

Agricultural Fungicides Shape Soil and Sediment Reservoirs of Multidrug-Resistant Fungi

Corresponding Author: Professor Qingxiang Yang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

What are the noteworthy results? These studies present data suggesting that agricultural fungicide use has increased the proportion of fungicide-resistant fungi in the environment.

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references. The work appears to be original, and would be of significance were the authors to address the many concerns that I have with the manuscript (see below). These concerns are partly simple writing, but also will require additional experimentation and validation.

Does the work support the conclusions and claims, or is additional evidence needed? Additional evidence is needed.

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

Yes, there are flaws (see below).

Is the methodology sound? Does the work meet the expected standards in your field? The methodology has problems both with the description of the methods, and the experiments that were performed, chiefly due to the lack of validation experiments that would support or refute the bioinformatics-based conclusions.

Is there enough detail provided in the methods for the work to be reproduced? No.

Additional comments for the authors:

Line 14: Throughout the manuscript, numerous errors in English language usage and grammar make the manuscript difficult to read. The authors should consider consulting with an advisor who has greater English language skills to clean up the text.

Line 126: What does QuEChERS represent?

Line 130: What was the eluate used for this gradient? What were the gradient concentrations (start and finish)?

Line 133: Please define 'ecological risk'.

Line 144: In actuality, you haven't examined the microbiome, but only the fungal component of it. Since bacterial can produce a variety of anti-fungal compounds, how do you know that the resistant fungi that you are seeing are resulting from selection for fungicide resistance, as opposed to resistance to bacterial compounds in the soil? Do bacterial antifungal compounds lead to cross-resistance with other fungicides? I think that you should know what the local bacterial microbiome looks like, in order to correlate this with the resistance profiles that you're seeing in the fungal populations.

Line 147: Specifically, was this the FastDNA Spin Kit for Soil from MP Biomedicals? This should be stated in the manuscript. What was the yield and purity of the DNA that was obtained? How was this DNA yield and quality assessed?

Line 150: How were the PCR amplicons purified and quantified? What library prep protocol was used to prepare libraries for the MiSeq? What were the characteristics of the resulting dataset (raw reads, reads that survived clean-up, etc.)?

Line 158: Is there a reference for the UNITE database?

Line 325 and Fig. 1: This figure legend is extremely non-informative; I have the same concern about all of the other figure legends in this manuscript. The lack of information in the figure legend requires the reader to constantly move back-and-forth

between the figure and the text. For instance, the abbreviations of the site names do not have their translated meanings in Fig. 1A, and do not have corresponding information about what these mean in the legend. Fig 1B has two 'B' panels, which is confusing and seems unnecessary, as the information in the first 'B' figure appears also to be contained in the second 'B' figure. The abbreviations for each of the tested fungicides also is lacking in the legend, which reduces the ease of interpretation of the figure.

Line 345: In several places, you use the term 'pathogenic' and apparently are referring to both plant and animal (and/or human) pathogens simultaneously. Surely there are differences between these pathogens, based on the host that each is infecting. Are there differences in these that are important for interpreting the text, particularly since you appear to be studying the effect of agricultural usage on fungicide resistance, and so presumably are most interested in plant pathogens.

Line 350 and Fig. 2: As with Fig. 1, this figure legend would benefit from additional text to explain the figure. The Chao1 panel in Fig. 2B seems unnecessary, as the data comparisons appear to be statistically insignificant, judging from the p-value shown. Thus, this Chao1 plot could be deleted from the figure, and this observation merely noted in the text. Also, the difference between these two alpha diversity measurements are not commented upon in the text. Why do you show both of these? This is important for interpreting the diversity measurements. Lastly, I am not entirely sure what Fig. 2C is showing. Is this an aggregate of all data on fungicide-resistant strains? Why are you not showing the data for each sampling site? Are there differences in these sampling sites? Fig. 2C would benefit from a much more detailed description in the figure legend.

Line 396 and Fig. 3: The figure legend for this figure also is extremely non-informative. Why are panels B and C included in this figure, without explanation?

Line 410: All of these binding estimates were done in silico; you did not actually measure binding affinity. Therefore, the changes in binding affinity that you discuss in the text are based on modeling, not on actual measurements. Did you collect any real data that substantiated these in silico predictions?

Line 213: Your conclusion of enhanced efflux activity was predicted from the increased transcriptome levels of these efflux pumps, but not directly measured. What data do you have that directly supports the increased efflux pump activity that you think is occurring?

Line 426: Do you have any data directly demonstrating that an increased flux of secondary metabolites has occurred? This conclusion appears to be an interpretation of the transcriptome data; although this is reasonable, it does not necessarily follow, and your conclusion needs to be substantiated by direct measurements.

Line 542: This comment could suggest that your interpretation of the consequences of the mutations that you have observed may be irrelevant; as noted above, direct measurements of these phenotypic changes would strengthen your conclusions considerably, instead of relying merely on in silico predictions alone.

Line 546: Again, how do you know that these mutations resulted in resistance? This is inferred, rather than directly demonstrated.

Reviewer #2

(Remarks to the Author)

The contribution broadly surveyed isolates from agricultural soils and comprehensively assayed their fungicide resistance, being also able to pinpoint likely cellular/molecular mechanisms that underlay the resistance. The contribution is well-delivered, exciting and timely, particularly in the context of One Health and currently limited options for treatment of human fungal infections. Overall the contribution is well written but suffers from some overstatements in its interpretation/narrative.

The manuscript outlines that fungicide exposures coordinately upregulate virulence genes and mycotoxin biosynthetic clusters and follows with mycotoxin phenotype assays that it claims indicate greater toxin production in the (multi-)drug resistant isolates. This is surprising and equally alarming. However, a closer look at the data indicates that the greater toxin production seems relatively rare, often only one in three of the analyzed isolates. The authors may wish to soften their narrative to avoid misrepresentation of their data. This narrative and subsequent conclusions should be rephrased and also accurately represented in the abstract.

There is also a problem with so broadly relying on ITS for species identification. The narrative that focuses on exclusively ITS-based identification of isolates in diverse genera (*Aspergillus*, *Fusarium*) represent overstatements similar to the toxin production. This limitation should be acknowledged and the binomials softened with "cf" for Latin "confer" referring to "compare with." For example, use *Aspergillus* cf. *fumigatus*.

It is odd that the results include no mention of the MiSeq metabarcoding data. Why are these included in the materials and methods?

MINOR COMMENTS:

Line 14: "Multi-fungicide"

Line 16: define MDR; "were resistant"

Line 18: omit "were"?

Graphical abstract: "enhanced MDR fungus"

Graphical abstract: 204 of 349 were multidrug resistant?

Line 29: These are "putative" phytopathogens or "genera that include many phytopathogens" consider this also for human pathogens.

Line 37: I argue that this is expected. Alas, this research "confirms" ag landscapes as reservoirs.

Lines 117-8: This may be inaccurate. Were the samples really at -20°C before and during transport?

Line 144: fungal or microbial community? Fungal community may be more accurate.

Line 147: identify kit.

Line 158: identify UNITE version and provide reference.

Line 193: medium is PDA with 1/2 dextrose, correct? Please state so.

Lines 199-201: identification may be an overstatement as ITS is poor marker for many fungi, e.g., *Fusaria*. Rephrase to "taxon assignment"

Lines 208-9: please describe how the spore suspensions were obtained, state the volume of suspension used. Also, were there no non-sporulating cultures among the MDR fungi?

Lines 221-4: this puzzle me - why not use a cell spreader?

Lines 237-9: Were these isolates acquired in this study or a mix that included references that were listed above?

Line 256: simply "reads" or "sequences"

Line 260-1: which reference? Conspecific? The analyses include three genera.

Line 316: Is the ASB concentration truly zero or below detection level.

Lines 330-1: Are these means across the soils and sediments of only in soils?

Line 333: "that of"

Lines 340-1: "were the communities" something is missing here.

Lines 344-5: Avoid over-generalization: these are genera with pathogens.

Fig. 2C: 145 genera. Also specify that these are the cultures and not your eDNA communities.

Fig. 2A: is this prevalence across samples or isolates? Please clarify.

Line 371: isolates within the clinically significant genus

Line 372: avoid overstatements. ITS-based ids may not be accurate for large genera like *Aspergillus*.

Line 384: "pathogens"

Line 393: "and" should not be in italics.

Line 407: the qPCR is confirmatory - use "confirmed"

Line 433: "wild-type" seems incorrect here. Consider non-MDR strain vs. MDR strain.

Lines 449-451: In Fig. 7 the greater toxin production seems relatively rare often only one in three of the analyzed isolates.

The authors may wish to soften their narrative to avoid misrepresentation of their data. Note that this also should be rephrased in the abstract.

Line 539: Remove comma ","

Lines 569-572: The greater mycotoxin production was not universal, but relatively infrequent. Please soften the narrative and acknowledge this. Note that the final conclusions later in the paragraph appear akin to cherry-picking a storyline that is not totally accurate.

Reviewer #3

(Remarks to the Author)

The manuscript "Agricultural Fungicide Overuse Shapes Soil and Sediment Reservoirs of Multidrug-Resistant and Virulent Fungi" explores the community composition in soils from China and analyses their fungicide concentrations, linking those to fungal species and resistant isolates found within these soils. These findings would add to the data of identifying environments that act as reservoirs for drug-resistant fungi which can cause infections in humans. This topic is much of interest and has clear implications from environmental microbiology to human health. However, the analyses performed here and conclusions drawn are inconclusive and sometimes plain wrong or impossible. I have listed these below

Major comments:

- The methodology for determining of the fungicides and their concentrations is not shown. It is stated "the method was validated for specificity, sensitivity and accuracy, with detection limits..." No data is shown to support this statement. At least some standard curves are expected, and limit of detections need to be calculated. There are standard methods of performing these and these need to be done, for example in *The Fitness for Purpose of Analytical Methods* (2025)
- ITS1 sequencing can't describe most of these fungi to their species level and only to their genus or section level. Therefore, figure 3 is inaccurate and should not be included at all. For example, in *Aspergillus* species this is shown in <https://doi.org/10.3114/sim.2007.59.05>
- Calling the fungi that grow on these concentrations resistant is not correct. Growth on one concentration that has been taken from the field application levels are not transposable to growth under laboratory media. A susceptible control and resistant ones should be included to determine levels per species for these drugs.
- The mycotoxin levels in these fungi are not somewhat surprising. Especially finding Aflatoxins and Ochratoxins in species that do not produce these compounds. Yet, they are somehow detected at different levels, while they should all be 0. There seems to be an issue here with the assay and this data is not convincing.

Minor comments:

- L45-46 A nice commentary to reference here is Van Rhijn et al 2025 (Lancet)
- L57-59 MDR has not spread, MDR species have become more common
- L61-63 They don't mirror these in environmental fungi, more MDR species are found which is the same but they have different species they act upon.

- L64 Agricultural fungicide... this trend. This is still a hypothesis and not proven as of yet.
- L76 Ref 15 does not show this.
- L263 here it says log2FC 1 and results say 2. 2 should be used and please check if 1 is an error.
- L286 Homology models, homology models using one mutation is not really good practice, please mention these limitations.
- L312-313 so not all samples were measured. Why is that and are only samples that were all measured included?
- Fig 2C. This is not really clear and an unusual way of showing mycobiome data. Please show all data with stacked bar chart divided per sample per location
- L363 to be triazole resistant, EUCAST or CLSI MICs should be done if that's claimed.
- Fig 3A Why the half circle, this can just be done by a normal stacked bar chart.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

I am reading the revision of the manuscript I reviewed earlier. The contribution has been substantially modified, now includes additional experiments and has gone through a language editing. Although the presentation has been revised, these are still a few issues that need attention - please see the minor comments below.

The included multi-locus analyses substantially strengthen the taxon inferences, even if these only include a limited number of MDR strains. Further, I appreciate the care that the authors have taken to soften their verbiage to avoid overstatements. I have pointed out one where they might address this issue further.

One reviewer presents an argument that the antifungal resistance can emerge as a result of bacterial antifungals that they produce. I agree with the authors that bacterial antifungals are most likely spatially very heterogeneously distributed, on average in low concentrations and therefore unlikely to account for the multidrug resistance.

MINOR COMMENTS

Line 18: define MDR in highlights. Also, focus on results/conclusions rather than methods. What to the transcriptome data tell?

Graphical abstract: Correct text - "Genes for virulence and toxin production"

Lines 26-27: Redundant. Rephrase "Multiple fungicides..."

Lines 31-32. Remove cf., it is only used if followed by a specific epithet.

Lines 33-34: but not clinical fungicides?

Line 55: no need to redefine IFI

Line 55: candidiasis and aspergillosis need not be in italics

Line 56: Does candidiasis have a mortality rate >40%? This may be true for immunocompromised individuals, but maybe not across all candida infections. Please confirm.

Lines 66-67: "agroecosystems"

Lines 120-121: "ensure spatially representative sampling"

Lines 136-137: The Limit of Detection (LOD) and Limit of Quantification (LOQ)...

Line 179: what chemistry, 2x250, 2x300 cycles?

Lines 229-230: please provide reference for the primers.

Line 233: "six additional"

Lines 233-234: is this the complete manufacturer info?

Line 468: culture-derived

Line 508: You included also LSU marker, this is not protein coding locus.

Line 676: omit "total"

Line 695: Overstatement. It remains unconfirmed if these strains are indeed associated with invasive infections. They do represent genera that do so.

Line 777: shorten "agroecosystems"

Reviewer #3

(Remarks to the Author)

The revised manuscript "Agricultural Fungicide Overuse Shapes Soil and Sediment Reservoirs of Multidrug-Resistant and Virulent Fungi" has been much improved and now shows a more consistent story that is in line with the data. However, several key points remain that need to be addressed, although this can be done textually and no more experimentation is required.

Major comment:

Throughout the manuscript, human fungal pathogens and evolution of resistance has been mentioned. However, this data does not support any virulence data or potential for evolution of resistance. Many fungal genera are intrinsically resistant, so please make sure this is shown throughout the manuscript. Also, many of the genera contain fungal species that are pathogens, but the species are not present/shown. Only the case for *A. fumigatus*

L1-2 Title: "Fungicide Overuse" is not really shown. Also "Virulent Fungi" are not directly shown in this article. A more appropriate title that is true to the data would be: Agricultural Fungicides Shape Soil and Sediment Reservoirs of Multidrug-Resistant Fungi

L335-339 Please give the workflow, including packages, versions and parameters.

L341 Same here, what was the reference-free workflow

L347 I can't seem to find the primers used for qPCR validation, please include a supplemental table.

L401 "Reliability" reliability is not really tested, please remove.

L403 "81 samples" were more samples tested that came back with no fungicides at all? The text seems to imply, which would be very interesting to include here.

L440-442 It does not show selection for resistance. It could merely be that these fungi are intrinsically resistant. Please remove this sentence.

L503-506 Just using genera to compare is not really good practice. Most of these fungal genera are huge and only several species are pathogens. Please remove.

L520-522 Related to human clinical pathogens, but these species are not. Remove sentence and say that Twenty environmental fungal isolates were selected.

L570 What I'm missing here is that cyp51a was sequenced? I assume? And these mutations were found? If not, then this result should be removed.

L594 resistance evolution. No data on evolution is shown. Most of these fungal species are intrinsically resistant to many of these fungicides.

L632-633 nothing is shown about virulence. Therefore, remove.

L651 chronic fungicide application. This is not shown, only soil residues. Please amend

L667 same here and throughout the discussion.

L721-723 These have been linked to resistance, so please amend. This has been done in <https://journals.asm.org/doi/10.1128/aem.02539-20>

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Dear Editors and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "**Agricultural Fungicide Overuse Shapes Soil and Sediment Reservoirs of Multidrug-Resistant and Virulent Fungi**" (ID: NCOMMS-25-81428). Those comments are highly valuable and very helpful for our further revising and future study. We have studied the comments carefully and have made a major revision accordingly. The revised portions are marked in red fonts in the paper.

According to the reviewers' suggestions, we have made major revisions mainly in the following 5 aspects:

(1) In the result and Method parts: We supplemented 3 new functional experiments: MICs of agricultural fungicides and clinical antifungals (supplemented in Fig. 3C, 3D), Flow cytometer testing results to indicate the efflux activities of MDR strains (supplemented in Fig. 4D), and further taxon identification on the representative MDR isolates through multilocus phylogenetic analysis (supplemented Data in Fig. S2).

(2) In the Materials and Methods section: More details were provided to make the description clearer and improve its reproducibility.

(3) The previous Fig. 1B, Fig. 2C and Fig. 3A were removed. Supplemented Fig. 2 C and Fig. 2D, Fig. 3C and Fig. 3D, Fig. 4D, Fig. S2, Table S1, Table S3, Table S8, Table S9 in the revised version. All the figures were redesigned and their legends rewritten to be fully self-contained and publication-ready.

(4) We revised some conclusions to avoid overstatement and unsupported extrapolation.

(5) After revision, the manuscript was sent to a professional English-language editing service (EditorBar) again to edit and polish the English.

More detail corrections and the point to point responds to the reviewer's comments are as the flowing:

Response to reviewers' comments

Reviewer #1 (Remarks to the Author):

Question: Line 14: Throughout the manuscript, numerous errors in English language

usage and grammar make the manuscript difficult to read. The authors should consider consulting with an advisor who has greater English language skills to clean up the text.

Answer: Thanks a lot. The revised manuscript has been thoroughly edited and polished by a professional English-language editing service (EditorBar) to improve the language. We hope the English could be up to the standard.

Question: Line 126: What does QuEChERS represent?

Answer: Sorry for this. The acronym “QuEChERS” stands for “Quick, Easy, Cheap, Effective, Rugged, and Safe.” We have now supplemented its full name at its first appearance in the text.

Question: Line 130: What was the eluate used for this gradient? What were the gradient concentrations (start and finish)?

Answer: We are so sorry for this mistake. In this study, an isocratic elution was used throughout the chromatographic run. The mobile phase consisted of 60% acetonitrile and 40% water at 0.8 mL/min. We have corrected the description as indicated in red fonts.

Question: Line 133: Please define 'ecological risk'.

Answer: Thank you for kind suggestion. Ecological risk refers to a likelihood of a fungicide residue affecting the indicator organisms (such as earthworm, algae, et al.) in the ecological system. Here, the ecological risk was represented by RQ_i . $RQ_i = MEC / PNEC$. (MEC, the measured residues at each sampling site were designated as the measured environmental concentration. PNEC, the Predicted No-Effect Concentration (PNEC) was derived from the No-Observed-Effect Concentration (NOEC) for these species obtained from the PPDB database). The definition of ecological risk was supplemented in the text as indicated in red fonts.

Question: Line 144: In actuality, you haven't examined the microbiome, but only the fungal component of it. Since bacterial can produce a variety of anti-fungal compounds, how do you know that the resistant fungi that you are seeing are resulting from selection for fungicide resistance, as opposed to resistance to bacterial compounds in the soil? Do bacterial antifungal compounds lead to cross-resistance with other fungicides? I think that you should know what the local bacterial microbiome looks like, in order to

correlate this with the resistance profiles that you're seeing in the fungal populations.

Answer: You are very right. There are variety of bacteria in soil and some of them could produce antifungal metabolites. However, in this study, we think that the resistant fungi and their cross-resistance were mainly induced by the fungicide residues or fungicide application process for the following reasons.

- (1) Compared to fungicide residues, the concentrations of various antifungal compounds produced by indigenous bacteria should be too low to induce fungi resistance.
- (2) Although we did not perform 16S rRNA bacterial profiling in this project, some evidences indicate that the observed MDR patterns cannot be explained by bacterial-derived antifungal compounds. Bacterial-produced antifungal compounds (e.g., phenazines, lipopeptides, volatiles) are structurally unrelated to azoles, benzimidazoles, and methoxyacrylates. Yet our isolates display: resistance to five agricultural fungicides, cross-resistance to clinical triazoles (VO, FLU), and multidrug efflux signatures typical of fungicide-driven resistance, not bacterial metabolite resistance. Bacterial-derived antifungal compounds generally do not induce sterol pathway upregulation, Cyp51 mutations, or clinically relevant azole resistance.
- (3) According to available literature, bacterial antifungal compounds rarely induce broad-spectrum MDR; instead, they act on membranes or signaling pathways and typically do not drive Cyp51 mutations or efflux super-induction. Cross-resistance between bacterial metabolites and azoles has not been reported in *Aspergillus*, *Fusarium*, or *Talaromyces*. Conversely, fungicide-induced cross-resistance to clinical antifungals is well documented, which matches the patterns we observed.

We have supplemented the above the points and your considerations in the discussion part as indicated in red fonts.

Question: Line 147: Specifically, was this the FastDNA Spin Kit for Soil from MP Biomedicals? This should be stated in the manuscript. What was the yield and purity of the DNA that was obtained? How was this DNA yield and quality assessed?

Answer: Thank you for your kind suggestions. Yes, the DNA was extracted using the

FastDNA™ Spin Kit for Soil (MP Biomedicals). We have also added information on DNA yield and purity. Across samples, the DNA yield ranged from approximately 100–200 ng/μL, with A260/280 ratios between 1.8 and 2.0, indicating high purity suitable for downstream PCR amplification. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) for accurate quantification and purity assessment. These details have been incorporated into the revised Methods section to ensure transparency and reproducibility.

Question: Line 150: How were the PCR amplicons purified and quantified? What library prep protocol was used to prepare libraries for the MiSeq? What were the characteristics of the resulting dataset (raw reads, reads that survived clean-up, etc.)?

Answer: Thanks for your kind suggestions. We have supplemented the corresponding details in the Methods section as indicated in red fonts. The ITS1 amplicons were purified using AMPure XP magnetic beads (Beckman Coulter, USA) and quantified with a NanoDrop 2000 fluorometer (Thermo Scientific, USA). Sequencing libraries were prepared using the Illumina TruSeq DNA PCR-Free Library Prep workflow, following the manufacturer's protocol, including end-repair, A-tailing, adapter ligation, and size selection. We have also added a description of the dataset characteristics: For each sample, 66,336–151,926 raw paired-end reads were obtained, of which an average of 90.79% passed quality filtering and denoising in QIIME2, yielding 2884 high-quality amplicon sequence variants (ASVs).

Question: Line 158: Is there a reference for the UNITE database?

Answer: Thank you for the suggestion. A citation for the UNITE database has now been added.

Abarenkov K, Nilsson R H, Larsson K H, et al. The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: sequences, taxa and classifications reconsidered[J]. *Nucleic Acids Research*, 2024, 52(D1): D791–D797.

Question: Line 325 and Fig. 1: This figure legend is extremely non-informative; I have the same concern about all of the other figure legends in this manuscript. The lack of information in the figure legend requires the reader to constantly move back-and-forth

between the figure and the text. For instance, the abbreviations of the site names do not have their translated meanings in Fig. 1A, and do not have corresponding information about what these mean in the legend. Fig 1B has two 'B' panels, which is confusing and seems unnecessary, as the information in the first 'B' figure appears also to be contained in the second 'B' figure. The abbreviations for each of the tested fungicides also is lacking in the legend, which reduces the ease of interpretation of the figure.

Answer: We thank the reviewer for this constructive comment. In the revised manuscript, all figure legends have been thoroughly rewritten to provide comprehensive and self-contained information, including definitions of site abbreviations and fungicide abbreviations. In addition, the redundant second “B” panel in Fig. 1 has been removed to avoid confusion.

Question: Line 345: In several places, you use the term 'pathogenic' and apparently are referring to both plant and animal (and/or human) pathogens simultaneously. Surely there are differences between these pathogens, based on the host that each is infecting. Are there differences in these that are important for interpreting the text, particularly since you appear to be studying the effect of agricultural usage on fungicide resistance, and so presumably are most interested in plant pathogens.

Answer: We highly appreciate your insightful comment. We have revised the manuscript to specify the pathogenic host category as “plant-pathogenic fungi” and “opportunistic human pathogens”.

Question: Line 350 and Fig. 2: As with Fig. 1, this figure legend would benefit from additional text to explain the figure. The Chao1 panel in Fig. 2B seems unnecessary, as the data comparisons appear to be statistically insignificant, judging from the p-value shown. Thus, this Chao1 plot could be deleted from the figure, and this observation merely noted in the text. Also, the difference between these two alpha diversity measurements are not commented upon in the text. Why do you show both of these? This is important for interpreting the diversity measurements. Lastly, I am not entirely sure what Fig. 2C is showing. Is this an aggregate of all data on fungicide-resistant strains? Why are you not showing the data for each sampling site? Are there differences in these sampling sites?. Fig. 2C would benefit from a much more detailed description

in the figure legend.

Answer: Thanks for your kind suggestions. The figure legend of Fig. 2 has been substantially revised to provide a clearer and more detailed explanation of each panel. The Chao1 plot in Fig. 2B has been removed. Regarding Fig. 2C, the panel has been completely redesigned. We now present (i) genus-level community composition of total fungal communities and (ii) genus-level composition of cultivated fungicide-resistant fungi as stacked bar charts. The revised figure and its legend now have been clearly described as what is being shown and how the data were generated.

Question: Line 396 and Fig. 3: The figure legend for this figure also is extremely non-informative. Why are panels B and C included in this figure, without explanation?

Answer: We appreciate the reviewer's suggestion. Considering other two reviewers' suggestions, we removed the previous Fig.3A and supplemented Fig. 3C Fig. 3D and to indicate the MIC values of MDR isolates. In addition, the figure legends have been described in details to clearly indicate the antifungal susceptibility profiles of MDR isolates to both agricultural fungicides and clinical antifungal agents.

Question: Line 410: All of these binding estimates were done in silico; you did not actually measure binding affinity. Therefore, the changes in binding affinity that you discuss in the text are based on modeling, not on actual measurements. Did you collect any real data did substantiate these in silico predictions?

Answer: We highly appreciate your concern and agree that the binding estimates we report are based on in silico molecular docking rather than direct biochemical measurements. To address this clearly, we have revised the text to specify that these values represent predicted interaction strengths, not experimentally determined binding affinities. Although we did not perform assays such as recombinant Cyp51 inhibition, ergosterol quantification, or ITC-based binding analysis, our computational predictions are strongly supported by multiple independent experimental datasets. Specifically, resistant isolates exhibited clear phenotypic resistance to multiple fungicide classes, possessed amino acid substitutions within Cyp51 that map to functionally relevant regions of the enzyme, and showed robust transcriptomic activation of ergosterol biosynthesis and multidrug efflux pathways—biological responses that are well-

established consequences of reduced drug–target interaction. Thus, while the docking results themselves are predictive, they are consistent with and reinforced by the observed molecular and phenotypic signatures of azole resistance. We now explicitly acknowledge this distinction in the manuscript and highlight that future biochemical assays will be valuable for validating these structural predictions.

Question: Line 213: Your conclusion of enhanced efflux activity was predicted from the increased transcriptome levels of these efflux pumps, but not directly measured. What data do you have that directly supports the increased efflux pump activity that you think is occurring?

Answer: Thank you for your thoughtful comment. In response, we have performed additional experiments using a flow cytometry–based rhodamine 6G efflux assay to directly measure drug efflux activity. The results show that fluorescence intensity decreased significantly in resistant strains following antifungal exposure, whereas quality control strains exhibited higher intracellular fluorescence, indicating limited efflux. These data provide direct experimental support for enhanced efflux pump activity and are now included in Fig. 4D.

Question: Line 426: Do you have any data directly demonstrating that an increased flux of secondary metabolites has occurred? This conclusion appears to be an interpretation of the transcriptome data; although this is reasonable, it does not necessarily follow, and your conclusion needs to be substantiated by direct measurements.

Answer: Thank you for your important comment. It is true that our inference of increased secondary metabolite flux was based primarily only on the evidences at transcript level, not direct metabolites measurements. Although the secondary metabolite biosynthetic gene clusters were generally up-regulated under the exposure of fungicide, the present results only implied a potential trend. Further direct measurements were highly required. Considering the assay methods for different secondary metabolites were not available in our lab at the moment, we revised the conclusion and corresponding discussion parts to make the conclusion more reasonable.

Question: Line 542: This comment could suggest that your interpretation of the

consequences of the mutations that you have observed may be irrelevant; as noted above, direct measurements of these phenotypic changes would strengthen your conclusions considerably, instead of relying merely on in silico predictions alone. Line 546: Again, how do you know that these mutations resulted in resistance? This is inferred, rather than directly demonstrated.

Answer: We appreciate your comments. In the revised manuscript, we have weakened the description on these substitutions and clarified that their potential effects on azole binding are inferred from molecular docking analyses rather than from direct phenotypic measurements. We also acknowledge that further biochemical and genetic experiments will be required to validate the functional role of these mutations.

Reviewer #2 (Remarks to the Author):

Question: The manuscript outlines that fungicide exposures coordinately upregulate virulence genes and mycotoxin biosynthetic clusters and follows with mycotoxin phenotype assays that it claims indicate greater toxin production in the (multi-)drug resistant isolates. This is surprising and equally alarming. However, a closer look at the data indicates that the greater toxin production seems relatively rare, often only one in three of the analyzed isolates. The authors may wish to soften their narrative to avoid misrepresentation of their data. This narrative and subsequent conclusions should be rephrased and also accurately represented in the abstract.

Answer: We highly appreciate your comments. We have revised the abstract, discussion and conclusion to weakened the address on the toxin production.

Question: There is also a problem with so broadly relying on ITS for species identification. The narrative that focuses on exclusively ITS-based identification of isolates in diverse genera (*Aspergillus*, *Fusarium*) represent overstatements similar to the toxin production. This limitation should be acknowledged and the binomials softened with “cf” for Latin “confer” referring to “compare with.” For example, use *Aspergillus* cf. *fumigatus*.

Answer: Thank you for your kind suggestions. We agree that ITS alone is insufficient for reliable species-level identification for fungi. Therefore, we performed multi-locus

phylogenetic analyses on the 5 representative multidrug-resistant isolates using ITS and additional protein-coding genes. The identification results were supplemented in Fig. S2 and described in the text as indicated in red fonts. For the remaining isolates that were identified based only on ITS sequences, we have revised the manuscript to soften species-level assignments by using “cf.” (confer) to indicate tentative identification.

Question: It is odd that the results include no mention of the MiSeq metabarcode data. Why are these included in the materials and methods?

Answer: Sorry for this confusion. The fungal community analyses presented in Fig. 2—including alpha diversity, genus-level composition, and the distribution of resistant taxa—were all derived directly from the MiSeq ITS metabarcoding dataset described in the Materials and Methods section. In the revised manuscript, we have added the information in the legend of Fig.2 and the corresponding text as indicated in red fonts.

MINOR COMMENTS:

Question: Line 14: “Multi-fungicide”

Answer: Revised.

Question: Line 16: define MDR; “were resistant”

Answer: Revised.

Question: Line 18: omit “were”?

Answer: Revised.

Question: Graphical abstract: “enhanced MDR fungus”

Question: Graphical abstract: 204 of 349 were multidrug resistant?

Question: Line 29: These are “putative” phytopathogens or “genera that include many phytopathogens” consider this also for human pathogens.

Answer: Thank you for your kind suggestion. We have revised the text to avoid overgeneralization. The term “phytopathogens” and “human pathogens” has been replaced with “putative phytopathogens” or “genera that include phytopathogenic or opportunistic human pathogenic species”.

Question: Line 37: I argue that this is expected. Alas, this research “confirms” agricultural landscapes as reservoirs.

Answer: Thank you for your kind suggestion. We agree that agricultural environments

with long-term fungicide exposure are widely recognized as potential reservoirs of resistant fungi, and our findings therefore serve to support and quantify this expectation rather than to present it as a novel discovery. Therefore, we have revised the concluding sentence in the abstract to make it more accurate.

Question: Lines 117-8: This may be inaccurate. Were the samples really at -20°C before and during transport?

Answer: Thank you for this comment. We are sorry for the inaccurate description. In the revised manuscript, we clarify that the soil samples were kept in ice box during transport and were subsequently stored at -20 °C upon arrival at lab. This correction more accurately reflects the actual sample-handling procedures.

Question: Line 144: fungal or microbial community? Fungal community may be more accurate.

Answer: Thank you for your kind suggestion. We have revised “microbial community” into “fungal community”.

Question: Line 147: identify kit.

Answer: Thank you for the comment. The kit is now specified as the FastDNA™ Spin Kit for Soil in the manuscript.

Question: Line 158: identify UNITE version and provide reference.

Answer: Thank you for the comment. The UNITE version and references have been added to the text.

Question: Line 193: medium is PDA with 1/2 dextrose, correct? Please state so.

Answer: Thank you for this helpful comment. In the revised version, we now define this medium explicitly as “½-dextrose PDA” at its first appearance in the Methods section and use the same term consistently throughout the manuscript. The sentence in the fungicide-screening section has therefore been revised to: “Specifically, purified colonies were first inoculated onto ½-dextrose PDA medium containing the three triazole fungicides....”.

Question: Lines 199-201: identification may be an overstatement as ITS is poor marker for many fungi, e.g., fusaria. Rephrase to “taxon assignment”

Answer: Thank you for your kind suggestion. We have replaced the identification as

taxon assignment as indicated in red fonts.

Question: Lines 208-9: please describe how the spore suspensions were obtained, state the volume of suspension used. Also, were there no non-sporulating cultures among the MDR fungi?

Answer: Thank you for this valuable comment. In the revised manuscript, we now describe in detail how the fungal suspensions were prepared and specify the inoculation volume. For sporulating molds, spore suspensions were generated by scraping conidia from 7-day-old cultures, suspending them in sterile physiological saline, and adjusting the concentration to 10^6 spores/mL. For non-sporulating isolates (including yeasts and molds that did not produce conidia), cell suspensions were prepared from fresh colonies in physiological saline and adjusted to an equivalent density of 10^6 cells/mL. We have also specified that 100 μ L of each suspension was used to inoculate each plate.

Question: Lines 221-4: this puzzle me - why not use a cell spreader?

Answer: We are so sorry for this mistake. We actually used a sterile cell spreader to spread the spore suspension. We have revised the corresponding description as indicated in red fonts.

Question: Lines 237-9: Were these isolates acquired in this study or a mix that included references that were listed above?

Answer: Thank you for the question. The 3 isolates used for transcriptome sequencing were all newly isolated in this study from the environmental samples described in the Methods section. We have clarified this point in the revised manuscript to avoid ambiguity.

Question: Line 256: simply “reads” or “sequences”

Answer: Revised. Thank you.

Question: Line 260-1: which reference? Conspecific? The analyses include three genera.

Answer: Thank you for this important comment. In the revised manuscript, we clarify that only the *Aspergillus fumigatus* isolate was analyzed using a conspecific reference genome (*A. fumigatus* A1163; GCA_000150145.1). For the *Fusarium* and *Talaromyces* isolates, no validated reference genomes were available from the databases; therefore,

their transcriptomes were analyzed using a reference-free workflow (de novo transcript reconstruction), and gene expression levels were quantified from the reconstructed transcripts. This distinction has now been added to ensure transparency and reproducibility of the transcriptome analyses.

Question: Line 316: Is the ASB concentration truly zero or below detection level.

Answer: Thank you for pointing this out. The ASB concentration in sediments was not truly zero but below the analytical detection limit. We have revised the corresponding description in the text.

Question: Lines 330-1: Are these means across the soils and sediments or only in soils?

Answer: We are sorry for this confusion. The reported mean values represented separate averages for agricultural soils and for river sediments, not combined averages. To make this clearer, we have rewritten the sentence and listed the soil and sediment values separately to make it clearer.

Question: Line 333: “that of”

Answer: Revised.

Question: Lines 340-1: “were the communities” something is missing here.

Answer: Thank you for pointing out this mistake. Our intention was to indicate that these genera represented the dominant taxa detected in agricultural soils. We have revised the sentence as indicated in red fonts.

Question: Lines 344-5: Avoid over-generalization: these are genera with pathogens.

Answer: Thank you for this helpful comment. In the revised manuscript, we clarify that genera, *Aspergillus*, *Fusarium* and *Talaromyces* included pathogenic members, rather than implying that all members are pathogens. The sentence has been revised accordingly to avoid over-generalization.

Question: Fig. 2C: 145 genera. Also specify that these are the cultures and not your eDNA communities.

Answer: Thank you for your kind suggestion. Considering other reviewers' suggestions, we have reorganized Fig. 2, removed the previous Fig.2C, supplemented new Fig. 2C and Fig. 2D and revised the corresponding legends to make the results more readable.

Question: Fig. 2A: is this prevalence across samples or isolates? Please clarify.

Answer: Thank you for suggestion. The prevalence shown in Fig. 2A was calculated across samples rather than across individual isolates. We have revised the figure legend and the corresponding text to make it clearer.

Question: Line 371: isolates within the clinically significant genus

Answer: Thank you for kind suggestion. We have rewritten the sentence accordingly.

Question: Line 372: avoid overstatements. ITS-based ids may not be accurate for large genera like *Aspergillus*.

Answer: Thank you for this important comment. We agree that ITS alone is insufficient for reliable species-level identification in some genera. Therefore, we performed multi-locus phylogenetic analyses on the 5 representative multidrug-resistant isolates using ITS and additional protein-coding genes. The identification results were supplemented in Fig. S2 and described in the text as indicated in red fonts. For the remaining isolates that were identified based only on ITS sequences, we have revised the manuscript to soften species-level assignments by using “cf.” (confer) to indicate tentative identification.

Question: Line 384: “pathogens”

Answer: revised.

Question: Line 393: “and” should not be in italics.

Answer: revised.

Question: Line 407: the qPCR is confirmatory - use “confirmed”

Answer: revised.

Question: Line 433: “wild-type” seems incorrect here. Consider non-MDR strain vs. MDR strain.

Answer: revised.

Question: Lines 449-451: In Fig. 7 the greater toxin production seems relatively rare often only one in three of the analyzed isolates. The authors may wish to soften their narrative to avoid misrepresentation of their data. Note that this also should be rephrased in the abstract.

Answer: Thank you for your kind suggestion. We have revised the descriptions on toxin production in the whole text including abstract, results, discussion and conclusion to

make it more accurate. It is true that our inference of increased secondary metabolite flux was based primarily only on the evidences at transcript level, not direct metabolites measurements. Although the secondary metabolite biosynthetic gene clusters were generally up-regulated under the exposure of fungicide, the present results only implied a potential trend. Further direct measurements were highly required. Considering the assay methods for different secondary metabolites were not available in our lab at the moment, we revised the conclusion and corresponding discussion parts to make the conclusion more reasonable.

Question: Line 539: Remove comma “,”

Answer: revised.

Question: Lines 569-572: The greater mycotoxin production was not universal, but relatively infrequent. Please soften the narrative and acknowledge this. Note that the final conclusions later in the paragraph appear akin to cherry-picking a storyline that is not totally accurate.

Answer: Thank you for your kind suggestion. We have revised the descriptions on toxin production in the whole text including abstract, results, discussion and conclusion to make it more accurate.

Reviewer #3 (Remarks to the Author):

The manuscript "Agricultural Fungicide Overuse Shapes Soil and Sediment Reservoirs of Multidrug-Resistant and Virulent Fungi" explores the community composition in soils from China and analyses their fungicide concentrations, linking those to fungal species and resistant isolates found within these soils. These findings would add to the data of identifying environments that act as reservoirs for drug-resistant fungi which can cause infections in humans. This topic is much of interest and has clear implications from environmental microbiology to human health. However, the analyses performed here and conclusions drawn are inconclusive and sometimes plain wrong or impossible. I have listed these below

Answer: Thank you very much for your professional comments.

Major comments:

Question: The methodology for determining of the fungicides and their concentrations is not shown. It is stated "the method was validated for specificity, sensitivity and accuracy, with detection limits..." No data is shown to support this statement. At least some standard curves are expected, and limit of detections need to be calculated. There are standard methods of performing these and these need to be done, for example in The Fitness for Purpose of Analytical Methods (2025)

Answer: Thank you very much for your kind suggestions. We have included the standard curves, R^2 , LOD, LOQ, and recovery rates for these five fungicides in the supplementary data (Table S1).

Question: ITS1 sequencing can't describe most of these fungi to their species level and only to their genus or section level. Therefore, figure 3 is inaccurate and should not be included at all. For example, in *Aspergillus* species this is shown in <https://doi.org/10.3114/sim.2007.59.05>

Answer: We are grateful to your professional suggestions. We fully agree that the ITS region alone is insufficient for species-level resolution. For this, we have removed the phylogenetic tree based solely on ITS sequences (previous Figure 3A) to avoid conveying inaccurate species-level relationships. We supplemented identification results of multi-locus phylogenetic analyses for the five representative multidrug-resistant (MDR) isolates. A phylogenetic tree based on ITS and additional protein-coding genes were established to identify the 5 fungal strains (as indicated in supplementary data Fig. S2, Tab. S3). For the remaining isolates that were identified based only on ITS sequences, we have revised the expression to soften species-level assignments by using "cf." (confer) to indicate tentative identification.

Question: Calling the fungi that grow on these concentrations resistant is not correct. Growth on one concentration that has been taken from the field application levels are not transposable to growth under laboratory media. A susceptible control and resistant ones should be included to determine levels per species for these drugs.

Answer: Thank you for your kind suggestions. We agree that growth on a single fungicide concentration derived from field application levels is insufficient to define resistance and cannot be directly extrapolated to laboratory susceptibility. Therefore, in

the updated study, isolates were first screened on fungicide-amended media to obtain putative resistant fungi. Then, we did additional experiments, MIC assay on the putative resistant fungi using standardized broth microdilution following CLSI guidelines. Susceptible quality control strains for the main species were included to validate the assay and to enable species-level interpretation of susceptibility. Accordingly, we have supplemented these data to Fig. 3C and Fig. 3D in the revised version and revised the description in the text accordingly.

Question: The mycotoxin levels in these fungi are not somewhat surprising. Especially finding Aflatoxins and Ochratoxins in species that do not produce these compounds. Yet, they are somehow detected at different levels, while they should all be 0. There seems to be an issue here with the assay and this data is not convincing.

Answer: You are very right. We agree that the detection of aflatoxins and ochratoxins in non-producer species raised concerns about assay specificity in the initial version. We re-considered the initial testing results by using ELLISA method and re-assayed mycotoxin production using a more specific and widely accepted analytical approach based on HPLC. We found that ELLISA method was really not accurate and mycotoxin concentrations in the tested MDR isolates were consistently below the quantitation limits of the HPLC method. We are still optimizing the assaying method to improve the testing sensitivity, and studying which strains might produce toxins. Therefore, we removed this part of results and revised the corresponding description and conclusion to prevent overstatement of toxin production.

Minor comments:

Question: L45-46 A nice commentary to reference here is Van Rhijn et al 2025 (Lancet)

Answer: Thank you for your recommendation. The reference has been updated.

Question: L57-59 MDR has not spread, MDR species have become more common

Answer: Thank you for this helpful comment. We agree that the original expression incorrectly implied that MDR itself had “spread,” whereas current surveillance data indicate that MDR isolates have become more common in certain regions. We have revised the sentence to reflect this distinction.

Question: - L61-63 They don't mirror these in environmental fungi, more MDR species

are found which is the same but they have different species they act upon.

Answer: Thank you for this insightful comment. We agree that resistance patterns in clinical pathogens do not directly “mirror” those in environmental fungi because the species involved and the selective pressures differ. Our intention was to highlight the parallel increase in the frequency of MDR isolates in both settings, rather than to imply identical resistance profiles. We have revised the sentence to clarify that, while the MDR species differ, the increasing prevalence of multidrug-resistant isolates in agricultural environments parallels the rise of MDR pathogens in clinical contexts, suggesting that agricultural ecosystems may contribute to broader antifungal selection pressures.

Question: L64 Agricultural fungicide... this trend. This is still a hypothesis and not proven as of yet.

Answer: Thank you for this valuable comment. We agree that describing agricultural fungicide use as a “critical driver” overstates the current level of evidence. Although multiple studies support the agricultural-origin hypothesis, definitive causal proof is still accumulating. To avoid overinterpretation, we have revised the sentence to adopt more cautious wording, stating that agricultural fungicide use is a potential contributor or potential driver of this trend. This revision more accurately reflects the state of the evidence while preserving the context of current scientific understanding.

Question: L76 Ref 15 does not show this.

Answer: Thank you for pointing out this error. We have removed this reference.

Question: L263 here it says log2FC 1 and results say 2. 2 should be used and please check if 1 is an error.

Answer: Thank you for your kind suggestion. The mention of "1" in the text was an error. We have revised it.

Question: L286 Homology models, homology models using one mutation is not really good practice, please mention these limitations.

Answer: Thank you for kind suggestions. We agree that homology models incorporating individual mutations may not accurately capture the true local conformational changes, and docking results based on such models should be

interpreted with caution. In response, we have revised the Methods, results and discussion sections to explicitly acknowledge this limitation and clarify that the mutant homology models serve only as structural approximations. We also emphasize that the docking results provide preliminary hypotheses regarding altered fungicide affinity and that experimental validation is required to substantiate these predictions.

Question: L312-313 so not all samples were measured. Why is that and are only samples were all were measured included?

Answer: Thank you for raising this point. All 81 soil and sediment samples were measured for all five fungicides. However, some fungicides were under the detecting limit in some samples. Therefore, in the initial version, we use “ASB (41/81 samples)” to express the detection frequency. To avoid confusion, we have revised the text to explicitly state that all samples were analyzed and that the values represent detection frequencies as indicated in red fonts.

Question: Fig 2C. This is not really clear and an unusual way of showing mycobiome data. Please show all data with stacked bar chart divided per sample per location

Answer: We highly appreciate your suggestions. We have removed the initial Fig. 2C and replaced by the updated Fig. 2C and Fig. 2D: Fig. 2C, a stacked bar chart showing genus-level composition of total fungal communities for each sampling site and location, Fig. 2D, a stacked bar chart showing genus-level composition of fungicide-resistant fungal communities enriched by culture under fungicide selection. These revisions provide a clearer and more standard visualization of the community data. Thanks again.

Question: L363 to be triazole resistant, EUCAST or CLSI MICs should be done if that;s claimed.

Answer: Thank you for your kind suggestion. We agree that resistance claims should be supported by standardized susceptibility testing. Therefore, we supplemented MICs tests for the resistance isolates using a standardized broth microdilution method following CLSI guidelines. Quality control strains, including *Talaromyces purpureogenus* (CICC 41734/CGMCC 3.12064), *Meyerozyma guilliermondii* (LWCC 2042/ATCC 6260), *Aspergillus fumigatus* (GDMCC 3.627/ATCC MYA-3627), et al.,

were included to ensure method reliability. The Results were indicated in the updated Fig. 3C and Fig. 3D. The corresponding description and methods were also updated in the text accordingly as indicated in red fonts.

Question: Fig 3A Why the half circle, this can just be done by a normal stacked bar chart.

Answer: We thank you for this suggestion. Panel A of Fig. 3 has been removed in the revised manuscript. This panel originally aimed to show species-level classification within several major genera; however, these assignments were based solely on ITS sequences and therefore lacked sufficient taxonomic resolution for reliable species identification. Based on your suggestion, we have removed this panel and the corresponding text from the Results section. This modification does not affect the main conclusions of the study.

Dear Editors and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "**Agricultural Fungicide Overuse Shapes Soil and Sediment Reservoirs of Multidrug-Resistant and Virulent Fungi**" (ID: NCOMMS-25-81428A). The comments are valuable and very helpful for revising and improving our paper. We have studied the comments carefully and have made corresponding corrections. The revised portions are marked in red fonts in the paper. The detail corrections and the point to point responds to the reviewer's comments are as the flowing:

Response to reviewers' comments

Reviewer #2

Question: Line 18: define MDR in highlights. Also, focus on results/conclusions rather than methods. What to the transcriptome data tell?

Answer: Thank you for your suggestion. We have revised the highlights to define MDR and to emphasize the key findings rather than the methodology.

Question: Graphical abstract: Correct text - "Genes for virulence and toxin production"

Answer: Thank you for your correction. According to the journal's requirement, the Graphical abstract has been removed from the final version of the manuscript.

Question: Lines 26-27: Redundant. Rephrase "Multiple fungicides..."

Answer: Thanks. We have revised the sentence

Question: Lines 31-32. Remove cf., it is only used if followed by a specific epithet.

Answer: Revised.

Question: Lines 33-34: but not clinical fungicides?

Answer: Thank you for your comment. In this study, MDR refers specifically to resistance across multiple agricultural fungicides, as clinical antifungal susceptibility was not assessed for this dataset. We have revised the text to clearly define MDR in this context.

Question: Line 55: no need to redefine IFI

Answer: Revised.

Question: Line 55: candidiasis and aspergillosis need not be in italics

Answer: Revised.

Question: Line 56: Does candidiasis have a mortality rate >40%? This may be true for immunocopromized individuals, but maybe not across all candida infections. Please confirm.

Answer: Thank you for pointing this out. We have revised the sentence to make the expression more accurate.

Question: Lines 66-67: “agroecosystems”

Answer: Revised.

Question: Lines 120-121: “ensure spatially representative sampling”

Answer: Revised.

Question: Lines 136-137: The Limit of Detection (LOD) and Limit of Quantification (LOQ)...

Answer: Revised.

Question: Line 179: what chemistry, 2x250, 2x300 cycles?

Answer: Thank you for pointing this out. The sequencing configuration (2 × 250 bp paired-end) has now been added in the text.

Question: Lines 229-230: please provide reference for the primers.

Answer: Thank you for pointing this out. A citation for the primer has now been added.

Question: Line 233: “six additional”

Answer: Revised.

Question: Lines 233-234: is this the complete manufacturer info?

Answer: Thank you for pointing this out. We have revised the manuscript to provide the complete manufacturer information for the DNA extraction kit.

Question: Line 468: culture-derived

Answer: Revised.

Question: Line 508: You included also LSU marker, this is not protein coding locus.

Answer: Thank you for this correction. The text has been revised.

Question: Line 676: omit “total”

Answer: Revised.

Question: Line 695: Overstatement. It remains unconfirmed if these strains are indeed associated with invasive infections. They do represent genera that do so.

Answer: Thank you for your comment. We have revised the sentence as “Notably, although not to species, the MDR strains identified here fall in the genera that are associated with invasive infections in immunocompromised hosts, such as *Meyerozyma*, *Aspergillus*, and *Fusarium*.”

Question: Line 777: shorten “agroecosystems”

Answer: Revised.

Reviewer #3 (Remarks to the Author):

Question: Throughout the manuscript, human fungal pathogens and evolution of resistance has been mentioned. However, this data does not support any virulence data or potential for evolution of resistance. Many fungal genera are intrinsically resistant, so please make sure this is shown throughout the manuscript. Also, many of the genera contain fungal species that are pathogens, but the species are not present/shown. Only the case for *A. fumigatus*.

Answer: Thank you for your comments. We agree that our study did not include direct assessment of fungal pathogenicity or the evolution of resistance. Accordingly, we have carefully revised the manuscript throughout and removed or rephrased statements related to “human fungal pathogens” and “evolution of resistance”

Question: L1-2 Title: “Fungicide Overuse” is not really shown. Also “Virulent Fungi” are not directly shown in this article. A more appropriate title that is true to the data would be: Agricultural Fungicides Shape Soil and Sediment Reservoirs of Multidrug-Resistant Fungi

Answer: Thank you for this important suggestion. Following your recommendation, we have revised the title to more accurately reflect the scope of our data and conclusions.

Question: L335-339 Please give the workflow, including packages, versions and parameters. L341 Same here, what was the reference-free workflow

Answer: Thank you for this comment. We have revised the Methods section to provide a detailed transcriptomic analysis workflow, including the software tools, versions, and

key parameters used for both reference-based and reference-free analyses.

Question: L347 I can't seem to find the primers used for qPCR validation, please include a supplemental table.

Answer: Thank you for pointing this out. The primers have been supplemented in Table S10.

Question: L401 "Reliability" reliability is not really tested, please remove.

Answer: Thanks. It has been removed.

Question: L403 "81 samples" were more samples tested that came back with no fungicides at all? The text seems to imply, which would be very interesting to include here.

Answer: Thank you for this comment. Sorry for the ambiguity in the original wording. A total of 81 samples were used to detect the fungicide residues in this study. We have revised the text to clarify this point.

Question: L440-442 It does not show selection for resistance. It could merely be that these fungi are intrinsically resistant. Please remove this sentence.

Answer: Thanks. It has been removed.

Question: L503-506 Just using genera to compare is not really good practice. Most of these fungal genera are huge and only several species are pathogens. Please remove.

Answer: Thanks. You are very right. It has been removed.

Question: L520-522 Related to human clinical pathogens, but these species are not. Remove sentence and say that Twenty environmental fungal isolates were selected.

Answer: Thanks. It has been removed.

Question: L570 What I'm missing here is that cyp51a was sequenced? I assume? And these mutations were found? If not, then this result should be removed.

Answer: Thank you for this important comment. The cyp51 sequences analyzed in this study were derived from transcriptome assemblies rather than from targeted PCR amplification and Sanger sequencing. However, based on the amino acid sequences translated from the transcriptome and comparing with those of the standard strains, we did find some mutations in cyp51 gene. To clarify this, we have revised corresponding sentences as indicated in red fonts.

Question: L594 resistance evolution. No data on evolution is shown. Most of these fungal species are intrinsically resistant to many of these fungicides.

Answer: Thank you for your comment. We have revised the text to remove references to “resistance evolution” and instead describe the observed resistance-associated mechanisms.

Question: L632-633 nothing is shown about virulence. Therefore, remove.

Answer: Thanks. It has been removed.

Question: L651 chronic fungicide application. This is not shown, only soil residues. Please amend.

Answer: Thank you for your comment. We have revised the text to focus on the observed fungicide residues and their association with changes in fungal communities and antifungal resistance, avoiding unsupported inference regarding application patterns.

Question: L667 same here and throughout the discussion.

Answer: Thank you for the comment. We have thoroughly revised the manuscript and corrected all similar expressions to avoid unsupported statements.

Question: L721-723 These have been linked to resistance, so please amend. This has been done in <https://journals.asm.org/doi/10.1128/aem.02539-20>

Answer: Thank you for pointing out that these mutation sites have previously been reported to be associated with fungal drug resistance. To avoid overstatement, we have removed language describing these variants as “novel” and have revised the text to clarify that the structural and docking analyses are predictive in nature and require further validation.