developing countries due to cost, registration issues, and lack of safe intravenous administration.

Resistance Mechanisms

Due to the limited number of antifungals used for cryptococcal infections, *C. neoformans* has developed ways to become drug resistant. In addition, through mechanisms such as increased Erg11 production and overexpression drug efflux pumps, *C.* neoformans can gain cross-resistance to more than one antifungal drug (Bastos et al., 2018). *C. neoformans* has proven to be a very flexible fungus when it comes to environmental stressors and those changes are inheritable.

The main category of resistance mechanisms is genomic plasticity that allows *C. neoformans* to survive Fluconazole treatment. *C. neoformans* is able to develop azole resistance through a process called heteroresistance, where subpopulations of cells gain resistance to the drug (Iyer, 2021). This occurs when the cells become aneuploid. The most common form of aneuploidy that gets selected in the presence of Fluconazole is a disomy of chromosome 1. Chromosome 1 contains *ERG11* and the efflux pump genes, therefore the resistant cells have increased levels of the Fluconazole target (Erg11) and have a higher rate of Fluconazole expulsion. The actual mechanism of aneuploidy formation is unclear, but aneuploids are observed in populations who have been exposed to sub-optimal amounts of drug and gained resistance. Another way resistance can be gained is through a hypermutator phenotype, where the mutation rate is about 200 times higher than an average cell; the result can be a rapid gain of resistance (Gambhir et al., 2022; Iyer, 2021). A final way is through movement of transposable elements, which can inactivate genes or lead to a higher gene expression (Gusa et al., 2020).

Morphological changes can also result in resistance. One of the main virulence factors of *C. neoformans* is its polysaccharide capsule. By changing the thickness of the cell wall and/or capsule, *C. neoformans* can gain resistance to antifungals (Iyer, 2021). Titan cells have all also

been implicated in increased resistance, mostly due to their thicker cell wall. In addition, Titan cells produce daughter cells that are aneuploid, which could also contribute to resistance (Zafar et al., 2019).

Azole Compounds

Azole compounds are important in the realm of antifungal drug treatments. The azole compounds currently in clinical use have an azole ring with two or three nitrogen atoms. Compounds with two nitrogen atoms are classified as imidazoles (Sheehan, 1999). Compounds with three nitrogen atoms are classified as triazoles. In general, imidazoles are used for superficial infections whereas the triazoles are used against both superficial and systemic infections. One of the reasons azole compounds are such a popular antifungal agent is the high affinity to the fungal cytochrome P-450 enzymes over the human variants (Warrilow et al., 2013). Both human and fungal variants of cytochrome P-450 play important physiological roles. Therefore, low affinity towards the human variants makes azoles relatively safe drugs, compared to other classes of antifungals.

Resistance and Tolerance

Resistance and tolerance are often used in describing the effect of drugs on fungal cells. A qualitative definition of resistance refers to a case when a strain that has a higher minimum inhibitory concentration (MIC) for the drug than the references strain (Berman, 2020). Since MIC is not used in these experiments, a more qualitative approach was used, measuring by how well cells grow within the area affected by the drug.

Tolerance is the ability of a cell to grow at inhibitory concentrations of a drug (Yang, 2022). The term “grow” simply refers to it as continuing dividing. The speed at which it does this is not important, as tolerant cells often grow slowly. Unlike resistance, tolerance can have a range within a given cell population. Some cells will not be as tolerant as others, which can be seen by how well they survive and how quickly they grow at inhibitory concentrations. Importantly, unlike resistant cells, tolerant cells when re-exposed to the same concentration of the drug exhibit heterogeneity of growth, similar to the initial population.

Aims

Fluconazole is a drug used for treatment of cryptococcal meningitis in developing countries, such as South Africa. We examined azole compounds, a group to which Fluconazole belongs, to test how chemical structures affect development of tolerance and resistance in *C. neoformans.* The stability of the drugs was taken into consideration using a disk diffusion over time assay. Re-exposure to test for resistance and tolerance was an additional aspect that we examined. We found the azole compounds to have varying effects on *C. neoformans*, indicating that chemical structure plays a role in efficacy. Thus, alternative other azole compounds could potentially be used for treatment, in place of currently administered Fluconazole.

Materials & Methods

Strains, Media, and Growth Conditions

The strains used are listed in Table 1. The media used for most experiments was yeast extract peptone dextrose (YPD). The exception is the experiments in which *bub1*Δ was studied, for which a YPD medium supplemented with the NAT antibiotic selection was used. For all

experiments, an overnight culture was prepared by combining 2 mL of YPD media and a scraping, using a pipette tip, of the designated strain. The pipette tip was swirled in the media to get the cells in the solution. The culture was incubated at 30˚C in a rotor overnight. In the morning, the culture was removed from the rotor and the cells were refreshed using 2 mL of new media in a separate tube and adding 200 µL from the overnight culture. The culture was put back in the rotor at 30 C for ~4 hours before it was used.

Strain	Strain Label	Mating Type
H99	LK54	α
KN99	LK55	a
KN99	LK56	α
H99	LK354	α
*bub1*Δ	LK359	a

Table 1. Strains used for experimentation.

Hemocytometer

The hemocytometer was first cleaned with a Kim wipe and ethanol. 10 µL of the sample was put on the plate. The number of cells in a square were counted using 4 random squares. The average between the four would be found and then that average would be multiplied by 25 x 10,000. This gave the cell density in cells/mL. The goal was to obtain a cell density somewhere between 4.5 – 6.5 x 10^6 cells/mL.

Drug Stocks

All drug stocks, except for Fluconazole, were made doing the following. An Eppendorf tube was labeled and ~0.01 g of the powder version of the drug was measured. DMSO in the amount of 1000 µL (or stoichiometric equivalent, depending exactly how much drug was weighted) was added to the Eppendorf tube and vortexed. The resulting drug stocks were each 10

mg/mL. Preparation of the Fluconazole stock followed the same procedure except the powder measurement was ~0.05 g and add 1000 µL (or stoichiometric equivalent) of DMSO was added, followed by vortex. The Fluconazole stock was a 50 mg/mL solution. Each drug was aliquoted into 10 Eppendorf tubes, each with 100 µL.

Disk Diffusion Assay

The overnight culture samples were refreshed the same day and allowed to go back on the rotor for at least 4 hours. Plates were labelled and plated with 100 µL of 4.5 – 6.5 x 10^6 cells/mL. Glass beads were added to the plate and every plate was shaken for 30 seconds. They were allowed to dry for 5 minutes. The drug stocks were thawed to make the working concentrations. The working concentrations for each drug are listed in Table 2. All disk diffusion assays used DMSO as a control. DMSO was the solvent each drug was dissolved in.

Azole Compound	Stock Concentration (mg/mL)	DMSO Added (µL)	Stock Added (µL)	Final Working Concentration (mg/mL)
Isavuconazole	1	22	8	0.2670
Voriconazole	1	28	2	0.0667
Cyproconazole	1	24	6	0.2000
Tebuconazole	1	24	6	0.2000
Myclobutanil	1	22	8	0.2670
Propiconazole	1	24	6	0.2000
Difenoconazole	1	99	1	0.0100
Efinaconazole	1	99	1	0.0100
Ketoconazole	1	25	5	0.1670
Fluconazole	50	18	12	20

Table 2. Working concentrations for equalized zones of inhibition. Included are the original stock concentration, the proper dilutions, and the concentration of the final sample.

Please note that the concentrations in Table 3 were used for LK354, LK55, and LK359 (Figures 6, 7, and 8, respectively). At the time, equalized zones had not yet been achieved. These were the concentrations that yielded the closest result to equalized zones.

Azole Compound	Stock Concentration (mg/mL)	DMSO Added (µL)	Stock Added (µL)	Final Working Concentration (mg/mL)
Isavuconazole	1	26	4	0.1330
Voriconazole	1	26	4	0.1330
Cyproconazole	1	25	5	0.1667
Tebuconazole	1	26	4	0.1330
Myclobutanil	1	20	10	0.3333
Propiconazole	1	26	4	0.1330
Difenoconazole	1	99	1	0.0100
Efinaconazole	1	99	1	0.0100
Ketoconazole	1	25	5	0.1670
Fluconazole	50	18	12	20

Table 3. Working concentrations for LK354, LK55, and LK359. Included are the original stock concentration, the proper dilutions, and the concentration of the final sample.

Under the fume hood, a pair of tweezers were used to put a paper disk, standing upright, in the center of the area designated for that drug. The working concentration of the drug was vortexed and 10 µL was pipetted into the top of the paper disk. The disk stood for a few seconds before being knocked over and patting it down to the plate using the pipette tip. The same procedure was performed for every drug tested. Each plate was allowed to dry for 10 minutes and then flipped over. The plates were stored in room temperature conditions, with light having no effect.

Re-exposure Test

The disk diffusion assay plates were allowed to grow until there was a clear zone of inhibition, which usually occurred by day 2 or 3. A sterile wooden toothpick was used to pick up

cells all throughout the middle of the zone of inhibition. Middle refers to the space between the edge of the paper disk and the edge of the zone of inhibition. Four swipes were taken and put into 200 µL of YPD. 100 µL of the sample was plated on a new plate using glass beads and 30 seconds of shaking. They were dried for 5 minutes before being flipped over. They were allowed to grow until visible colonies formed.

A toothpick was used to create an overnight culture for each drug. The next morning, the cultures were refreshed and allowed to sit for at least 4 hours. Each was measured to have a cell density of 4.5 – 6.5 x 10^6 cells/mL. The samples were then tested in the disk diffusion assay again.

Drug Stability Test

Under the fume hood, a pair of tweezers was used to put a paper disk, standing upright, in the center of the area designated for that drug. The working concentration of the drug was vortexed and 10 µL was pipetted into the top of the paper disk. The disk stood for a few seconds before knocking it over and patting it down to the plate using the pipette tip.

Still under the fume hood, a dot of 2 µL of 1000 cells/mL was added to the plate. There is one closer and one farther from the paper disk. This same procedure was used for adding dots at day 7 and day 10. The image below shows a diagram of the set-up.

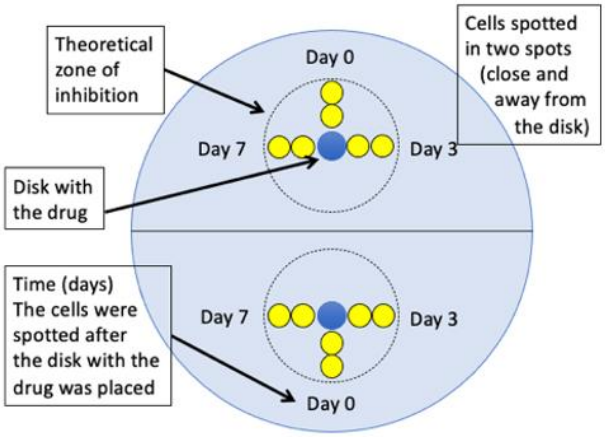
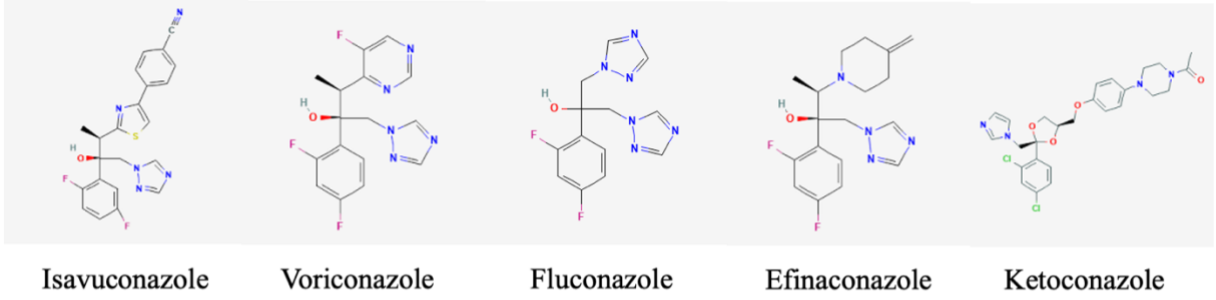


Figure 1. Drug Stability Test Lay-Out.

Results

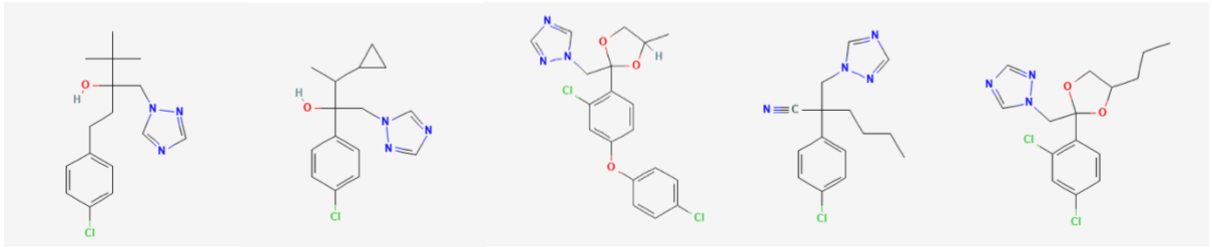
Azole Compound Structures

Medicinal Azole Compounds



Isavuconazole Voriconazole Fluconazole Efinaconazole Ketoconazole

Agricultural Azole Compounds



Tebuconazole Cyproconazole Difenoconazole Myclobutanil Propiconazole

Figure 2. Azole Compound Chemical Structures. Azole compounds are divided based on their use, medicinal or agricultural. Chemical structure images provided by the National Center for Biotechnology Information.

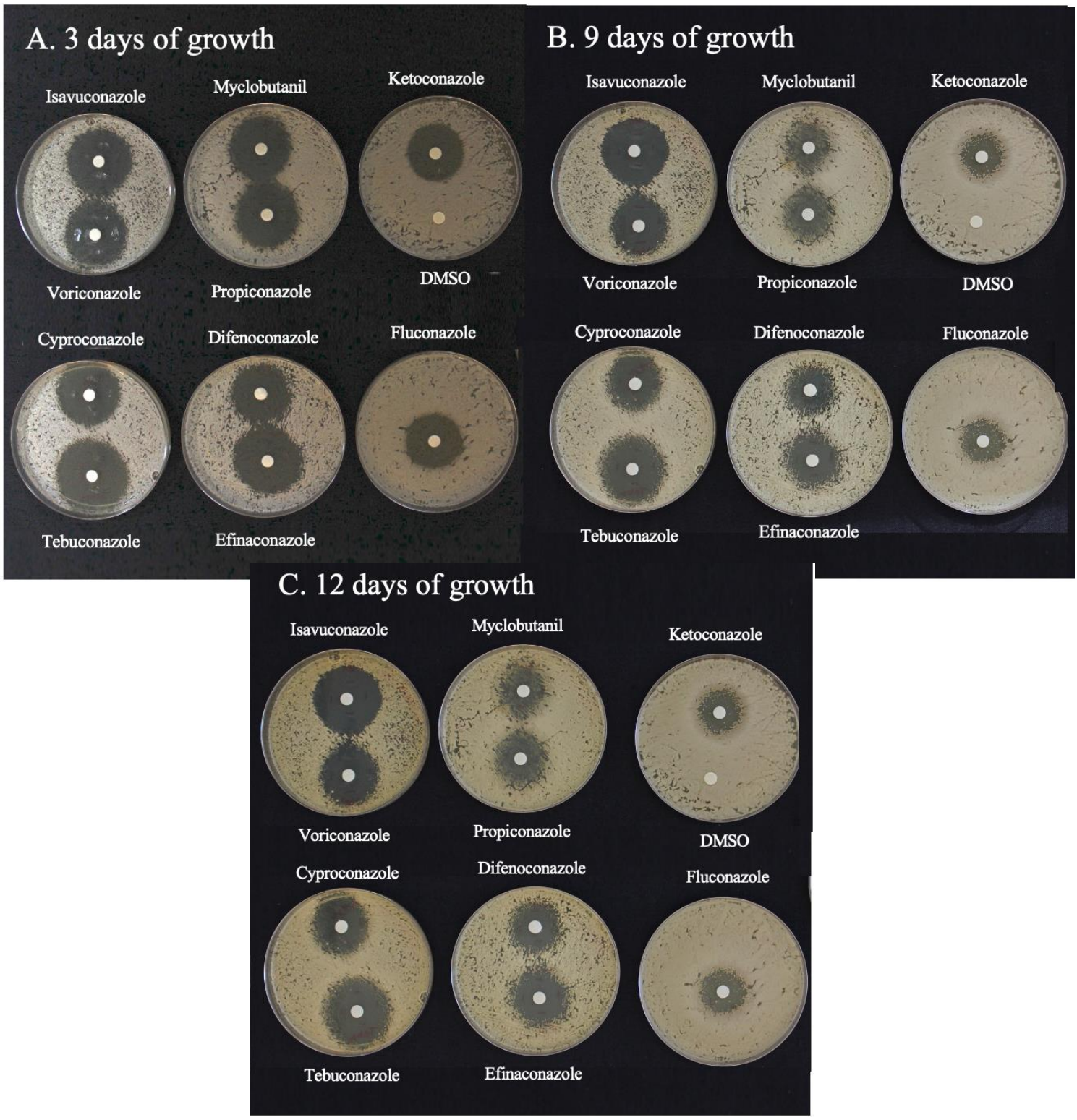
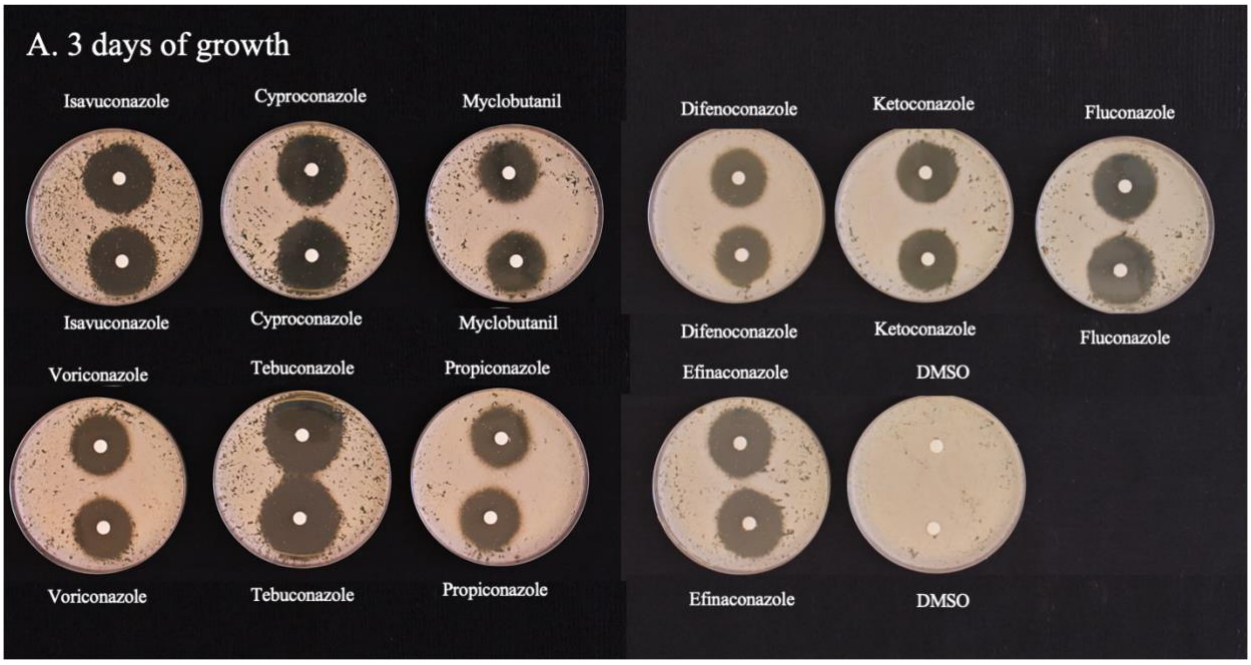


Figure 3. H99α (LK54) Disk Diffusion Assays. Wild-type H99α (LK54) was plated on YPD and exposed to an azole compound. All plates had ingrowth of the zone of inhibition and/or cells present within the zone of inhibition. Isavuconazole is notably the only exception to this.



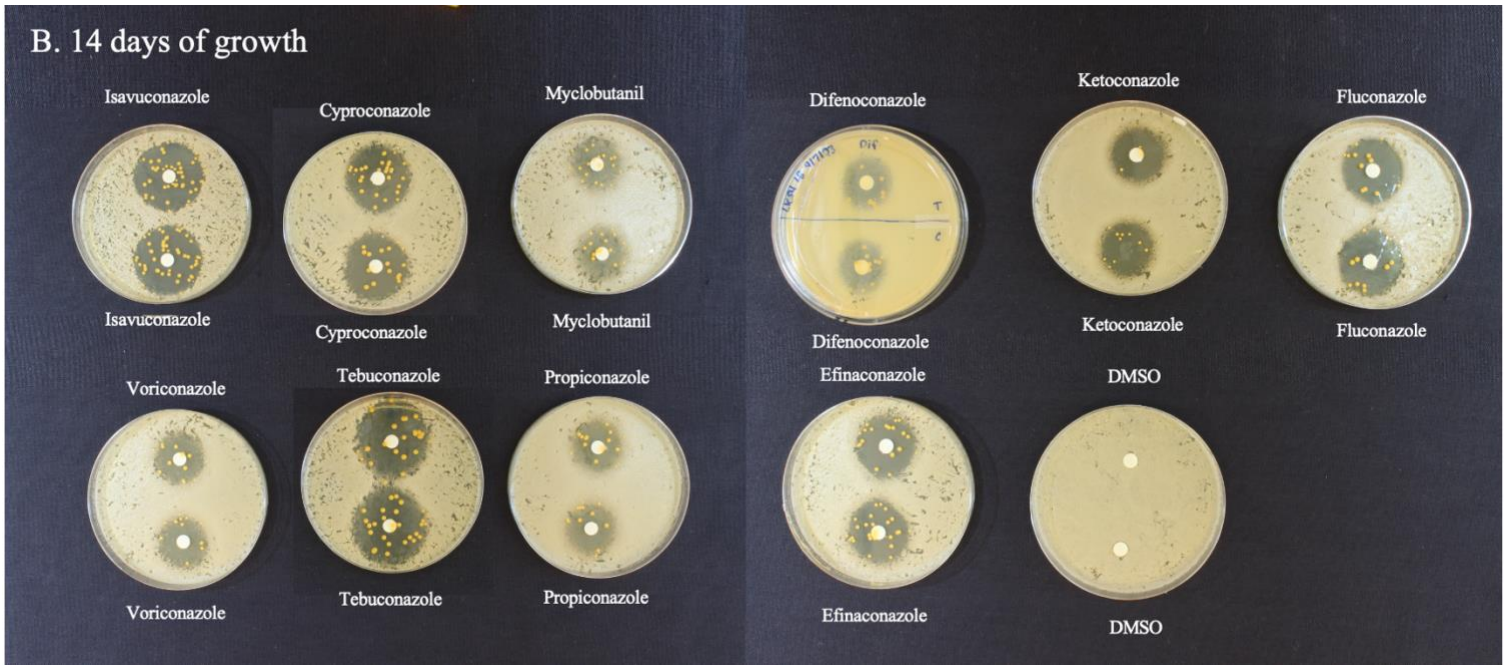


Figure 4. H99α (LK54) Disk Diffusion Assays. Wild-type H99α (LK54) was plated on YPD and exposed to azole compounds. Each plate has one drug tested twice. Note that there is contamination on every plate. Also note that the Ketoconazole plate in B is missing a disk; this disk was moved upon transportation of the plate to take the picture. There is no effect from the missing disk. H99α reacts consistently to the azole compounds.

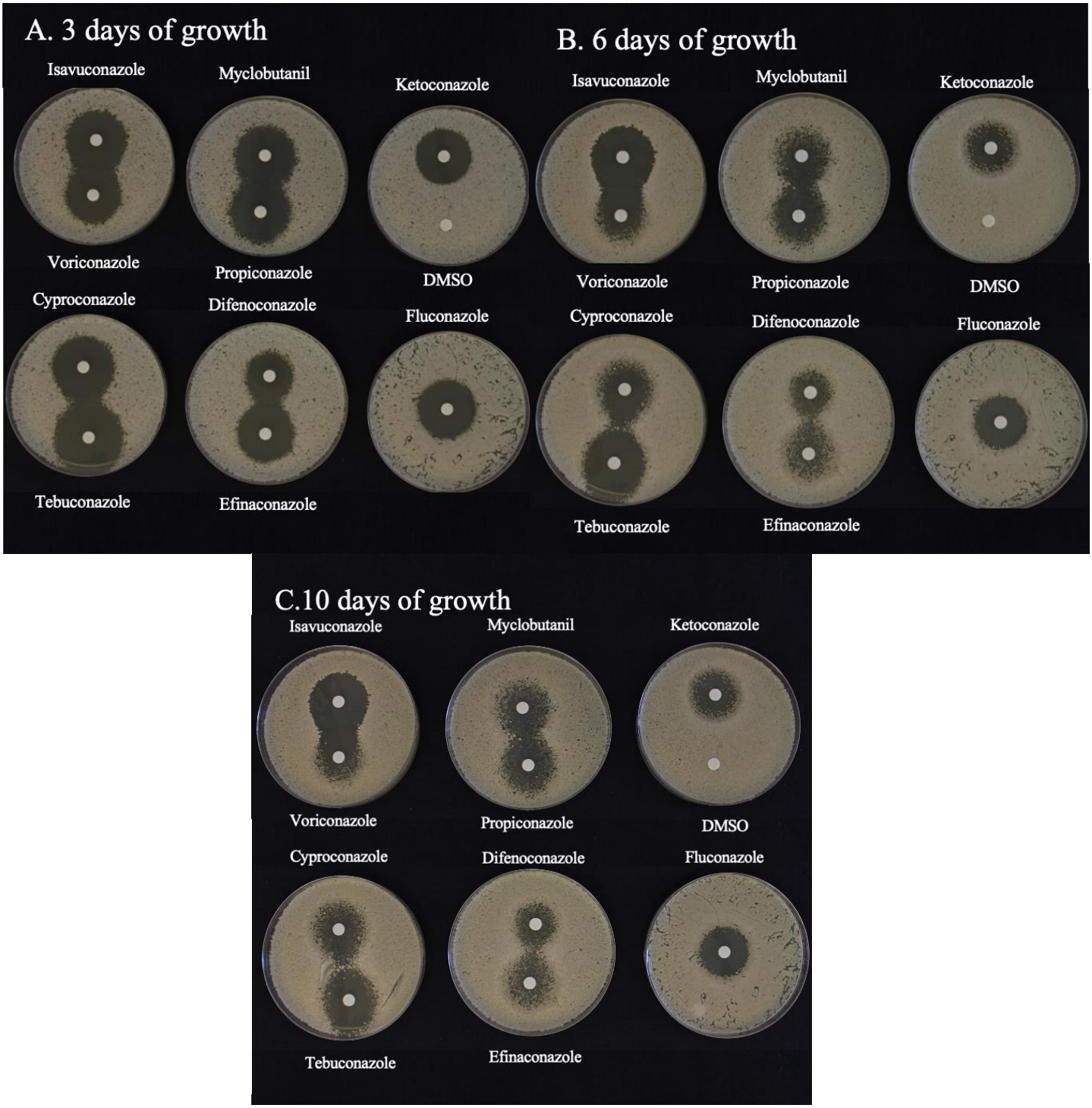


Figure 5. KN99α (LK56) Disk Diffusion Assays. Wild-type strain KN99α (LK56) was plated on YPD and tested against the azole compounds. Over time, all zones of inhibition had ingrowth of cells and/or cells growing within the zone. The exception is Isavuconazole, which didn't shrink in size and kept the zone's integrity.

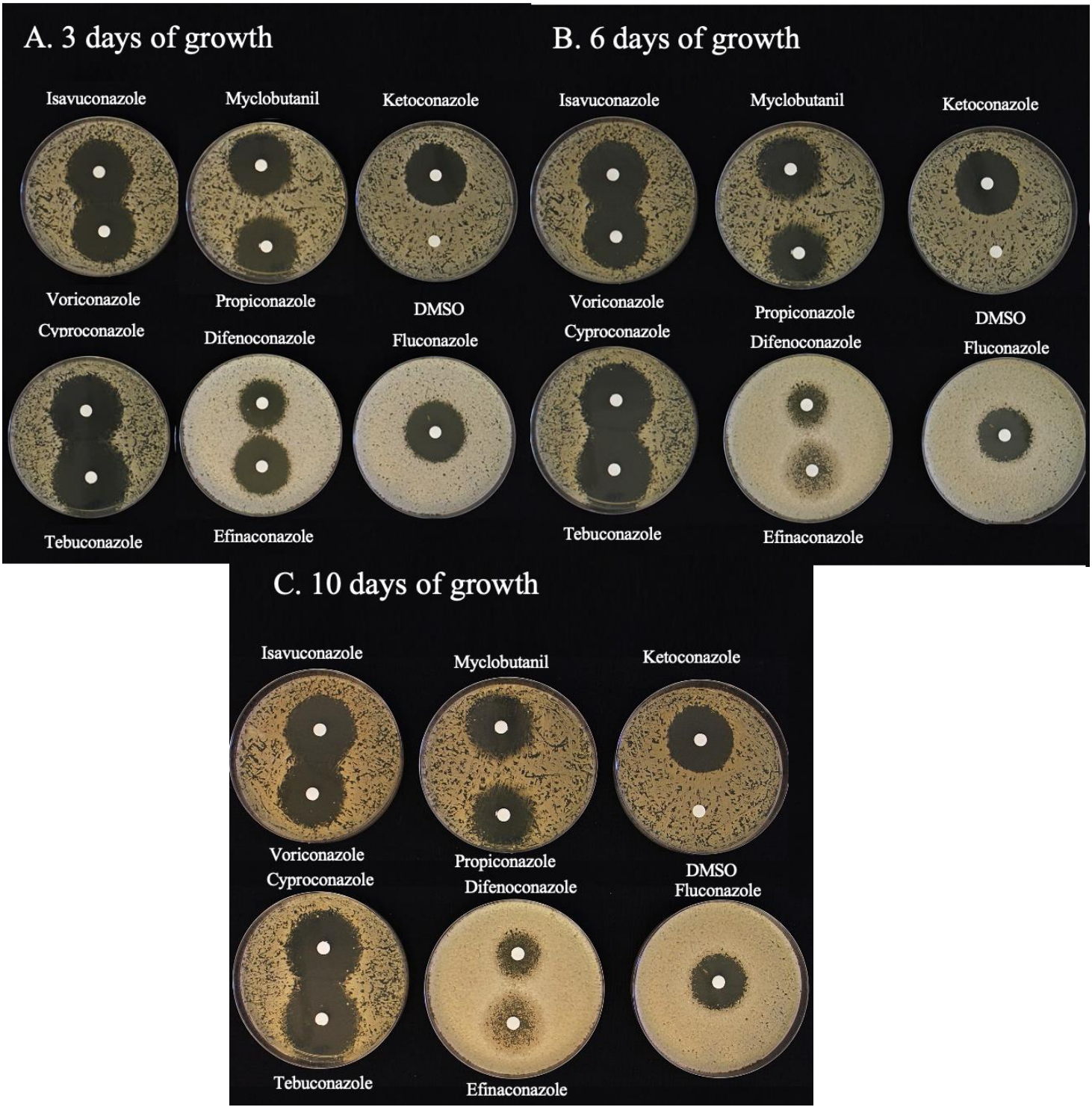


Figure 6. KN99a (LK55) Disk Diffusion Assays. Wild-type strain KN99a (LK55) was plated on YPD and tested against the azole compounds. Over time, most zones of inhibition had ingrowth and resistant cells growing within the zone. The exceptions are Isavuconazole, Voriconazole, Myclobutanil and Ketoconazole. For the duration of the experiment, there was no change to the zone of inhibition or its integrity.

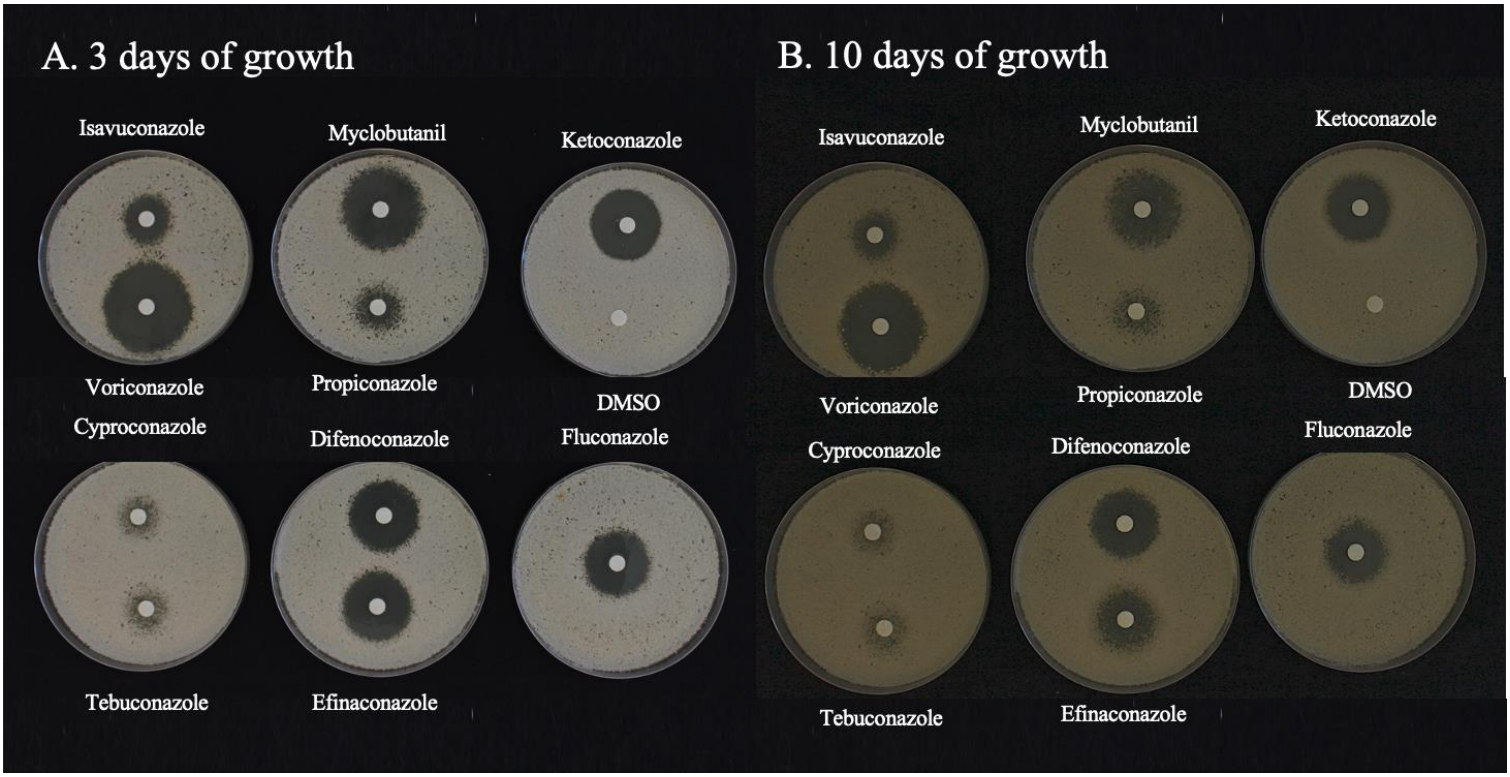


Figure 7. H99α (LK354) Disk Diffusion Assays. Wild-type strain H99α (LK354) was plated on YPD and tested against the azole compounds. Cyproconazole, Propiconazole, Isavuconazole, and Tebuconazole had little effect on the *Cryptococcus* cells. Over time, all zones of inhibition had ingrowth of cells and/or cells growing within the zone. The exception is Isavuconazole, which didn't shrink in size and kept the zone's integrity.

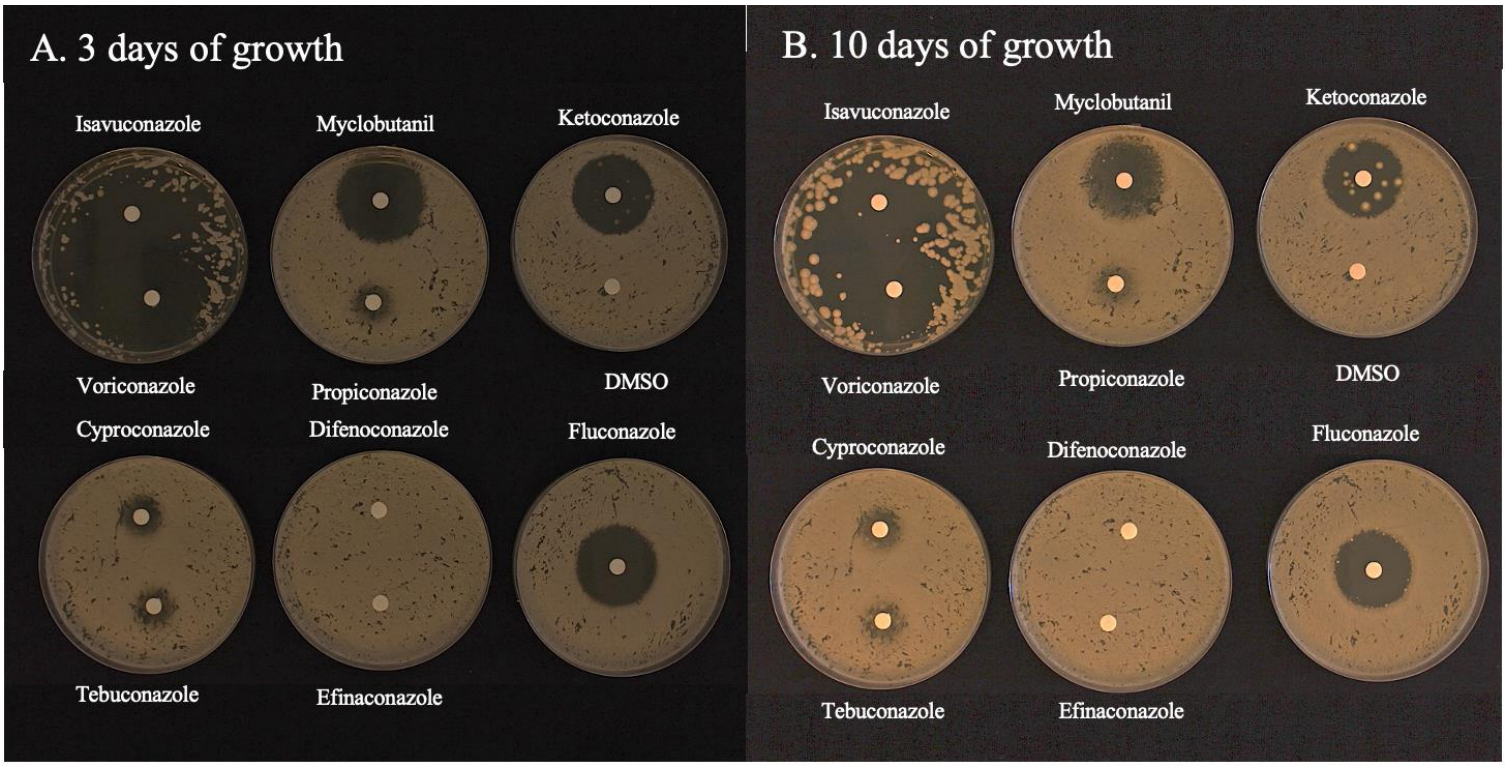


Figure 8. KN99a (LK55) Disk Diffusion Assays. Wild-type strain KN99a (LK55) was plated on YPD and tested against the azole compounds. The azole compounds did not work very well against KN99a. There was no effect by Difenoconazole and Efinaconazole. Fluconazole was the best of the azole compounds. Note that there was contamination on the plates. In addition, the plate with Isavuconazole and Voriconazole did not have the cells properly spread.

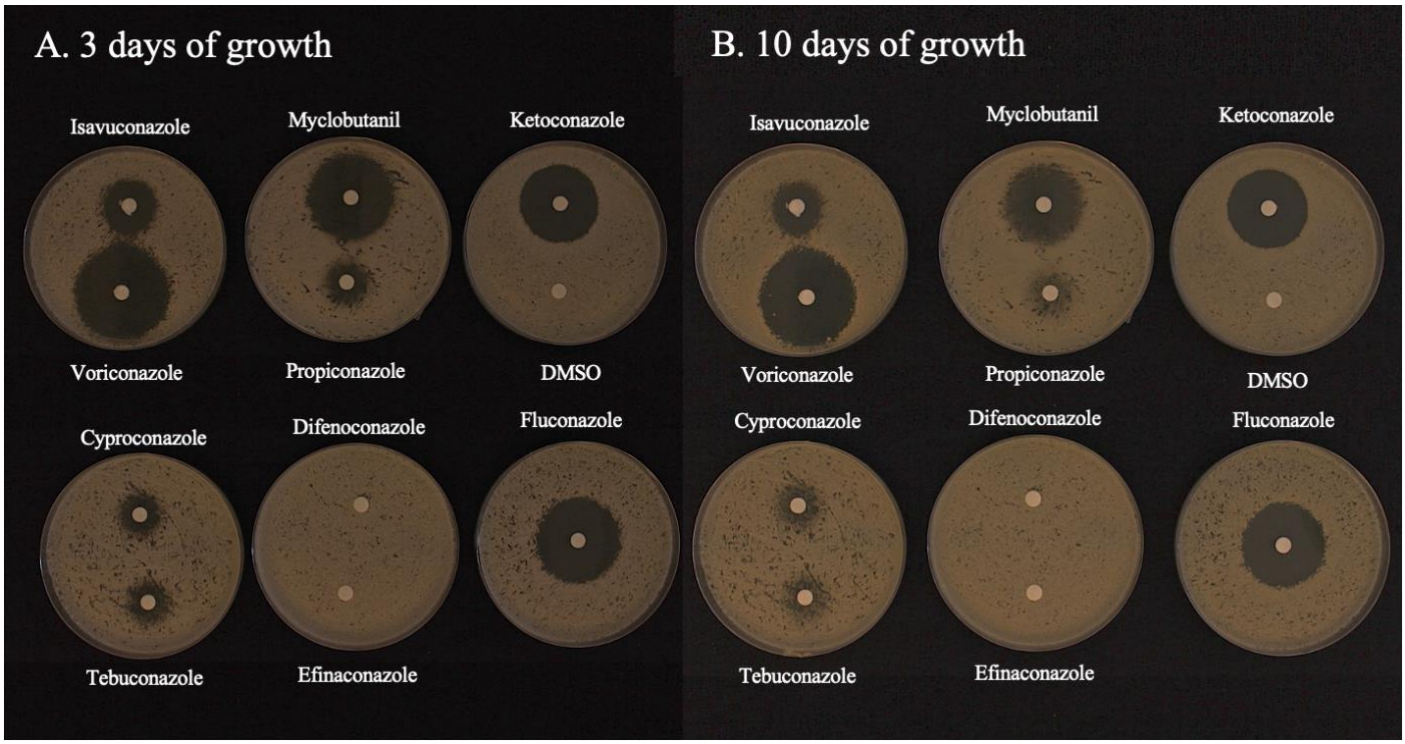


Figure 9. bub1Δ (LK359) Disk Diffusion Assays. A strain lacking Bub1 (LK359), which is a component of the spindle assembly checkpoint (SAC), was plated on NAT plates and tested against the azole compounds. Strain *bub1*Δ is mating type a. Ketoconazole, Fluconazole, and Isavuconazole were effective in both preventing resistant cell growth and preventing ingrowth of the zone of inhibition. Difenoconazole and Efinaconazole had no effect.

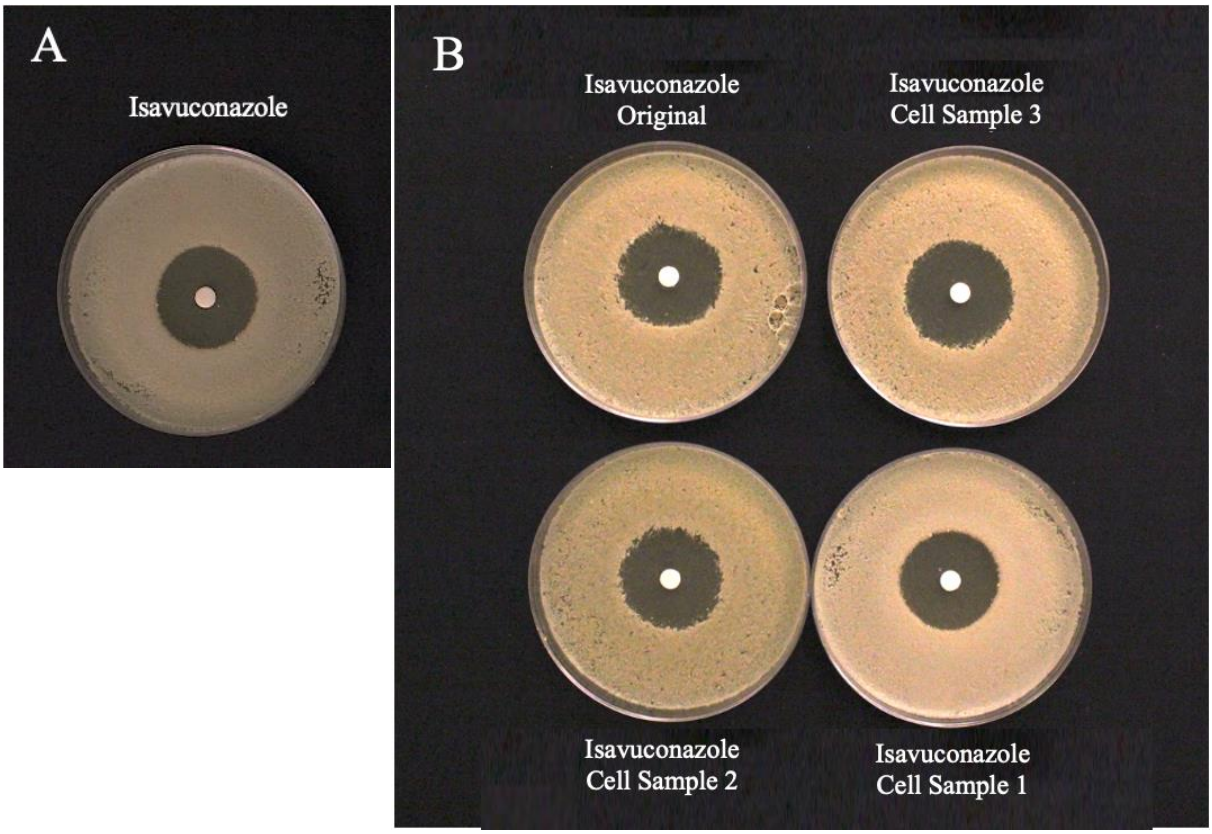


Figure 10. Disk diffusion assays to test resistance activity of azole compounds. **A.** H99α disk diffusion assays were performed by exposing strain H99α (LK54) to Isavuconazole. **B.** Swabs were taken from the plate shown in A and grown on YPD plates until there were visible colonies. Samples from three random colonies were collected. The cells were grown in media and re-exposed to Isavuconazole. There was still a very clear zone of inhibition, and the size hadn't been affected by the fact that these cells previously survived Isavuconazole.

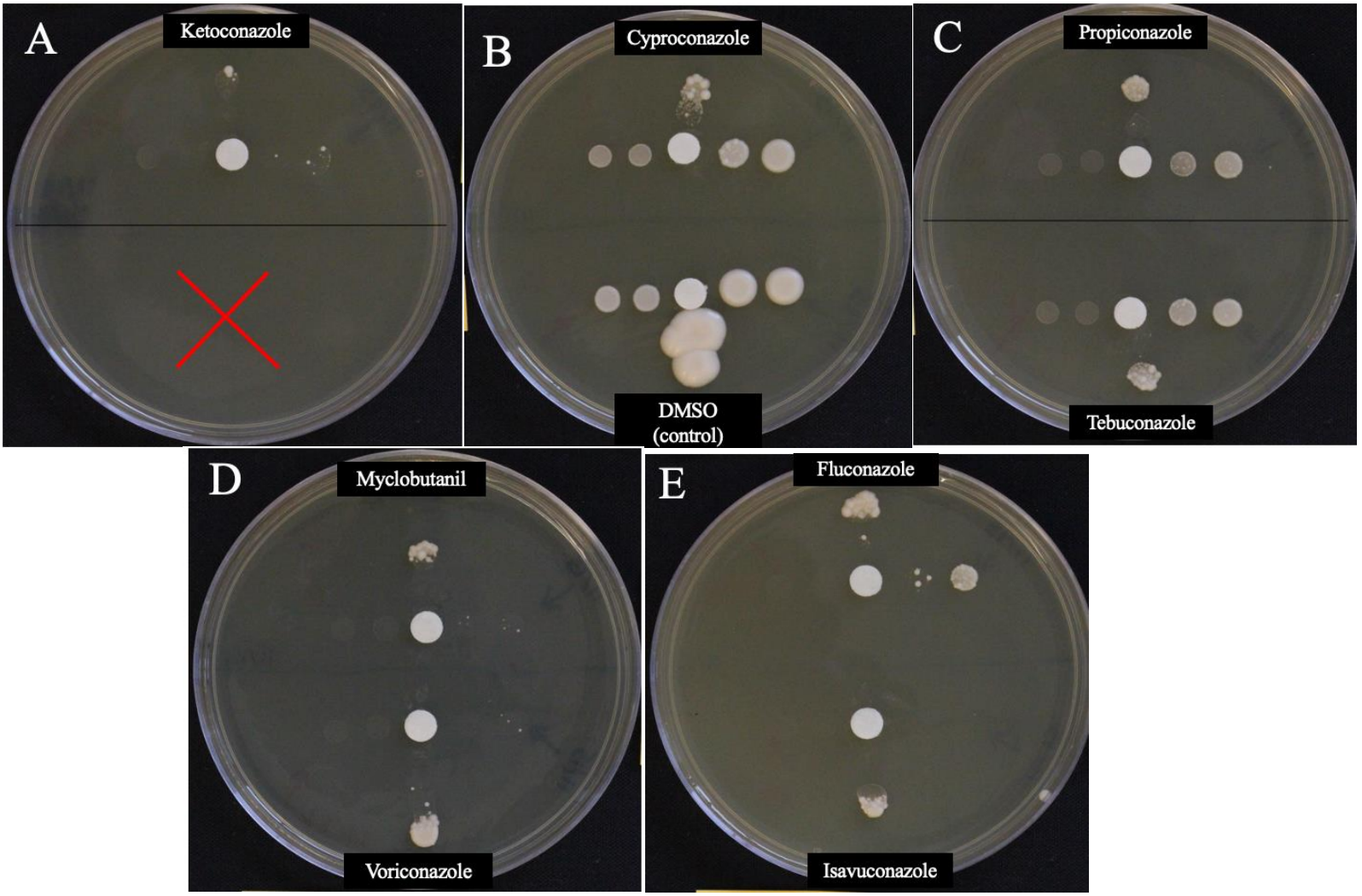


Figure 11. Drug stability - disk diffusion assay over time. Azole compounds were tested for stability by exposing *C. neoformans* cells (H99α, LK54) at different time points. The first time point was the same time the disk with the drug was placed. The second time point was 3 days after the drug was added to the plate. The third time point was 7 days after the drug was added to the plate. **A**. Ketoconazole is relatively stable. **B.** Cyproconazole had a very low stability. **C.** Propiconazole and Tebuconazole had a relatively low stability. **D.** Myclobutanil and Voriconazole have high stability. **E.** Fluconazole and Isavuconazole have high stability. It appears that Isavuconazole, Myclobutanil, Voriconazole, and Ketoconazole have better stability than Fluconazole.

All azole compounds share a heterocyclic ring containing nitrogen. The azole compounds used are divided into two categories of azole compounds, medicinal and agricultural as shown in Figure 2. Medicinal azole compounds are used for treatment of fungal diseases in humans and include Isavuconazole, Voriconazole, Fluconazole, Efinaconazole, and Ketoconazole. Agricultural azole compounds are used as fungicides against diseases in plants and include Tebuconazole, Cyproconazole, Difenoconazole, Myclobutanil, and Propiconazole.

Wild-type H99α (LK54) was tested against different azole compounds using a disk diffusion assay (Figure 3). The samples were allowed to grow for 12 days in total. Once the zone of inhibition was visible on Day 3, it was clear that all the zones were relatively the same size. There was little variance in the size overall therefore each drug started at an equal potency. By day 12, the effectiveness over time of each azole compound could be evaluated. Isavuconazole was the stand-out drug, as the zone of inhibition stayed the same size and no resistant colonies appeared within the zone of inhibition. Voriconazole and Tebuconazole retained their size but had resistant colony growth. All other drugs, including Fluconazole had major ingrowth with tolerant/resistant colonies. H99α (LK54) was tested again to see if there was variance in the size of zones of inhibition and reaction of the cells to the azole compounds (Figure 4). The zones were found to be consistent across all azole drugs, including later time points.

Another wild-type strain, KN99α (LK56), was tested against different azole compounds to determine if the results were strain-specific (Figure 5). By day 3, all the zones were still similar in size. Two exceptions are Efinaconazole and Difenoconazole. They had slightly smaller zones with KN99α than they did with H99α. When comparing day 10 of KN99α with day 12 of H99α, there are a greater number of differences (please take note that there is a two-day difference). Isavuconazole remained a stand-out drug with no shrinking or resistant colonies in

its zone of inhibition. Voriconazole, Tebuconazole, Cyproconazole, Difenoconazole and Efinaconazole were less effective on KN99α than H99α. Ketoconazole and Fluconazole were more effective on KN99α over time. There was still a shrinking of the zone of inhibition, but it was less than with H99α and less resistant colonies appeared.

KN99a (LK55) was tested against different azole compounds to determine if mating type had an impact on how the azole compounds affected the cells (Figure 6). Upon day 3, all the zones were relatively equal, except Difenoconazole and Efinaconazole. The zones were very similar in size to day 3 of KN99α (LK56). Day 10 revealed multiple differences between the mating types. All drugs except Difenoconazole and Efinaconazole had larger zones of inhibitions with no ingrowth and very few had resistant colonies growing in the zone.

H99α (LK354) was tested against azole compounds (Figure 7). The working concentrations used were not the concentrations that caused equalized zones. This is meant to give a comparison for KN99a and *bub1*Δ (LK359), which used the same exact concentrations. LK354 was used because it had a closer genetic profile to LK359.

KN99a (LK55) was tested against different azole compounds to give a comparison for *bub1*Δ, which is also mating type a (Figure 8). There was an issue with plating cells on the plate containing Isavuconazole and Voriconazole, therefore those will not be considered. Overall, the zones were similar to H99α (LK354). There was one distinct difference. Difenoconazole and Efinaconazole had absolutely no effect on the KN99a cells. Note that some plates have contamination present.

The *bub1*Δ mutant (LK359) was tested against different azole compounds. *bub1*Δ cells were grown on NAT plates. Isavuconazole and Voriconazole both performed well, with keeping zone integrity and size. Nothing else can be said as the Isavuconazole and Voriconazole samples

for KN99a were plated incorrectly. Consistent with KN99a, Difenoconazole and Efinaconazole had no effect on *bub1*Δ cells. Myclobutanil performed better with *bub1*Δ than it did with KN99a. It kept its zone size better with no resistant cells growing. When compared to H99α, *bub1*Δ was more susceptible to the azole compounds, except Cyproconazole, Tebuconazole, Efinaconazole and Difenoconazole.

Figure 10 gives a summary of re-exposing H99α (LK54) cells to Isavuconazole. Isavuconazole was able to maintain the exact same size of the zone of inhibition, even upon re-exposure of the cells. There were also no resistant cells that grew in any of the zones of inhibition of the Isavuconazole plates.

H99α (LK54) was used to test the stability of select azole compounds over time (Figure 11). The azole compounds excluded from this experiment were Difenoconazole and Efinaconazole because all other azole compounds had equalized zones of inhibition. At the time of experimentation, the drug dilutions we had available were still too potent. Ketoconazole, Isavuconazole, Myclobutanil, and Voriconazole were more stable than Fluconazole over the 10-day period. Propiconazole, Tebuconazole, and Cyproconazole were less stable than Fluconazole over the 10-day period.

Discussion

Isavuconazole was the drug that worked for all strains and mating types, often being superior to Fluconazole. At a much lower concentration, it was able to prevent resistant cells and tolerance. Additional drugs that worked better than Fluconazole were Cyproconazole, Voriconazole, Tebuconazole, Efinaconazole and Difenoconazole. In addition, the effects of all the azole compounds were consistent across experiments. There was no variance in the size of

the zone of inhibition when the concentration of the drug remained the same. The disk diffusion assay provided a great qualitative base to further investigate how azole compounds affect *C. neoformans.* Through re-exposure to the same drug, it was also found that Isavuconazole did not lose its effectiveness. Drug stability was another point to consider as some of the ingrowth of cells could have been due to the drug breaking down rather than the colonies becoming tolerant. In general, the agricultural drugs were less stable than the medicinal drugs.

In addition to finding what drugs worked, it was also important to determine if strain and mating type would have an influence on the efficacy of the azole compounds. The strain (KN99α vs H99α) changed how the azole compounds affected the cells. Many that worked well for the H99α didn't work as well, including Voriconazole, Cyproconazole, and Tebuconazole. Difenoconazole and Efinaconazole surprisingly had no effect on KN99α cells even though they worked very well against H99α. Mating type had a large impact on the effect of the drugs. Mating type a was more susceptible to the azole compounds as compared to mating type α.

The *bub1*Δ mutant strain was an interesting addition to the strains that were tested. This strain had a deletion of the BUB1 protein. This protein is a part of the spindle assembly checkpoint (SAC) and helps ensure spindle assembly is completed correctly (Leontiou et al., 2022). Without it, there is a higher potential of aneuploidy cells forming. The theory with Fluconazole is that aneuploids are resistant, therefore having a strain that would have increased aneuploids would be a good tool to determine if this theory is true (Berman & Harrison, 2019). If there are more colonies that are resistant compared to wild-type KN99α, then this would prove the theory correct. Unfortunately, since this experiment was only done once and there were issues with plating the KN99α, we cannot conclude much about the results of eliminating BUB1 from the data. In order to make more conclusive data, there would need to be multiple biological

replicates of disk diffusion assays with *bub1*Δ and KN99α followed by a counting of resistant colonies that appear within the zone of inhibition.

Conclusion

Overall, alternative azole compounds do have a potential to be used in addition to Fluconazole against *C. neoformans*. Isavuconazole is the best drug out of those tested, with it being the most effective and stable as well as the lack of ability of *C. neoformans* cells to gain resistance, even upon re-exposure. Other viable drugs are Voriconazole, Efinaconazole and Difenoconazole. In addition, mating type and strain do have an impact on how well an azole compound works against the cells. The sensitivity is different for H99α, KN99α, and KN99a, even though all of them are wild-types. Finally, agricultural azole compounds are less stable overall than medicinal azole compounds.

Future directions include finding the MIC value for each azole compound (Archibald et al., 2004). This will allow a quantitative assessment of the azole compounds and help to refine the equalized zones of inhibition. In addition, further investigation into the effect of mating types and strains on azole compounds is necessary. There also should be more testing done into whether azole compounds kill or inhibit *C. neoformans* as well as why the cells gain tolerance to some of the azole compounds (Pfaller et al., 2004). A step forward in this would be growing the cells in liquid media and testing over generations if resistance is gained, if it can be lost, and whether any cells remain living.

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