# Sub-lethal fungicide concentrations both reduce and stimulate the growth rate of non-target soil fungi from a natural grassland

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Conventional agriculture has relied extensively on the use of fungicides to prevent or control crop diseases. However, some fungicides, particularly broad-spectrum fungicides, not only eliminate target pathogens but also non-target and beneficial soil microbes. This scenario is not only limited to agricultural soil, but this may also potentially occur when neighboring environments are contaminated by fungicides through spray drift. Although concentrations may be sub-lethal, the chemicals may accumulate in the soil when used continuously resulting in more toxic effects. In this study, the effect on the colony extension rate of 31 filamentous soil saprobic fungi, initially isolated from a protected grassland ecosystem, were analyzed under fungicide treatment. These isolates were considered naive (no deliberate exposure), hence presumed to have not developed resistance. Two currently used fungicides with different modes of action were added to Potato Dextrose Agar at varying concentrations. Results showed a wide range of tolerance and sensitivity to isopyrazam and prothioconazole. Fungi belonging to the phylum Basidiomycota were most negatively affected by both fungicides. Phylum Mucoromycota were the most tolerant to prothioconazole while isolates belonging to phylum Ascomycota differed in their responses to both fungicides. Negative effects on the growth rate were more pronounced at higher concentrations except for a few isolates that were inhibited at $1 \, \text{mg} \cdot \text{L}^{-1}$. A slightly positive effect was also observed in three of the isolates under fungicide treatment. Lastly, the negative impact of fungicides was not associated with the growth strategy of the fungi, whether fast growing or slow growing, rather it is isolate-specific and phylogenetically conserved. The results of this study demonstrate that co-occurring fungi differ in their sensitivity to fungicides even without prior exposure. This difference in sensitivity among co-occurring fungi may result in shifts in community composition of the soil fungal community to the detriment of the more sensitive isolates.

# Introduction

Fungi, together with bacteria dominate soil microbial biomass (Fierer, 2017; He et al., 2020). They are found in virtually all types of soil ecosystems such as grasslands, forests, and agriculture. They are important in organic matter decomposition and nutrient cycling, biocontrol and regulation, and supporting ecosystem functions (Frac et al., 2018). Even though several species are associated with disease, many fungi serve as indicators of soil health (Ding et al., 2022).

Soil fungal biodiversity is altered by several factors including other organisms, soil properties (e.g., pH, salinity, water holding capacity), and chemical amendments like fertilizers and pesticides (Frąc et al., 2018; Jiang et al., 2021). Pesticides are widely accepted plant protection products commonly applied to maximize crop yield by preventing or controlling plant diseases (Bünemann et al., 2006; Wiik and Rosenqvist, 2010; Turkington et al., 2012; Caldwell et al., 2017). Farmers rely on intensive farming practices to increase productivity and profitability by minimizing crop loss. These include the use of fungicides to combat fungal-associated plant diseases (Wegulo et al., 2011; Fones et al., 2020). Despite public concerns on the impact of these chemicals to health and environment, pesticide sales remain constant within the 2011–2018 period, with fungicides and bactericides as the most sold group (Eurostat, 2020).

Prothioconazole and isopyrazam are foliar fungicides currently used in agriculture (Fungicide Resistance Action Committee, 2022). Prothioconazole is a broad-spectrum systemic fungicide belonging to the chemical class triazolinthione (United States Environmental Protection Agency, 2007). It is most commonly used to control diseases of cereals (Edwards and Godley, 2010; Sanssené et al., 2011) and other crops (Lonergan et al., 2015; Miller, Standish and Quesada-Ocampo, 2020). As a sterol 14α-demethylase inhibitor (DMI), it inhibits the formation of ergosterol, a major component of fungal cell membrane, thereby stopping fungal growth (Revie et al., 2018). Isopyrazam is a carboxamide that is also used against diseases of various crops (Pérez-Vicente, 2012; Ajigboye et al., 2014). It inhibits the enzyme succinate dehydrogenase (SDHI) which is crucial in the tricarboxylic cycle (TCA) associated with fungal respiration (Santísima-Trinidad et al., 2018).

Undeniably, fungicides have been useful in minimizing crop loss and increasing plant yield *via* the control and prevention of plant diseases. However, other non-target species may also be affected by the prolonged use and overuse of fungicides. Accumulation of foliar fungicides that enter into the soil may affect the microbiota that play an important role in decomposition, nutrient cycling, or production of important metabolites for plant growth (Karlsson et al., 2014; Baćmaga et al., 2016; Manoharan et al., 2017; Pánková et al., 2018; Santísima-Trinidad et al., 2018; Baćmaga et al., 2019; Baćmaga et al., 2020; Carpio et al., 2020; Meena et al., 2020; Roman et al., 2021; Edlinger et al., 2022; Noel et al., 2022; Sim et al., 2022; Yao

et al., 2022). Some of these organisms are even found to have antagonistic activities against pathogens that could rather be tapped to maximize the effect of existing fungicide application strategies (Tagawa et al., 2010; Patkowska et al., 2019; Chen et al., 2021). Like agricultural ecosystems, grasslands also benefit from soil microorganisms to maintain ecosystem functions or services. The structure of microbial communities in grassland ecosystems is affected by animal activities like grazing and human activities including long-term application of fertilizers (Cassman et al., 2016; Huhe et al., 2017; Liao et al., 2021). In turn, microbial composition, the presence of plant pathogens and plant-fungal interactions shape plant diversity and biomass production (Huhe et al., 2017). Land-use conversion of grasslands to agriculture have inadvertently exposed surrounding natural ecosystems to fungicides and other chemicals. Even nature conservation areas adjacent to agricultural fields are not exempted from this. Pesticide residues including fungicides are found on insect bodies collected from nature reserves all over Germany, including Oderhänge Mallnow where the fungi used in this study were collected (Brühl et al., 2021). Indirect contamination of neighboring habitats may also be possible *via* spray drift or the transmission of pesticide droplets through air to non-target sites (Van Den Berg et al., 1999; United States Environmental Protection Agency, 2007, OCSPP, 2015).

Grassland studies involving fungi mostly focused on their biodiversity and role in soil health (Yang T et al., 2017; Yang Y et al., 2017; Navrátilová et al., 2019; Ceulemans et al., 2019; Kang et al., 2020; Wu H et al., 2021; Yu and Dijkstra, 2020; Yu et al., 2021; Zhang et al., 2021). So far, only a few studies have focused on the effect of fungicides on the grassland soil fungal community (Frank et al., 2003; Daniel et al., 2015; Sułowicz et al., 2016; Sułowicz and Piotrowska-Seget, 2016; Pánková et al., 2018) and even fewer on grasslands within nature reserves. In the study of Brühl et al. (2021) mentioned above, it clearly showed that pesticide contamination poses a risk on non-target organisms inside a protected habitat adjacent to areas where conventional agriculture is being practiced. In the present study, soil fungi previously isolated from a protected grassland environment were used to evaluate the potential effect of fungicides on naive (i.e., not deliberately exposed to fungicide) fungal isolates. These isolates come from three fungal phyla, namely Ascomycota, Mucoromycota and Basidiomycota. Their tolerance against two currently used fungicides in agriculture was determined at different concentrations. Although these fungicides were not among the reported residues detected in insect bodies living in the grassland area where these fungi were isolated (Brühl et al., 2021), traces of exposure *via* spray drift from nearby farms cannot be excluded. This set of fungi (referred to as Rillig Lab Core Set, RLCS) was previously evaluated for different trait characteristics and growth strategies (Andrade-Linares et al., 2016; Veresoglou et al., 2018; Lehmann et al., 2019; Camenzind et al., 2020). These well-characterized isolates were

used to test the following hypotheses: 1) the application of the fungicides prothioconazole and isopyrazam will negatively affect growth of fungal isolates from a natural grassland, with stronger detrimental effects at higher fungicide concentration, 2) the isolates will respond differently against the two fungicides having different modes of actions, 3) slow-growing fungi will have better tolerance to fungicides than fast-growing ones, and 4) responses are phylogenetically conserved.

## Materials and methods

### Fungal isolates

Thirty-one fungal strains were previously isolated from a natural semi-arid grassland situated inside a natural reserve in Mallnow Lebus, Brandenburg, Germany (52°27.778′N, 14°29.349′E), as described in Andrade-Linares et al. (2016). These comprised species from Ascomycota, Basidiomycota and Mucoromycota (Supplementary Table S1).

### Determination of minimum inhibitory concentration

To determine the concentration of the fungicides that will be used in the experiment, a preliminary test to establish the MIC was performed using the broth microdilution technique. This is a reference method that makes use of microdilution plates (e.g., 96-well plate) containing a series of two-fold dilutions of the antifungal drug, inoculated with the test organism and evaluated visually or spectrophotometrically (Pfaller et al., 2000). Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that causes a specified reduction in visible growth (CLSI, 2008). Eight species from the three fungal phyla were grown on PDA plates at 25°C for 7 days. To dislodge the spores, 5 ml of sterile distilled water with 0.1% Tween 80 (v/v) was pipetted over the mycelium and the surface was gently rubbed with a sterile glass rod. The liquid containing the spores was harvested and vortexed for 10 s to distribute the spores evenly. An aliquot of 2,000 µl was obtained from the collected suspension and centrifuged at 12,500 rpm for 5 min. Afterwards, 1,800 µl of the suspension was discarded and the remaining 200 µl were used to count the number of spores using a Neubauer counting chamber. Final spore concentration was adjusted to $4.0 \times 10^4$ (Balouiri, Sadiki and Ibnsouda, 2016). Stock solutions of the fungicides prothioconazole (Sigma-Aldrich) and isopyrazam (Sigma-Aldrich) were prepared using 100% dimethyl sulfoxide (BioChemica, Germany) and were further diluted to 2-fold of the final working concentration (30 mg·L⁻¹). On the first well, 200 µl of the fungicide solution was pipetted. This served as the fungicide control (blank well). Aliquots of 100 µl Potato Dextrose Broth were dispensed on wells 2–12. A two-fold serial dilution was employed using a multichannel pipette from well 1–11 giving final

concentrations ranging from 0.015 to 16 mg·L⁻¹. From the prepared suspension of the test organisms, 100 µl was inoculated from wells 2–12. The last well served as growth control well containing only the test organism and PDB. Plates were incubated at 25°C for 48 h. Following incubation, the wells were visually inspected for growth and absorbance (OD) was measured at 570 nm using a microplate reader (Bio-RAD, United States). Prior to calculating the MIC, the optical densities (ODs) of the blank (uninoculated well) were first subtracted from the ODs of the inoculated wells. The percentage of growth were then calculated for the individual wells using the formula: $OD_{treatment} / OD_{growth\ control} \times 100$ (Espinel-Ingroff and Cantón, 2007; Meletiadis et al., 2017). The concentration of the well that with an OD of ≥50% less than the growth control (Pfaller et al., 1995) was recorded as the $MIC_{50}$.

### In vitro assessment of the effects of fungicides on the growth of the test fungi

A mycelial plug of 6-mm diameter of fungal mycelium was obtained from the margin of an actively growing culture and placed at the center of Potato Dextrose Agar supplemented with fungicides. The inoculated plates were then individually sealed with parafilm and incubated in upright position at 25°C for 7 days. Concentrations of 1, 3 and 5 mg·L⁻¹ were chosen as treatment conditions based on the MIC tests and published literature (Hof, 2001). These concentrations were considered sub-lethal for most of the tested isolates, therefore allowing measurable effects within the experimental period instead of completely preventing fungal growth. Plates without fungicide were included as controls for each treatment. Three replicates were prepared for all the organisms and for each concentration (including the controls). Colony diameter was measured after 3, 5, and 7 days. Two perpendicular measurements were made for every plate and subsequently averaged to calculate the colony area. Colony extension rate (mm²·d⁻¹) was computed following the equation: $Colony\ extension\ rate\ (mm^2\ day^{-1}) = Area_{(f)} - Area_{(i)} / (Day_{(f)} - Day_{(i)})$, where day 3 and day 7 values were considered as initial and final readings, respectively, except for isolates RCLS01 and RCLS02. For these two isolates, day 5 measurements were considered final reading due to the rapid growth of the mycelia reaching the edge of the Petri dish before the 7th day.

Similar experiments were also conducted at temperatures of 30°C and 35°C (results are presented in Supplementary Materials, Supplementary Figure S5; Supplementary Table S6).

### Data analysis

Colony extension rate was calculated as the response variable. Since the correlation of the colony extension rate to concentration mostly followed non-normal distributions and showed heteroskedasticity, generalized linear mixed models (GLMM) were used to analyze the effects of these treatment conditions using the

TABLE 1 Minimum inhibitory concentrations (MIC$_{50}$) of the representative isolates belonging to three different phyla.

| Species | Phylum | MIC$_{50}$ (mg L$^{-1}$) | |
|---|---|---|---|
| | | Isopyrazam | Prothioconazole |
| RLCS01 (*Mucor fragilis*) | Mucoromycota | 8 | 8 |
| RLCS05 (*Fusarium* sp.) | Ascomycota | >8 | >8 |
| RLCS11 (*Mortierella alpina*) | Mucoromycota | <0.02 | <0.02 |
| RLCS17 (*Clitopilus* sp.) | Basidiomycota | >8 | 8 |
| RLCS18 (*Fusarium gibbosum*) | Ascomycota | >8 | >8 |
| RLCS19 (*Umbelopsis isabellina*) | Mucoromycota | 4 | 2 |
| RLCS24 (*Metarhizium marquandii*) | Ascomycota | 8 | 2 |
| RLCS32 (*Fusarium oxsyporum*) | Ascomycota | >8 | >8 |

function *glmmPQL*() in R (package MASS, Venables and Ripley, 2002). Concentration and fungicide types were used as fixed factors and species identity nested within phylum were used as random factors to analyze the collective response of all fungi to the two fungicides. For individual fungal isolates, replicate was considered a random factor. Gamma distribution with log link was used to deal with skewed but non-zero inflated data. In cases where skewness was due to many zeros (e.g., no growth), quasipoisson was used instead.

Effect sizes of fungicide concentrations were calculated for each isolate using the natural logarithm of the response ratios of the treatment mean and control mean, LRR = ln ($\bar{X}_t / \bar{X}_c$) (Lajeunesse, 2015). To determine whether the two fungicides with different mode of action differ in their effect sizes, a paired non-parametric Wilcoxon test was implemented using the wilcox. test (). Further, the effect sizes on the three phyla were also compared at each fungicide concentration using analysis of variance and TukeyHSD test. To test whether the effect sizes were more pronounced for fast-growing than for slow-growing fungi, a correlation test between the effect sizes and the growth rate under control conditions was performed for all treatments. Since the assumptions of normality and heteroscedasticity were violated, a non-parametric Kendall test was followed using the *cor. test*() in R. This was further analyzed using linear models coupled with phylogenetic generalized linear models (pgls) using the caper package (Orme et al., 2018) to account for phylogenetic dependencies.

Finally, the phylogenetic signal of the treatment effects of the different concentrations were analyzed based on Blomberg's K statistics (Blomberg et al., 2003; Keck et al., 2016) using the function *phyloSignal* (Keck et al., 2016). All statistical analysis were done using R version 4.0.5 (R Core Team, 2021).

# Results

The broth microdilution assay provided a range of minimum concentration that inhibits the growth of the eight isolates from the three phyla (Table 1; Supplementary Figure S2). These MICs
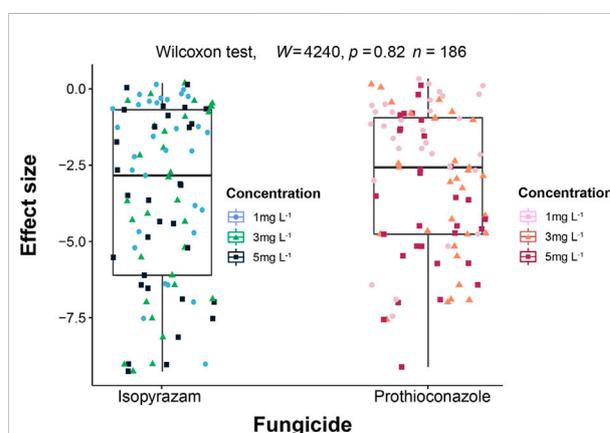


FIGURE 1
Comparison of the effect sizes on fungal colony extension rate between isopyrazam and prothioconazole. Effect sizes (ES) are calculated as the natural logarithm of the response ratios of the treatment and control mean values (LRR = ln ($\bar{X}_t / \bar{X}_c$). Data points presented in each box plot represent the ES for individual isolate treated with 1 mg L$^{-1}$ (red circle), 3 mg·L$^{-1}$ (yellow triangle) and 5 mg·L$^{-1}$ (green square) of the fungicide (Wilcoxon test, $p$ = 0.82).

were used as the basis for the range of concentrations used to determine the effect of fungicides on the growth of soil fungi in terms of colony extension rate. The MIC$_{50}$ of the representative isolates displayed the variability in tolerance and sensitivity among the isolates in this pre-test.

In the absence of fungicides, colony extension rate of the 31 soil fungi tested ranged from 7.2 to 1,341.07 mm$^2$·day$^{-1}$ with the fastest growing species belonging to phylum Mucoromycota (Supplementary Figure S3). Overall, the two fungicides did not differ ($p$ = 0.82) in their effect on fungal colony extension rate (Figure 1).

However, when effects were compared at the phylum level under different fungicide concentrations, results revealed different responses to the two fungicides. Among the three
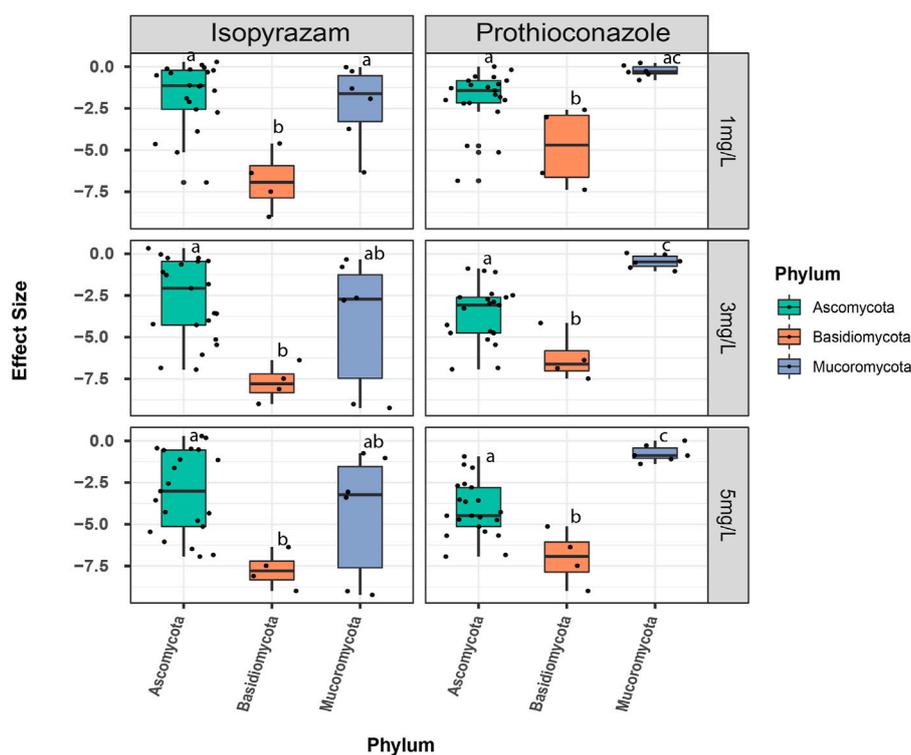
**FIGURE 2**
Comparison of effect sizes of the fungicide concentrations at the phylum level. Effect sizes (ES) are calculated as the natural logarithm of the response ratios of the treatment and control mean values (LRR = ln $(\bar{X}_t/\bar{X}_c)$). Dots represent ES for individual isolate. Different letters denote significant difference between groups ($p < 0.05$).

phyla, the strongest negative effect of prothioconazole occurred for Basidiomycota (Figure 2). On the other hand, Mucoromycota were least negatively affected by this fungicide. At 1 mg·L$^{-1}$, effect sizes on Mucoromycota and Ascomycota were not significantly different ($p = 0.092$, Figure 2; Supplementary Table S2). However, at higher concentrations, the effects differ significantly between these two phyla with greater negative effect on the latter (3 mg·L$^{-1}$: $p = <0.001$ and 5 mg·L$^{-1}$: $p = <0.001$, Supplementary Table S2). With isopyrazam, Basidiomycota remained the most negatively affected under all concentrations. The fungicide effect on phylum Mucoromycota and Ascomycota varied widely but there was no significant difference between these two phyla (1 mg·L$^{-1}$: $p = 0.834$; 3 mg·L$^{-1}$: $p = 0.516$ and 5 mg·L$^{-1}$: $p = 0.539$, Supplementary Table S2).

Looking into the individual isolates, the increasing concentration of fungicide elicited variable responses from the 31 fungi belonging to different classes and orders from these three fungal phyla. In most species tested, colony extension rate (CER) decreased with increasing fungicide concentration (Figure 3). Five isolates (RLCS09, RLCS16, RLCS23, RLCS28, and RCLCS29) were highly sensitive to isopyrazam, such that the addition of 1 mg·L$^{-1}$ completely inhibited their growth. Similarly,

1 mg·L$^{-1}$ of prothioconazole inhibited the growth of four isolates, namely, RLCS27, RLCS28, RLCS29, and RLCS31. In general, isolates belonging to Basidiomycota (RLCS09, RLCS16, RLCS17, and RLCS29) were the most sensitive to both fungicides. Their CER were reduced significantly at all concentrations. Fungi in Ascomycota also showed high sensitivity to the two fungicides with RLCS23 being the most sensitive. In the Ascomycota, isolates belonging to class Sordariomycetes (RLCS05, RLCS07, RLCS13, RLCS18, RLCS24, RLCS25, and RLCS32) were the most tolerant to isopyrazam together with RLCS12. Contrary to the predicted outcome, RLCS25 (*Gliomastix sp.*) had higher CER at 5 mg·L$^{-1}$ of isopyrazam, resulting in a positive effect size. Similarly, CER of RLCS31 (*Cyphellophora sp.*) also slightly increased when treated with isopyrazam. Under prothioconazole treatment, fungi belonging to phylum Mucoromycota (RLCS01, RLCS02, RLCS03, RLCS11, RLCS15, and RLCS19) were the most tolerant whereas greatest reduction was detected for RLCS27, RLCS28, RLCS29, and RLCS31. Interestingly, isolate RLCS19 (*Umbelopsis isabellina*) even had higher CER at all concentrations of prothioconazole compared with the control (Figure 3).

To evaluate whether the effect of fungicide was more pronounced for fast-growing fungi than for slow-growing
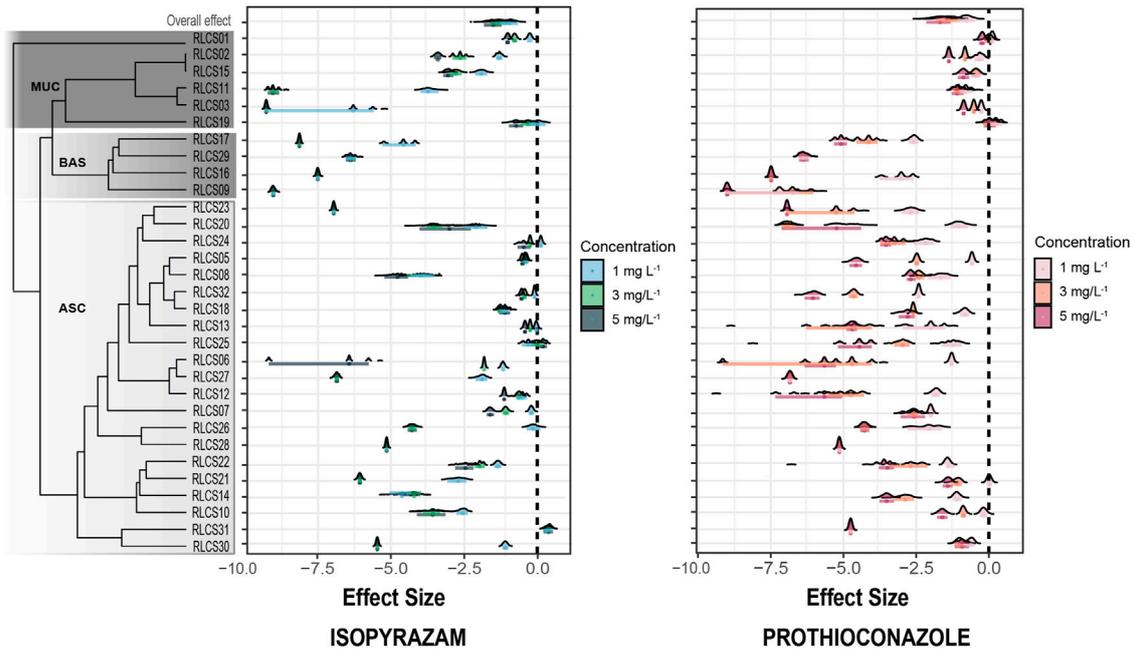
**FIGURE 3**
Effect sizes of fungicide treatment on the colony extension rate (CER) of individual isolates. Effect sizes (ES) are calculated as the natural logarithm of the response ratios of the treatment and control mean values (LRR = ln $(\bar{X}_t/\bar{X}_c)$. Dots represent the effect sizes for individual isolate. Kernel density plots display the data distributions including the 95% confidence intervals as represented by the bars. Phylogenetic affiliations of the isolates are indicated on the left, with the three phyla (MUC, Mucoromcota; BAS, Basidiomycota; ASC, Ascomycota) indicated by shading. The overall effect of each fungicide is also included on the top row of each panel. Dashed line represents zero effect size.
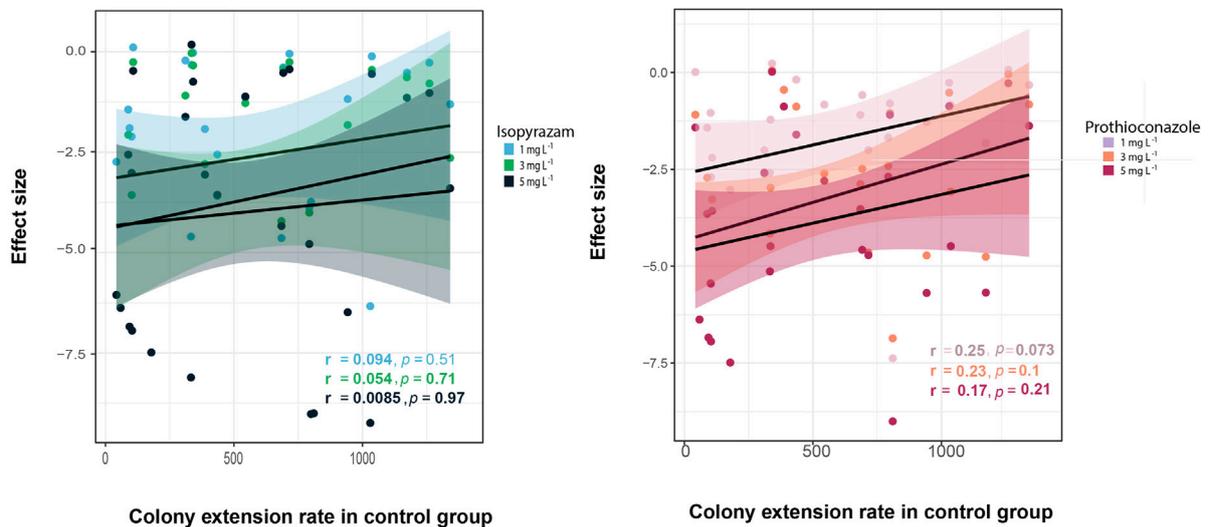


**FIGURE 4**
Correlation between the effects sizes of the fungicides and the colony extension rate (mm$^2$·day$^{-1}$) in the control group. Dots represent effect sizes for individual isolates under certain concentration of isopyrazam (blue) and prothioconazole (light red). Increasing fungicide concentration is depicted by increasing color intensity. Linear regression (black lines) and confidence interval (colored band) in every treatment are presented. Correlation coefficients (r) and significance level (p < 0.05) are included in each panel.

isolates, the relationship between the fungicide effect size and the colony extension rate under control conditions was investigated. A Kendall test revealed a weak, non-significant correlation between the growth rate and the treatment effects (Figure 4). The same relationship was observed when phylogenetic dependence was considered (Isopyrazam at 1 mg·L$^{-1}$: $R^2$ = 0.057, $p$ = 0.1036; at 3 mg·L$^{-1}$: $R^2$ = 0.052, $p$ = 0.1141; at 5 mg·L$^{-1}$: $R^2$ = −0.029, $p$ = 0.722; and Prothioconazole at 1 mg·L$^{-1}$: $R^2$ = 0.1021, $p$ = 0.045; 3 mg·L$^{-1}$: $R^2$ = −0.005, $p$ = 0.365; 5 mg·L$^{-1}$: $R^2$ = 0.095, $p$ = 0.051, Supplementary Table S3).

Effect sizes of fungicide treatments on colony extension rate were also assessed for phylogenetic signal using Blomberg's K statistics (Supplementary Table S4). Moderate phylogenetic signals were detected on the effect sizes on colony extension rate of isopyrazam under all concentration treatments (1 mg·L$^{-1}$: $K$ = 0.474, $p$ = 0.016; 3 mg·L$^{-1}$: $K$ = 0.465, $p$ = 0.014; 5 mg·L$^{-1}$: $K$ = 0.512, $p$ = 0.003) as well as at lower concentration of prothioconazole (1 mg·L$^{-1}$: $K$ = 0.349, $p$ = 0.087). At higher concentrations of prothioconazole, high phylogenetic signals were detected (3 mg·L$^{-1}$: $K$ = 0.902, $p$ = 0.001; 5 mg·L$^{-1}$: $K$ = 0.925, $p$ = 0.001).

In the parallel experiment using 30°C and 35°C, prothioconazole and isopyrazam did not differ in their over-all effect sizes despite the difference in their modes of action (Supplementary Figure S4). However, there was a remarkable decrease in colony extension rate at higher temperature, with the greatest reduction seen at 35°C even without the addition of fungicides. The reduction was further exacerbated by the addition of fungicides in both temperature conditions (Supplementary Table S6). These results provide insight on how increasing temperature in the environment might aggravate the impact of fungicides on non-target soil organisms.

## Additional observations

Although not quantified, it was also observed that mycelium pigmentation changed in the presence of fungicides as the case of isolates RLCS05, RLCS06, RLCS10, and RLCS12 (Supplementary Figure S4). Mycelia color of these isolates became lighter when grown on either prothioconazole or isopyrazam. In particular, isolates RLCS06 and RLCS12 form visible asci on their mycelium (Camenzind et al., 2022) but the application of fungicides could have suppressed the formation of ascospores resulting in reduced coloration.

## Discussion

### Differential susceptibility to the fungicides

Despite being isolated from the same grassland ecosystem and presumed to not have been particularly exposed to fungicides

previously, these saprobic, non-target soil fungi have a wide range of tolerance and sensitivity to fungicides. The lowest concentration that inhibits 50% of growth (MIC$_{50}$) based on the reduction in turbidity (OD reading), ranged from <0.02 to >8.0 mg·L$^{-1}$. This differential susceptibility implies that exposure to fungicides could potentially affect diversity and structure of microbial communities in the soil. Although at low concentrations, some pesticides may persist in soil for years (Chiaia-Hernandez et al., 2017). Repeated fungicide application may lead to accumulation in soil subsequently affecting beneficial soil organisms (Lewandowska and Walorczyk, 2010; Pelosi et al., 2021). In a study conducted in European agricultural topsoil, as low as 0.1 mg·kg$^{-1}$ to more than 2.0 mg·kg$^{-1}$ of different pesticides were detected either as a single residue or a mixture of several pesticides, of which broad-spectrum fungicides were among the most frequently found (Silva et al., 2019). These concentrations are comparable to the experimental concentrations used in this study.

## Implication of differential sensitivity on potential soil-borne pathogens

Although isolated from grassland ecosystem, some of these fungi are potential plant pathogens such as RLCS01 - *Mucor fragilis* (Ghuffar et al., 2018; Wu X et al., 2021; Khan and Javaid, 2022), RLCS05, RCLCS08, RLCS13, RLCS18, and RLCS32—*Fusarium sp.* (Michielse and Rep, 2009; Nag et al., 2022), RLCS07—*Truncatella angustata* (Arzanlou et al., 2013; Wenneker et al., 2016; Sokolova et al., 2022), RLCS10—*Alternaria sp.* (RLCS10, De Mers, 2022; Htun et al., 2022; Martinko et al., 2022; Xu et al., 2022), RLCS22—*Paraphoma chrysanthemicola* (Wang et al., 2022), and RLCS23—*Paramyrothecium sp.* (Haudenshield et al., 2018; Pinruan et al., 2022; Withee et al., 2022),. These isolates were more tolerant to the sub-lethal concentrations of either prothioconazole or isopyrazam used in this study. Thus, differential sensitivity among isolates implies that growth and proliferation of potential pathogens that are more tolerant to the fungicides may be favored over the less tolerant ones.

## Isolate-specific responses on sub-lethal fungicide concentrations

In a similar study involving different azole derivatives, fungicide efficiency also varied against azole-sensitive fungal strains ranging from 0.075 to 8 mg·L$^{-1}$ (Hof, 2001). As hypothesized, the increasing fungicide concentrations led to a decrease in colony extension rate in most of the isolates. However, in a few instances, isolates had a slightly increased growth rate (CER) when grown on fungicide-amended PDA such as the case of RLCS25, RLCS31 in isopyrazam and RLCS19 in

prothioconazole. One possible explanation for this response is the use of sub-lethal concentrations of fungicides. Stimulatory effects of sub-lethal concentrations of fungicides have been reported by either increasing mycelial growth (Garzón et al., 2011; Zhou et al., 2014) or virulence (Audenaert et al., 2010; Cong et al., 2019) of fungal pathogens. Sub-lethal doses of prothioconazole also induced $H_2O_2$ production, which in turn trigger the production of mycotoxin (Audenaert et al., 2010).

Isolates of the phylum Mucoromycota showed remarkable tolerance to prothioconazole, a fungicide that inhibits ergosterol synthesis in the cell membrane of fungi. This observed tolerance can be intrinsic to these isolates due to the lack of ergosterol in most of them (Weete et al., 2010). However, in the case of Mucorales, despite ergosterol being the major sterol in its membrane (Weete et al., 2010; Müller et al., 2018), high tolerance to the azole fungicide was observed as in the case of RLCS01 (*Mucor fragilis*). As previously reported in some clinically important Mucoromycetes, azoles such as isavuconazole, voriconazole, itraconazole, and posaconazole are also inactive against most tested species (Alastruey-Izquierdo et al., 2009; Caramalho et al., 2017; Borman et al., 2021). *Mucor* sp. In particular had >16 mg·$L^{-1}$ $MIC_{90}$ for all azoles mentioned (Borman et al., 2021). This response implies other mechanisms are in place in these isolates to evade fungicide effects without necessarily developing fungicide resistance. One of which is altering the ergosterol biosynthetic pathways to convert intermediate products to ergosterol preventing severe damage to the membrane (Müller et al., 2018). For example, Müller and his team (2018) reported that the Mucorales species *Rhizopus arrhizus* has an increased actual amount of ergosterol relative to the biomass when exposed to sub-lethal concentration of posaconazole. They noted an increase in eburicol, another ergosterol precursor aside from lanosterol during the treatment. This led to an increased total amount of ergosterol, preventing detrimental damage to the membrane, and consequently overcoming fungicide effects.

Fungi in the Basidiomycota were the most sensitive to both isopyrazam and prothioconazole. The isolates, all in the class Agaricomycetes, are known to possess ergosterol as the major sterol in their membrane (Weete et al., 2010; McLaughlin et al., 2015). Basidiomycetes are essential drivers of carbon cycling in grassland ecosystem (Floudas et al., 2020; Lodato et al., 2021). Their lignolytic ability are responsible for the degradation of grass lignins, an important input in grasslands. Lignin decomposition consequently impact the quality and quantity of organic matter in the soil (Gilmullina et al., 2021) as well as the soil structure by enhancing soil aggregate formation and stability (Caesar-TonThat and Cochran, 2000; Lehmann et al., 2020). Hence, the high sensitivity of Basidiomycota to both fungicides may negatively impact these soil processes due to the eventual decline of these important terrestrial decomposers. Finally, Ascomycota had varying susceptibility to prothioconazole most likely due to the varying biochemical characteristics

including the major sterol produced by the fungi belonging to this group (Weete et al., 2010). Some isolates were also observed to have tolerance to isopyrazam. Succinate dehydrogenase inhibitors (SDHIs) like isopyrazam block the conversion of succinate to fumarate in the tricarboxylic acid cycle (TCA), thereby inhibiting respiration (Sierotzki and Scalliet, 2013). Although not tested in this study, several mechanisms of avoiding the toxic effects of fungicides are possible (Deising, Reimann and Pascholati, 2008). These mechanisms include efflux transporters that pump the fungicide molecules to the extracellular space, modifying plasma membranes to reduce permeability, synthesizing enzymes that degrade the fungicide or utilization of alternative metabolic pathways (Del Sorbo, et al., 2000; Löffler et al., 2000; Schnabel and Jones, 2001; Steinfeld et al., 2001; Deising, Reimann and Pascholati, 2008).

This isolate-specific responses to fungicide treatment did not support our hypothesis that slow-growing fungi are more tolerant to stress (Maynard et al., 2019), hence will be less affected by the fungicides than the fast-growing isolates. Our results rather showed a weak correlation between the growth strategy and treatment effect. Therefore, the effect magnitude of the fungicide treatment is not dependent solely on the colony extension rate. Analysis using Blomberg's K statistics revealed varying levels of phylogenetic signals across the different treatment conditions. Moderate signals were detected on low concentration (1 mg·$L^{-1}$) of prothioconazole and all concentrations of isopyrazam. Higher concentrations of prothioconazole (3 and 5 mg·$L^{-1}$), on the other hand, presented higher phylogenetic signals indicating that the treatment effect of this fungicide is phylogenetically conserved. This can be explained by the cell membrane composition of the isolates wherein some groups possess ergosterol while the others do not. On the other hand, isopyrazam targets the enzyme essential for respiration common to all fungi.

## Other non-target effects of fungicides

Also in the study, other non-target effects of fungicides were observed on some of the isolates. Changes in pigmentation was observed in some isolates (RLCS05, RLCS06, RLCS10, and RLCS12) treated with either isopyrazam or prothioconazole. Spore production and asci formation by RLCS06 and RLCS12 (*Chaetomium angustispirale*) may have been reduced upon fungicide exposure similar to several reported studies (Buck et al., 2011; Celar and Kos, 2016). On the contrary, Han et al. (2022) found that sub-lethal concentrations of two 14a-demethylase inhibitor fungicides promoted the spore production of the fungus *Colletotrichum* spp. This two-way effect of fungicide on growth and reproduction of fungi emphasized the need for critical evaluation of fungicide prior to usage. Aside from potential reduction in spores, species of

*Chaetomium, Fusarium* and *Alternaria* were also reported to produce the dark pigment, melanin (Anitha and Murugesan, 2008; Hu et al., 2012; Fernandes et al., 2016; Frandsen et al., 2016). Melanin provides protection against environmental stresses such as temperature, UV light and chemicals (Narsing Rao et al., 2017). Although not further explored in this study, the fungicides may have inhibited the synthesis of this pigment resulting in their noticeable decrease. Such an effect was also observed for several isolates of *Penicillium, Aspergillus* and other fungal species treated with fungicides due to the inhibition of 1,8-dihydroxynaphthalene (DHN)-melanin pathway (Wheeler and Kuch, 1995; Hamada et al., 2014). Several fungicides like tricyclazole, pyroquilon, phthalide, and carpropamid are used in agriculture due to their activity against melanin biosynthesis (Motoyama and Yamaguchi, 2003; Adeniyi et al., 2021; Fungicide Resistance Action Committee, 2022). Interestingly, in another study by Golubeva et al. (2020) on the same set of fungi, RLCS05 and RLCS12 were found to have darker mycelium when treated with copper suggesting that different environmental stressors produce different effects on the mycelial pigment. These results suggest that the change in pigmentation may be a signature non-target effects of chemical stressors, in this case, fungicide on some fungal species. With the limited studies currently available, these important responses are often overlooked in the study of the impact of fungicides on soil microbiota.

## Conclusion

In conclusion, the result of this study highlights the non-target impact of fungicides on soil fungi isolated from a grassland community inside a nature conservation area. The variable responses to two currently used agricultural fungicides are likely to underpin possible shifts in fungal community structure in the field. Due to innate sensitivity to specific fungicides, important soil fungi involved in decomposition and nutrient recycling may be eliminated completely or outcompeted by the more tolerant strains. In contrast, potential plant pathogens that showed high tolerance to the fungicides could possibly dominate the community. It is also important to emphasize the observed increase in growth rate of some isolates under fungicide treatment. Further investigation on the mechanism involved in the positive effect size of fungicide is necessary to understand whether stimulatory effects of sub-lethal concentrations of fungicides is truly at play in this scenario. It is also interesting to investigate the role of the evolution of volatile compounds in the presence of fungicides and how this affects interactions among microbes in the community. As studies on temperature effects on the development of antimicrobial resistance of pathogens are increasing, it is also important not to overlook the impact of fungicides on non-target soil

microbiota in future climate scenarios. Our controlled exposure study demonstrates the existence of differential effects of fungicides on non-target organisms, in this case soil fungi, that are essential for maintaining soil health.

## Data availability statement

Original datasets are available in a publicly accessible repository: The original contributions presented in the study are publicly available. This data can be found here: https://doi.org/10.6084/m9.figshare.20473161.v2.

## Author contributions

JC, TC, and MR designed the study. JC carried out the experiments, analyzed the data and wrote the first draft of the paper. TC assisted in analyzing the data. All authors revised the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.1020465/full#supplementary-material

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