Resistance of Environmental Fungi to Azole Drugs that are Used to Treat Fungal Infections Including Coccidioidomycosis

Ahlam Alamamy[a], Benjamin Whipkey*[a], Victoria Saez[b], & Antje Lauer*[a]*

[a] *California State University Bakersfield, Department of Biology, 9001 Stockdale Highway, Bakersfield, CA 93311,*
[b] *Bakersfield Community College, 1801 Panorama Drive, Bakersfield CA 93305*

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Students: aalamamy@csub.edu, bwhipkey@csub.edu*, victoria.saez2706@email.bakersfieldcollege.edu*
*Mentor: alauer@csub.edu**

ABSTRACT
The increasing use of fungicides in the agricultural hub of the Central Valley of California to fight plant pathogens has led to concerns about fungal pathogens developing resistance against these agents. The soil environment harbors many opportunistic fungal species that can cause disease in plants, animals, and humans. Among them are *Coccidioides* spp. known to cause Valley fever, an orphan disease, endemic to the arid regions of the Southwestern U.S. The disease is often misdiagnosed, delaying treatment with antifungal agents in the early stages of the disease, which has led to the dissemination of the disease in many patients. In this study we found and tested a large cache of fungal isolates, identified as members of ten fungal families and obtained from the air of Bakersfield, Kern County, CA. A large percentage showed strong resistance against three different azole drugs, namely fluconazole, itraconazole, and voriconazole, that are used to treat fungal infections including aspergillosis and Valley fever in humans and animals. Especially fluconazole, one of the most commonly used azole drugs prescribed for treatment, showed no or only minimal effect against most fungal isolates, in contrast to posaconazole which strongly reduced fungal mycelium growth of most isolates in azole challenge assays on Sabouraud Dextrose medium. These results were statistically significant. Two-way ANOVA were used to compare the effects of four azole drugs on fungal mycelium growth among members of ten fungal families. The ANOVA revealed a significant difference in efficacy when comparing the impact of individual drugs on fungal mycelium growth, $p < 0.05$. Post-hoc comparisons showed that posaconazole significantly inhibited fungal growth more than all other azoles ($p < 0.05$). Members of most fungal families tested showed a high measure of resistance to azole drugs and 20-39% showed even an increased growth in the presence of fluconazole. The results of this study are concerning in times where Valley fever incidence is increasing due to increased soil disturbance and climate change in the Central Valley of California.

KEYWORDS
Coccidioidomycosis; Valley fever; agriculture; fungal pathogen; azole drug; drug resistance; antifungal susceptibility; airborne fungal spores; fungal resistance mechanisms; fungicide

INTRODUCTION

The Southern San Joaquin Valley, as part of the Central Valley of California, is primarily known for its highly productive agriculture, and is one of the most fertile and important farmland regions in the United States.[1] The transition from valley grassland to industrial fields is a constant and major driver of emerging dust that negatively affects the air quality in the valley.[2] Unfortunately, arid soils in the Southwestern U.S. are home to the soil-dwelling fungus *Coccidioides*, the causative agent of Valley fever.[3] This disease, also known as coccidioidomycosis, is generally treated with a variety of azole drugs (imidazoles and triazoles). These antifungal agents block the lanosterol 14-α-demethylase enzyme essential for ergosterol biosynthesis, inhibiting the growth of the fungus. Therefore, they are widely used to inhibit fungal pathogens that are able to cause disease in humans and animals.[4,5]

The approval and use of azole antifungal drugs have significantly advanced the treatment of coccidioidomycosis and other fungal diseases. After clotrimazole (1973), miconazole (1979), and ketoconazole (1981), fluconazole, brand-named Diflucan, was the fourth azole drug that was approved by the Federal Drug Administration (FDA) in 1990 to treat systemic and superficial fungal infections in humans. Together with itraconazole that was approved in 1992, fluconazole belongs to the so-called early triazole drugs. Triazoles of the second generation include voriconazole (2002), and posaconazole (2006).[6] The only azole drug with fungicidal properties is voriconazole, which has been recommended to treat invasive mycoses, in contrast to all other azole drugs which are fungistatic in nature.[7] However, fluconazole is still the most used azole drug due to its affordability, wide tissue

penetration, and only mild side effects despite higher in vitro Minimum Inhibitory Concentrations (MICs) compared to other azole drugs.[8,9]

Azole antifungals, among them propiconazole, tebuconazole and epoxiconazole, are being used increasingly and excessively in agriculture to protect crops from fungal plant pathogens.[10-13] In Kern County, located in the Southern San Joaquin Valley, azole antifungals like tebuconazole (9,485 tons/year) and triflumizole (5,833 tons/year) are among the most commonly used azole fungicides[14] **Supplementary Figure S1 (Appendix)**. Additional azole antifungals such as prothioconazole, and difenoconazole which are known for their broad-spectrum efficacy against fungal pathogens are often used in seed treatments to control diseases caused by *Fusarium* and *Microdochium* and are classified within the demethylation inhibitors (DMI) group.[15-17] In addition, other non-azole fungicides such as pyrimethanil (3,933 tons/year), an aminopyrimidine to control *Botrytis cinerea* throughout the winemaking process in grapes, as well as penthiopyrad (10,683 tons/year), an aromatic amide, and member of the pyrazoles which is being used as a broad-spectrum anti-fungal in agriculture are often applied.[14]

The use of antifungals to combat fungal plant pathogens has increased significantly in California in the last decade[18] with numerous negative impacts on human health.[19] Not surprisingly, these management practices have led to the emergence of antifungal resistance among opportunistic fungal pathogens that can cause disease in humans, animals, and plants.[5,20-23] Consequently, soilborne opportunistic fungal pathogens that can threaten humans and animals, including *Coccidioides* spp., *Aspergillus fumigatus,*[24-27]*Histoplasma capsulatum,*[28] and *Cryptococcus* spp.,[29] are experiencing environmental pressures that may drive the development of resistance to antifungals, including azoles used to treat infections. In fact, this has been shown in some clinical studies with *Coccidioides* isolates obtained from patients, potentially leading to untreatable coccidioidomycosis, dissemination of the disease, increased healthcare costs and death for some patients.[30,31]

Significance of this study

Even though Kern County has had the highest incidence of coccidioidomycosis in California for many years[32] (**Figure 1)**, finding a potential link between treatment failure and pathogen resistance has not been the focus of ongoing research in recent years[33,34]. Our work will hopefully raise awareness of this problem.

The results of this study emphasize the need for new non-azole treatment and raise awareness about the consequences of long-term overuse of azole fungicides in industrial agriculture. Despite observations that treatments with fluconazole have become less efficient, it is still recommended as the first line of treatment for coccidioidomycosis and coccidioidal meningitis[35,36].

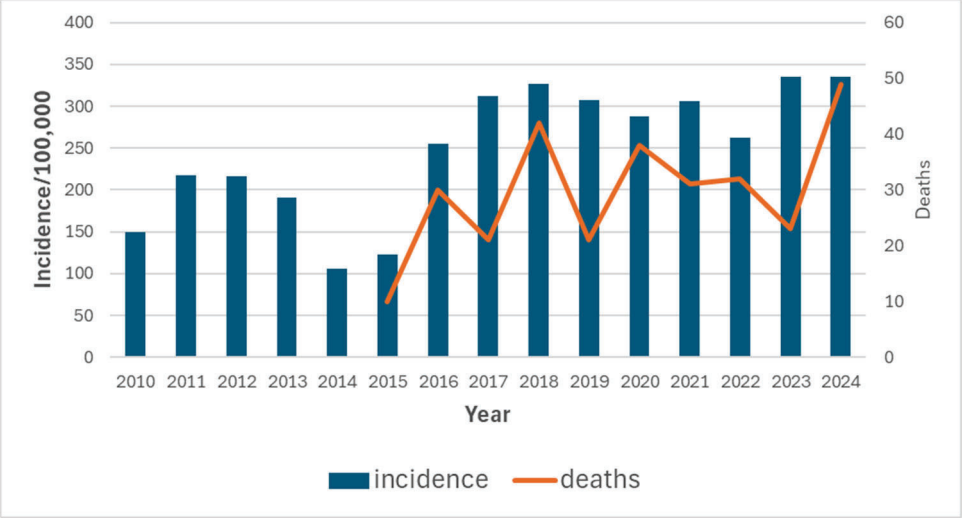

Figure 1. Reported Valley fever incidence and deaths in Kern County between 2010 and 2024. No individual data for deaths was available for the years prior to 2015.[37] As of May 2025, more than 3,100 cases (incidence 336) were confirmed in Kern County.[38]

Aims of this Study

The extent of azole drug resistance among *Coccidioides* spp., the causative agent of Valley fever, is currently unknown. Most recent reviews on the topic of disease prevention do not even mention the risk of increased azole drug resistance due to practices common in industrial agriculture in Kern County and other areas of the Central Valley where disease incidence is high and increasing.[39] We investigated the extent of antifungal resistance among common soil fungi, some of them opportunistic pathogens

to immunocompromised humans and animals, from airborne spores with a culture dependent method and exposed them to different types of azole drugs used to treat Valley fever and other fungal diseases. Environmental fungi that share the same habitat are exposed to the same environmental pressures as *Coccidioides* spp. and therefore can be seen as a proxy for the pathogen.

METHODS AND PROCEDURES

Sampling, Isolation, and Identification of Fungi

Sabouraud Dextrose (SabDex) media plates were used to capture airborne fungal spores from several locations in Bakersfield, CA, between 2021 and 2024. Sampling sites included both agricultural zones and urban areas. Fungal sampling was not performed in remote regions, where less azole exposure would be expected. All plates were incubated at room temperature for 10 days. All individual fungal colonies were transferred onto fresh SabDex plates until pure cultures were obtained. The plates did not contain antibiotics but relied on a low pH (5.6) to inhibit bacterial growth, as SabDex supports fungi at acidic conditions.[40] Although some bacterial colonies were occasionally observed, only fungal isolates were transferred to fresh plates until pure cultures were achieved. To identify fungal isolates, DNA was extracted from a 0.5 cm^2 piece of colony using the DNEasy PowerLyzer Microbial DNA extraction kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Subsequently, a 1,000 bp fraction of the 18S rDNA gene was amplified with primer pair NSA3/NLC2 using the Polymerase Chain Reaction following a published cycling protocol,[41] including positive and negative controls. All PCR products were evaluated for the correct size with 2% agarose gel electrophoresis and compared to a 100 bp DNA ladder (G2101, Promega). Aliquots of PCR products were purified with ExoSAPit (Affymetrix, Santa Clara, CA) and sequenced (Laragen, Inc., Culver City, CA). Sequences were then compared to entries in the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) of the National Institute of Bioinformatics (NCBI).[42] *C. posadasii* Δ *chs5* was obtained from Biodefense & Emerging Infections Research Resources Repository (BEI Resources) (Safety Level 2) and grown according to the recommendations in the product information sheet. This fungal strain was used as a positive control in all PCRs. Aseptic techniques were followed alongside safety guidelines during the collection process and during work in the laboratory.

Phylogenetic Analysis

PCR amplicons from all environmental fungal isolates were identified to the genus or species level using the online Basic Local Alignment Search Tool (BLAST) with DNA sequence matching available on the National Center for Biotechnology Information (NCBI) database.[43] Furthermore, all sequences were aligned with a selection of closest matches from NCBI GenBank and processed using MEGA 11 software.[44] A phylogenetic tree was constructed using the Neighbor-Joining method including bootstrapping with 100 replicates **Figure 4**. *Batrachochytrium dendrobatidis* was used as an outgroup.

Antifungal Challenge Assays

Fungal isolates were grown on SabDex medium that included 4.5 μg/ml of individual azole drugs that are used to treat fungal infections including Valley fever and Aspergillosis. The antifungal agents selected for this project included fluconazole, posaconazole, itraconazole, and voriconazole. Antifungal agents were dissolved in water or dimethyl sulfoxide (DMSO) and added to the medium after autoclaving when the medium had cooled down to 55 °C to avoid deactivation of the azole agents. Control plates without azole drugs were included in the assays, as well. The inoculum of fungal mycelium on each SabDex medium was 0.5 cm^2. All plates were secured with parafilm and incubated for 10 days at 23 ºC. After incubation, the size of fungal colonies was measured in millimeters (mm) and compared to the growth on the control plates. The effect of different azole drugs on mycelium growth of different fungal isolates was recorded. To evaluate the challenge assays, we proposed four categories: 1. (almost) completely inhibited (0-10% growth), 2. strongly inhibited (11-50% growth), 3. somewhat inhibited (51-80% growth), 4. (almost) no inhibition (81-100% growth) compared to the control plates. Fungal isolates that grew in yeast form were included in the azole drug challenge assays, using a simple streaking technique and assessing the amount of growth compared to the control plate after incubation.

Statistical Analysis

The analysis was designed to compare the effectiveness of four azole drugs to each other and across fungal isolates from ten taxonomic families. The following statistical approach was followed with analyses conducted in R (version 4.5.1). Prior to modeling, a Shapiro-Wilk test was used to assess the normality of drug effectiveness values across families and drugs. The majority of distributions were significantly non-normal, prompting the use of robust scaling to normalize the data. Robust scaling centers the data by the median and scales by the interquartile range (IQR) rather than standard deviation, thus preserving biologically meaningful outliers and values of zero while minimizing their influence on model fit.

A two-way ANOVA was performed on the normalized data to test the main and interaction effects of drug on isolates from different fungal families. Significance was evaluated with a Tukey HSD post-hoc test to compare the overall effectiveness of each drug to the other. To help determine the ordinal ranking of drug effectiveness, an estimated marginal means test (EMM) of the normalized data was used to gather adjusted means for each drug for comparison.

To confirm the results of the normalized findings, a two-way permutation ANOVA was conducted on the unnormalized percentage data to again search for main or interaction effects. Then a confirmatory pairwise post-hoc analysis was conducted for all families combined, as was done with the normalized ANOVA, but with *p*-values adjusted using the Benjamini-Hochberg false discovery rate (FDR) correction because of its appropriateness for exploratory analysis and permutation tests. Tests on the permutation data were set to perform 999 permutations. The median values of the percentage data for each drug were gathered and used for ordinal ranking. Lastly, the number and percentage of isolates were calculated for each fungal family ($n \geq 5$) that exhibited increased growth (>100%) in the presence of fluconazole, itraconazole, voriconazole or posaconazole relative to control conditions.

Safety

All work was performed in a microbiology laboratory equipped with a Purifier Class 2 Biosafety 2 Cabinet (LabConco, USA). This allowed us to work with non-pathogenic *Coccidioides posadasii* mutant and environmental fungi, including opportunistic pathogens that are not a threat to healthy humans.

RESULTS

Overall, pure cultures of 151 fungal isolates were obtained. Of these 124 (82%) were identified to the genus or species level belonging to 10 families within 8 fungal orders. Members of the *Pleosporales* (*Alternaria* spp.) were among the most common isolates, followed by members of the Eurotiales (*Penicillium* spp. and *Aspergillus* spp.) and Cladosporiales (*Cladosporium* spp.) **Figures 2 and 3**.

Figure 2. Example of a SabDex plate exposed to the air after incubation at room temperature for about 10 days, showing diverse fungal colonies that were isolated and included in this study.

A phylogenetic tree that includes representatives from diverse fungal families displays the isolated diversity as well **Figure 4**. Most fungal isolates are described as known plant pathogens in the literature, but some can be opportunistic pathogens to humans, being mainly a threat for those with reduced immune functions. Interestingly, some fungal isolates were related to biocontrol agents that have been used to successfully inhibit some plant pathogens that are responsible for major plant diseases **Supplementary Table S1 (Appendix)**. In addition to those species that were isolated more frequently, such as *Penicillium* spp., *Cladosporium* spp., *Aspergillus niger*, and *Alternaria alternata*, we identified a few isolates as closely related to pathogens of concerns, such as *Aspergillus fumigatus*, *A. flavus*, *Alternaria infectoria*, and *Fusarium oxysporum*. The fungal isolates that grew in yeast form were identified as closely related to *Aureobasidium pullulans*.

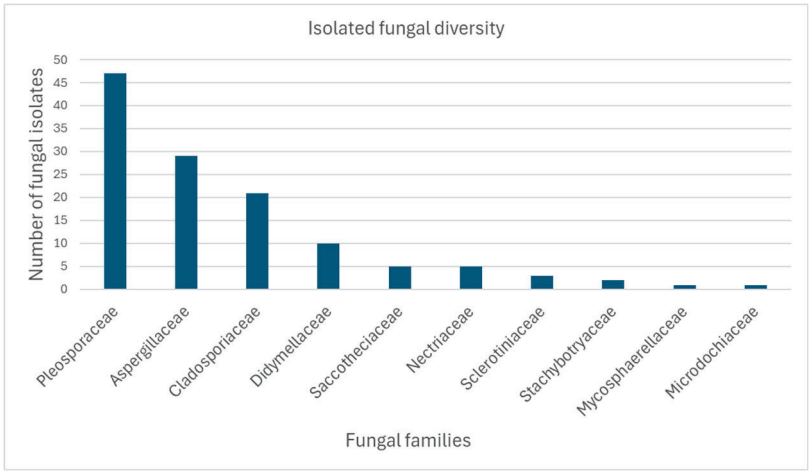

Figure 3. Overview of isolated fungal diversity (n=124) for 10 fungal families.

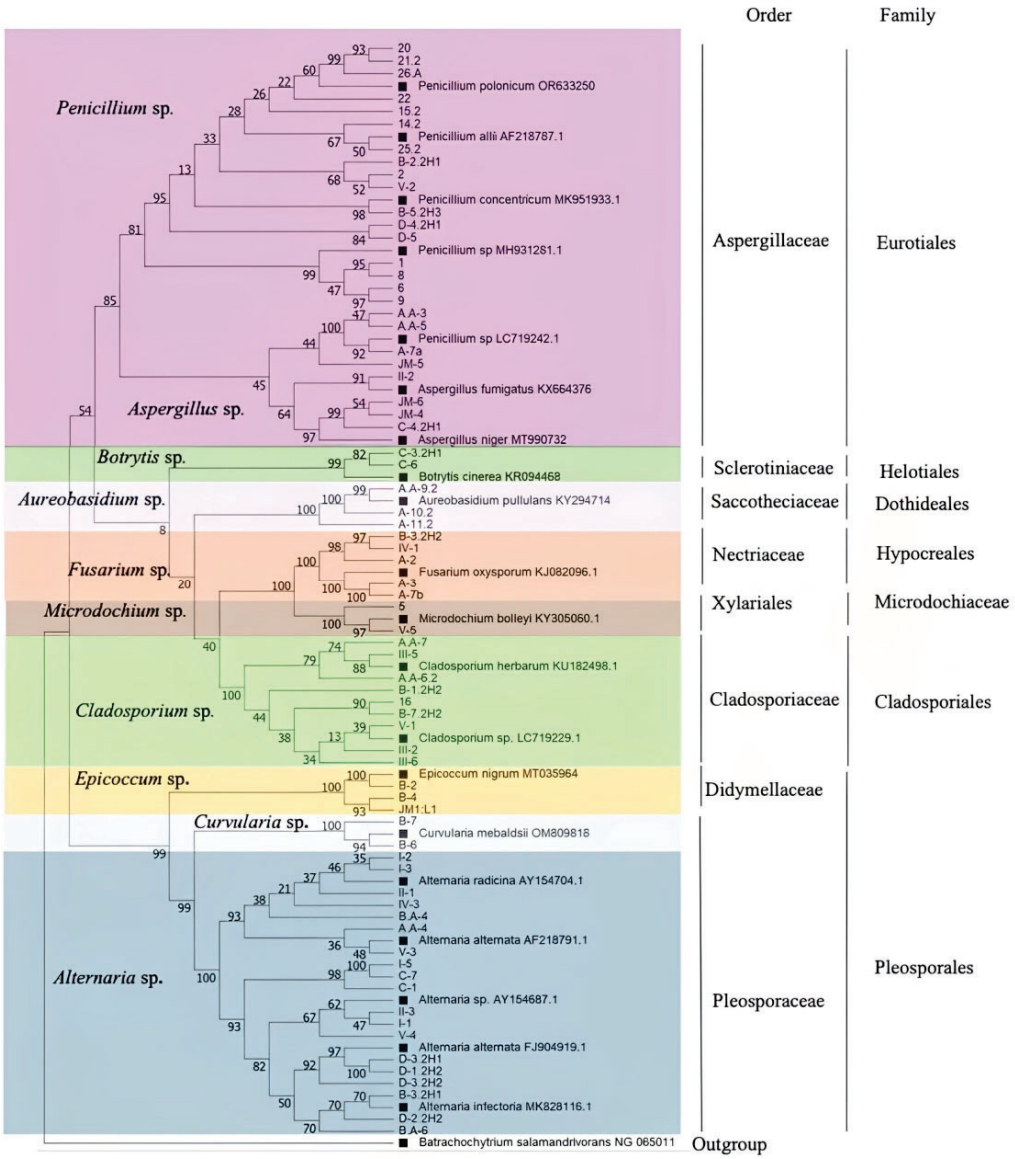

Figure 4. Phylogenetic tree of selected sequences (n=73) from representatives of all major fungal families, constructed using the Neighbor-Joining method with 100 bootstrap replications, showing the isolated diversity of fungi. *Batrachochytrium salamandrivorans* was used as the outgroup.

A selection of challenge assays against different azole drugs is shown in **Figure 5**. The *C. posadasii* control obtained from BEI Resources was completely inhibited by all azole drugs, except when exposed to fluconazole which was able to inhibit the growth of this fungus by 46.4% **Figure 6**. Negative controls on SabDex medium showed no negative effect of DMSO on fungal growth (not shown).

Challenge assays against four different azole drugs were completed for 124 identified isolates. We visually observed wide variation in fungal resistance to individual azole drugs among fungal isolates of all families. Fluconazole can be described as least effective, followed by itraconazole and voriconazole; posaconazole was visibly the most effective in inhibiting fungal growth **Figure 5 and 7**. However, most fungal isolates were able to grow at least to some degree in the presence of most antifungals, except when exposed to posaconazole which inhibited the growth of many fungi completely as seen in the last column of **Figure 5**, and by the clustering of means in the 0-40% growth range seen in **Figure 7**.

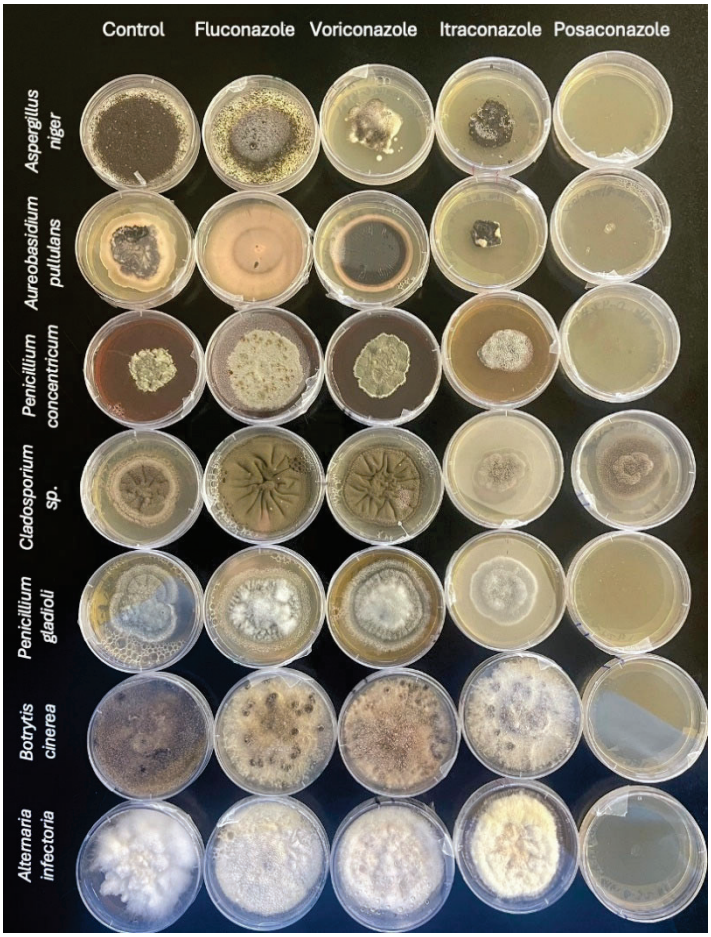

Figure 5. Selection of different fungal isolates growing on SabDex medium supplemented with different azole drugs. All fungal isolates shown were at least 98% related to entries in the GenBank nucleotide database.

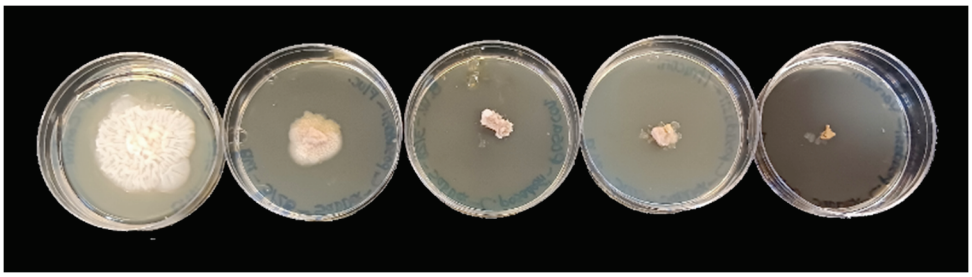

Figure 6. A challenge assay was performed on the non-pathogenic *Coccidioides posadasii* strain Δ *chs5*. The SabDex plates in order from left to right are the control, followed by fluconazole, posaconazole, itraconazole, and voriconazole. Growth only occurred on the control plate (28 mm) and the fluconazole plate (15 mm). The other plates show the inocula in the center from which no mycelium growth was able to develop.

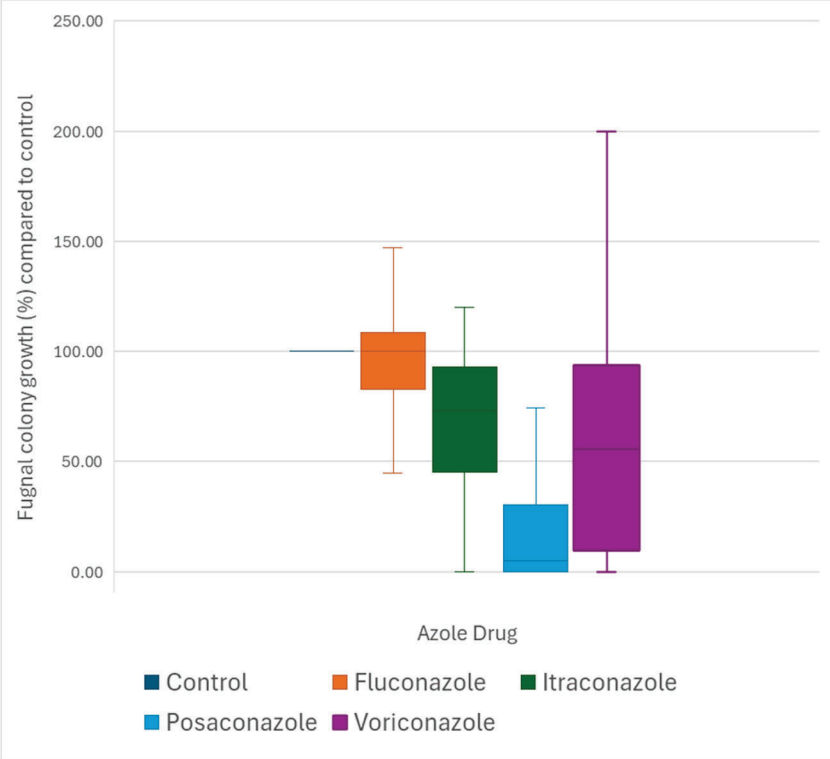

Figure 7. Bar plot showing the average growth (%) of fungal isolates exposed to four different azole drugs compared to growth on the control plates without any azole drug. The wide bars represent the clustered means of each drug, and the error bars represent the entire sample set for that drug, including outliers. A two-way ANOVA showed a significant difference between the drugs in affecting mycelium growth ($p < 0.05$), meaning the average reduction of growth (%) are not the same. Because $p < 0.05$, we reject the null hypothesis and conclude that the drugs differ in efficiency regarding fungal mycelium growth. Fluconazole showed the least inhibitory effect, with average fungal growth closest to the untreated control, while posaconazole was the most effective, significantly inhibiting mycelial development across most isolates.

Isolate percentage data was used to group them into levels that categorize azole's effectiveness, and a visually indicative color scheme was assigned for those categories. We then grouped them by drug, **Figure 8,** as well as by the top four fungal families in **Figure 9** for visual comparison of effectiveness.

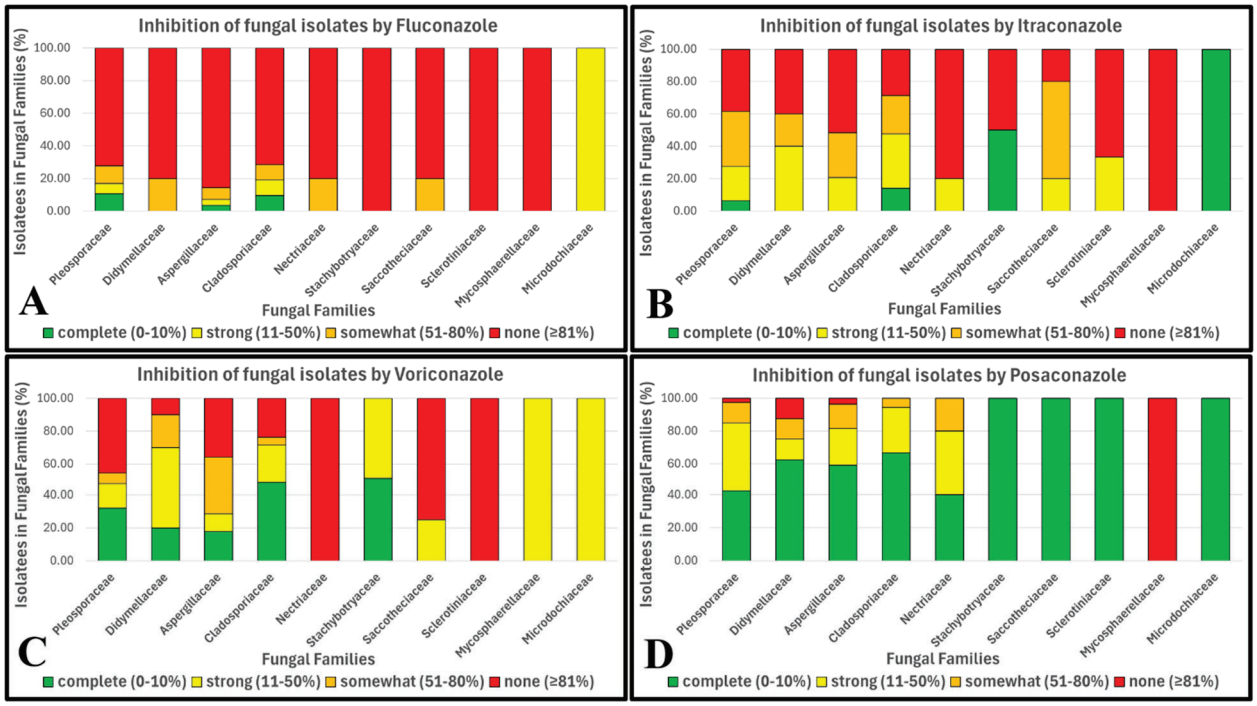

Figure 8. Efficiency of azole drugs to inhibit fungal mycelium growth (%) compared to control plates for each drug. **(A)** Fluconazole. **(B)** Itraconazole. **(C)** Voriconazole. **(D)** Posaconazole.

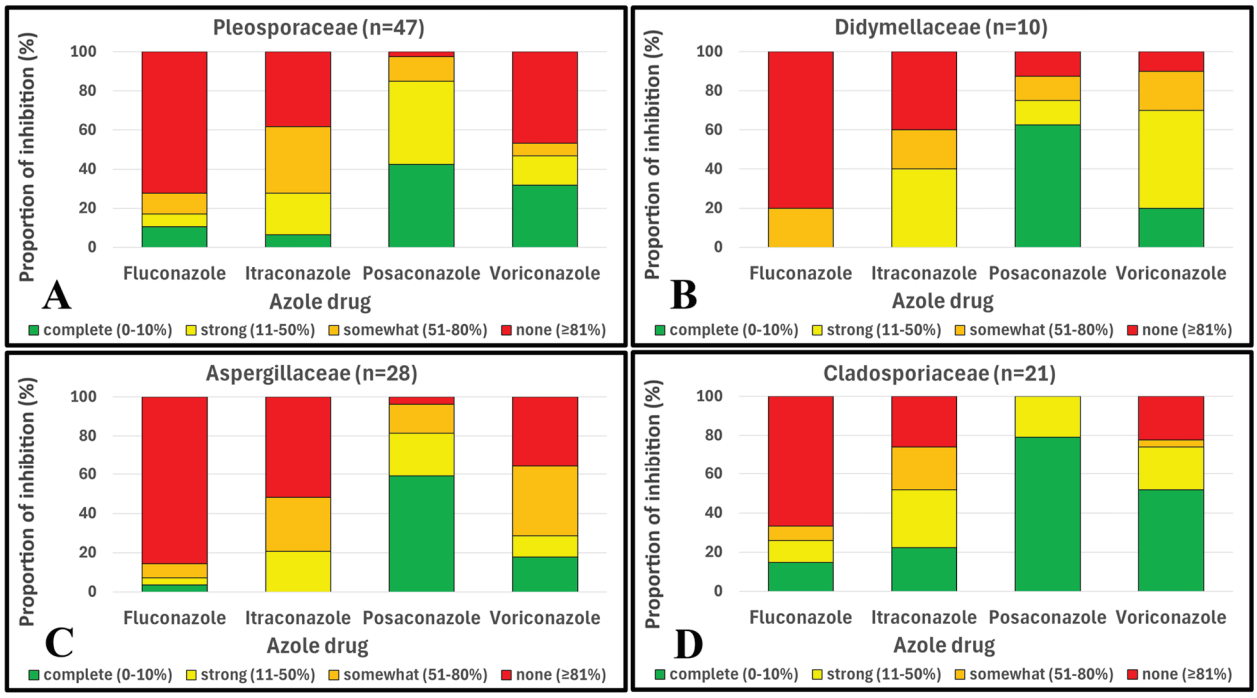

Figure 9. Efficiency of azole drugs to inhibit fungal mycelium growth (%) compared to control plates for the top four families. **(A)** Pleosporaceae. **(B)** Didymellaceae. **(C)** Aspergillaceae. **(D)** Cladosporiaceae.

Initial statistical analysis via normality tests **Supplementary Table 2 (Appendix)** revealed abnormal data that needed to be normalized before proceeding. A two-way ANOVA on the normalized data showed a significant difference between the drugs in affecting mycelium growth ($p < 0.0001$) **Table 1**, but no other significance was found. The permutation ANOVA also revealed only a significant difference in drug effectiveness ($p < 0.05$) **Table 1**. This suggests that the drugs each work the same irrespective of what family it is used on. The post-hoc pairwise tests for each ANOVA revealed a significant difference between the four

drugs with the same pattern emerging from the significant results found in nine of the twelve test levels **Table 2**. The statistics support the visual examination.

Normalized
Two-Way ANOVA

Factor	df	F-statistic	*p*-value
Drug	3	7.691	5.10×10^{-5}
Family	9	0.8868	0.5370
Drug x Family	27	0.8561	0.6763

Permutation
Two-Way ANOVA

Factor	df	F-statistic	*p*-value
Drug	3	7.186	0.0218
Family	9	0.8336	0.456
Drug x Family	27	0.6193	0.6901

Table 1. Two-way ANOVA tests compared azole drug effects on fungal mycelium growth. The analysis revealed significant differences between all drug combinations. A confirmatory two-way permutation ANOVA was done on the original percentage data. Drug selection was the only significant variable.

Drugs ranked by effectiveness	Estimated marginal mean	Median percent of growth	More effective drug
1st Posaconazole	1.948	5.00%	6
2nd Voriconazole	0.006954	55.91%	2
3rd Itraconazole	-0.1645	73.21%	1
4th Fluconazole	-0.2040	100.00%	0

Table 2. Effectiveness comparison and ordinal ranking. Estimated marginal means represents the normalized data (higher numbers means higher effectiveness), the simple median is the percentage of growth compared to the control and represents the permutated data (lower percent means more effective). The last column gives the pairwise results from both ANOVA post-hoc tests where the tallies represent a test that resulted in that drug being significantly more effective than the other (9 of 12 were significant).

Family (Order)	n	FLZ (n)	ITZ (n)	VOR (n)	FLZ (%)	ITZ (%)	VOR (%)
Pleosporaceae (Pleosporales)	47	16	5	6	34.0	10.6	12.8
Didymellaceae (Pleosporales)	10	2	0	0	20.0	0	0
Aspergillaceae (Eurotiales)	28-29	11	5	3	39.3	17.2	10.7
Cladosporiaceae (Cladosporiales)	21	7	2	2	33.3	9.5	9.5
Nectriaceae (Hypocreales)	5	1	1	1	20	20	20
Saccotheciaceae (Dothideales)	5	1	0	1	20	0	20

Table 3. Number and percentage of isolated members of six fungal families that showed increased growth (>100%) in the presence of fluconazole (FLZ), itraconazole (ITZ), and voriconazole (VOR) when compared to the control plate (included in this table are only fungal families with at least 5 isolates).

In the presence of fluconazole and voriconazole, a large number of fungal isolates produced larger colonies when compared to growth on control plates. This was especially evident in the presence of fluconazole, which enhanced the growth of a large number of isolates of the Pleosporaceae (34.0%), Cladosporiaceae (33.3%), and Aspergillaceae (39.3%) **Table 3**. Some of those fungal isolates that benefited from the addition of fluconazole in the SabDex medium also showed excessive growth when exposed to voriconazole and itraconazole. This increased growth was not observed in the presence of posaconazole. Fungal isolates that belonged to the families Stachybotryaceae (n=2), Mycosphaerellaceae (n=1), and Microdochiaceae (n=1) did not show any increased growth in the presence of any azole drug. The fungal family Sclerotiniaceae (n=3) did include some isolates that grew larger than the control on its fluconazole plate.

DISCUSSION AND CONCLUSION

Results of this study confirm that the majority of environmental fungi isolated from airborne spores in Bakersfield, Kern County, which is known for its large-scale industrial agriculture of various crops, have built some resistance to azole drugs that are commonly used to treat diseases like coccidioidomycosis (Valley fever) and aspergillosis. Clinically, this concentration is higher than typical therapeutic targets for most azoles. Itraconazole and voriconazole trough levels generally aim for 1–2 μg/mL, posaconazole for 0.5–1.5 μg/mL, and fluconazole for 2–4 μg/mL, making 4.5 μg/mL slightly above standard dosing but still within a tolerable range.[46] Different azole drugs have different Minimum Inhibitory Concentrations (MIC) on fungal isolates. We used concentrations of azole drugs in this study that were slightly higher than MICs documented in the literature to inhibit opportunistic fungal pathogens.[47] In vitro inhibition shows how fungi respond to antifungal drugs under controlled conditions, but it doesn't always predict how effective the drugs will be in the body, where factors like drug distribution, metabolism, and the host immune system plays a role. However, these studies are meaningful for initial investigations before being tested on a living organism which is essential for determining clinical effectiveness. Our results revealed that fluconazole, as one of the early azole drugs, was least effective against fungal isolates from all families included in this study. Results from challenge assays with other early azoles also showed a limited efficiency against members of most fungal families in contrast to posaconazole, which is a newer azole drug. Posaconazole appeared effective against most fungi which aligns with the concept that environmental fungi had less time to develop resistance mechanisms. Interestingly, we observed that strains belonging to the same fungal family and even within species showed great differences in their ability to grow in the presence of different azole drugs included in this study. We did not have enough fungal isolates to investigate if this observation was statistically significant. However, these findings indicate that various azole resistance mechanisms might be used by even closely related fungal species. As discussed, statistical results indicate that the effectiveness of azole drugs varied significantly in inhibiting fungal growth ($p < 0.0001$), with posaconazole providing the strongest inhibition of fungal growth across all families. No significant differences were observed based on fungal family or its interaction with drug type ($p > 0.05$). Post-hoc tests showed that posaconazole was the most effective drug, followed by voriconazole, itraconazole, and fluconazole being the least effective. In some families, fluconazole enhanced fungal growth, while posaconazole provided the strongest resistance across all families, indicating its broad-spectrum antifungal activity. Additionally, insufficient DNA extraction from fungal mycelium, contamination, or the fungal isolate's inability to be maintained in pure culture on SabDex medium prevented the identification of 18% of fungal samples.

Some mechanisms of fungal resistance to azoles are known, but not all have been studied extensively. One of the most prevalent mechanisms of azole resistance involves alteration or overexpression of the drug target gene, lanosterol 14α-demethylase ERG11/cyp51A/cyp51B, which has been studied in *Candida* and *Aspergillus* resistant isolates showing amino acid substitutions in regions close to the heme-binding site of the enzyme compared to non-resistant isolates.[26,45,48] Other mechanisms of azole resistance in filamentous fungi, especially *Aspergillus* spp., include efflux pumps, mechanisms of azole tolerance, upregulation of ergosterol biosynthesis with specific regulatory proteins, alternative ergosterol synthesis mechanisms, and likely yet unknown mechanisms.[49-54] Knowledge of resistance mechanisms among environmental fungi is limited, and no studies have been conducted on *Coccidioides*. The mechanisms of resistance among fungal isolates in this study were not investigated but will be included in future work.

The increase in azole drug resistance among opportunistic fungal pathogens in industrial agricultural areas and the impact on human health is of significant concern and is increasingly addressed in the literature and by healthcare providers.[5,18,55,56] The first publications on azole resistant *Candida albicans* strains emerged in the 1980's.[57-59] In the late 1990's, concerns were raised about *A. fumigatus,* one of the most ubiquitous of the airborne saprophytic but also opportunistic fungi, showing resistance to treatment with azole drugs.[60-63] At about the same time, observations from azole resistant *Coccidioides* strains, and other fungal species that are particularly dangerous to immune compromised hosts, such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitis*, among others, were reported.[64,65] Fungal resistance to azole fungicides were noticed among farmers even earlier[66] which resulted in ever-increasing amounts of fungicides being sprayed in traditional agriculture resulting in mounting environmental pressure to select

for azole resistant fungal species in the soil.[18,22,67] Kern County, located in the Central Valley of California, is no exception. Indeed, it is known as a hot spot for an emerging disease which has reached epidemic proportions: Valley fever.[68,69]

It is surprising, with mounting evidence in the literature, that fluconazole is still the most used antifungal agent to treat Valley fever and other fungal diseases in humans and animals. Several other azole drugs with increased efficiency were developed since the approval of fluconazole by the FDA and are being used to treat mycoses, but physicians agree that side effects are often more severe compared to fluconazole. Among them, itraconazole, which shows higher efficacy in treating skeletal diseases but has absorption issues and risks for heart failure patients. Voriconazole, another azole drug, is effective, even in meningitis cases, but its use is limited by toxicity, especially hepatoxicity and neurotoxicity, whereas posaconazole is used for refractory cases, with improved absorption and strong efficacy except in the CSF and with side effects mostly in the gastrointestinal system.[70,71] Increasing the dosage of fluconazole in long-term treatments, especially doses over 400-800mg/day often results in negative effects on the endocrinological and neurological system, and although not hepatotoxic, can damage the liver, as confirmed by elevated liver enzymes.[72] One of the newer approved broad-spectrum triazole antifungal agent isavuconazole that was approved in 2015, is being primarily used to treat invasive fungal infections.[73,74] Isavuconazole was approved in 2015 but was not included in this study. It has a broad distribution, but clinical data for coccidioidomycosis remains limited.[74] Regarding the effectiveness of inhibiting fungal growth, we observed that more recently approved FDA azole drugs exhibit greater inhibitory activity against fungal mycelium compared to those that were approved earlier. Summarily, the older fluconazole was the least effective compared to the newer posaconazole, which was able to inhibit the growth of most fungal isolates to some degree.

Furthermore, the observed enhanced mycelium growth of some isolates in the presence of fluconazole was interesting. This observation could be explained by development of resistance mechanisms, such as alterations in drug-target binding, efflux pump activity or metabolic changes that allow the fungus to tolerate or even thrive in the presence of a particular antifungal agent.[75] Posaconazole, however, is a more effective antifungal agent compared to the other drugs included in this study, which might be due to its broader spectrum effectivity, stronger binding affinity to fungal ergosterol, and its ability to inhibit the synthesis of key components in the fungal cell membrane.[76] Generally, *Coccidioides* isolates are not tested for azole resistance when treatment starts, which may allow a resistant fungal strain to become established in the host, maybe even disseminate, before treatment is adjusted. Healthcare providers are becoming aware of this problem,[77] but no newer drugs or vaccines are available at this time.[34]

The observation of fungal resistance among fungal isolates in our study can be explained by agricultural practices using antifungal treatments that are not selective against fungal pathogens but work against most soil fungi. Fungi can build resistance against these agents in diluted form, which has implications for public health because soilborne fungi known to cause disease in humans, such as *Coccidioides,* will be exposed to these fungicides as well and experience selective environmental pressure to become resistant.[10] The California Department of Pesticide Regulation has implemented policies to reduce pesticide exposures[77] and to promote Integrated Pest Management, a method that supports reduced pesticide use through alternative pest management strategies.[78]

As of 2025, four modes of action have been identified among fungal species to become resistant to azole drugs. On the population level, antifungal resistance to fungistatic azoles is based on mutations in certain genes due to environmental exposure and selective pressure on the fungal population in the soil. Known mechanisms of resistance include alterations in the target enzyme, efficient drug efflux, and adaptations to the ergosterol biosynthesis pathway.[25,48,79,80] Consequently, efforts are ongoing to identify and develop new drug candidates that can be used to fight fungal infections.[81]

It is reasonable to assume that *Coccidioides* and other soil borne opportunistic pathogens to humans are being exposed to fungicides used for crop treatment and seed preservation on a regular basis and thus have acquired increased resistance to these treatments which we will likely observe even more in the future. As of 2025, no vaccine to protect from Valley fever or Aspergillosis is available for humans,[82] and once promising alternative candidates to azole drugs, with fewer side effects, such as Nikkomycin Z (nikZ), a chitin synthase inhibitor, are not available yet.[68,83] Choosing a treatment that is effective in inhibiting the fungal pathogen in a patient, so that the immune system is not overwhelmed and can successfully eradicate the pathogen without causing damage to organs and nerves, especially when treatment is extended, has become a challenging dilemma. Consequently, many physicians have decided to increase the dosage of fluconazole instead of switching to using a newer azole drug that may have increased toxicity, following the Sanford Guide[84] or Johns Hopkins Guide for Antimicrobial Therapy.[85]

LIMITATIONS

This study focused on *in vitro* azole susceptibility of environmental fungi without determining minimum inhibitory concentrations (MICs) or analyzing genomic resistance mechanisms. 18% of isolates were not identified, due to insufficient DNA extraction

from fungal mycelium, contamination, or the inability to maintain isolates in pure culture on SabDex medium. Additionally, assays were performed without replicates, which may affect reproducibility. Despite these limitations, the findings provide meaningful insights into environmental azole resistance and establish a foundation for future studies to determine MICs for different species and strains and investigate underlying resistance mechanisms to better inform antifungal strategies. Furthermore, there might be fundamental differences between inhibition *in vitro* and *in vivo*, because the environment differs. However, *in vitro* studies as performed in this study are meaningful for initial investigations before being tested on a living organism—which is essential for determining clinical effectiveness.

FUTURE WORK

For future studies of azole resistance among fungal isolates that are of concern for human health, we consider using liquid cultures supplemented with fungal spores and azole drugs, followed by measuring the inhibition of growth with a spectrophotometer,[86-88] in addition to the plate assay, as used in this study. This will also allow us to determine the Minimum Inhibitory Concentration (MIC) of each drug that is able to significantly inhibit a fungal isolate. Investigations on natural resistance towards azole drugs or fungicides among isolates from California's Central Valley is challenging due to the region's long history of azole use. Comparison of isolates from non-agricultural areas with those collected in this study to better understand the role of natural resistance could be pursued. Furthermore, we are planning to collaborate with another institution to evaluate the susceptibility of azole antifungal drugs against *Coccidioides* in a BSL-3 laboratory setting. Of interest would also be investigating the modes of action our fungal isolates used to resist exposure to different azole drugs using primers for genes that code for proteins involved in these resistance mechanisms. Results from these studies will also be shared with Kern County Public Health and the Valley Fever Institute in Bakersfield, CA.

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ABOUT THE STUDENTS

Ahlam Alamamy is an undergraduate at California State University and majoring in Biology with a Bio-technology concentration. She has a strong passion for science and a deep curiosity for how the world works, especially through the lens of biology and medicine. She is determined to pursue a career in medicine and is eager to engage in further research along the way as she continues her studies

Benjamin Whipkey is a senior Microbiology student at California State University of Bakersfield. After graduating in 2026, he plans to pursue a master's degree there as well. After college he plans to teach and conduct research in various subfields of biology. As a long-time naturalist and animal rescuer, his life goal is to create a sanctuary for differently-abled cats and small animals.

Victoria Saez is a recent graduate from the Research Laboratory Technology Program at Bakersfield College. She is passionate about science and has a great interest in pursuing scientific research in the future, with a desire to contribute to medical advancements.

PRESS SUMMARY

Coccidioidomycosis (Valley fever) is a fungal disease endemic to the southwestern United States, particularly California's Central Valley. It is often misdiagnosed, delaying antifungal treatment and increasing the risk of severe illness. Fluconazole, the most widely used antifungal for Valley fever, is losing effectiveness. In this study, we isolated and identified 151 airborne fungi from Bakersfield, CA, an agricultural region with high Valley fever incidence. Many isolates showed strong resistance to commonly

prescribed azole drugs, especially fluconazole. Alarmingly, some fungi grew better in the presence of the drug. Posaconazole, a newer antifungal, significantly inhibited growth across most fungal families tested. These findings suggest that environmental exposure to azole fungicides in agriculture may be driving resistance among soil-dwelling fungi, including potential pathogens. This study emphasizes the urgent need to reassess treatment protocols and monitor antifungal resistance in areas where Valley fever is endemic and agricultural fungicide use is high.