Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

# Investigating the Impact of Anthropogenic Change on Soil Microbiome Functioning and Crop Health

Jake Mc Entagart

MSc Thesis

2025

# Declaration

I have read and understood the College / University statement on plagiarism.

I declare that the attached dissertation is my own, original work undertaken in partial fulfilment of my degree. I have made no use of sources, materials, or assistance other than those which have been openly and fully acknowledged in the text. If any part of another person's work has been quoted, this either appears in inverted commas or (if beyond a few lines) is indented. Any direct quotation or source of ideas has been identified in the text by author, date, and page number(s) immediately after such an item, and full details are provided in a reference list at the end of the text.

I understand that any breach of the fair practice regulations may result in a failure of this dissertation and that it could also involve other repercussions. I understand also that too great a reliance on the work of others may lead to a low mark.

Jake Mc Entagart

# Acknowledgments

I would like to express my gratitude to Dr Siobhán O'Brien, the O'Brien research group and the staff and students at the Moyne Institute of Preventive Medicine, for their unwavering support and guidance. I am also deeply grateful to Matthew Kelbrick, Jamie Hall, Edward Cairns, and Sharon Zytynska at the University of Liverpool for generously offering their time, assistance, and for providing access to their laboratory facilities, which was essential to the completion of my research. Additionally, my gratitude goes towards Matthew Kelbrick for evolving the soil communities in which the isolates were sourced. Finally, I would like to thank the Sunflower Charitable Foundation and the IRC for funding this project.

# Table of Contents

# Abstract

Anthropogenic change poses significant challenges for society today. Soil microbial communities are under constant anthropogenic stress from pollution, rising climates, pesticide use, and deforestation to name a few. We have little idea how anthropogenic stress shapes soil communities, and the impact that stress can have on soil community functioning. This study examines the impact of fungicide and temperature stress, individually and in combination, on the phenotypes of soil communities. Evaluations included their phenotypic adaptation to these stressors, virulence, antibiotic resistance, and their capacity to enhance plant growth and defence mechanisms. Results revealed that isolates from soil communities evolved under fungicide stress exhibited heightened adaptation to both fungicide and temperature stress compared to those from the stress-free control and temperature-only evolved communities. Virulence assays using wax moth larvae (*Galleria mellonella*) indicated that fungicide-evolved soil communities displayed reduced virulence towards their host compared to ancestral or control evolved communities. Conversely, isolates from the increasing temperature evolved communities predominantly showed greater antibiotic susceptibility compared to evolutionary control isolates. Furthermore, fungicide-evolved communities conferred improved barley (*Hordeum vulgare*) shoot height after 20 days. Together, this study highlights that anthropogenic stressors can in some cases markedly affect the phenotype of soil communities, with impacts for crop growth, antimicrobial resistance, and virulence. Further work should determine how anthropogenic stress affects crop production, indirectly, *via* changes to the soil microbiome.

# Introduction

## 1. Microbial communities in the Anthropocene

The soil microbiome is a vast interconnected community. There is a large, complex biodiversity within the soil as it is composed of every family from the tree of life, such as, bacteria, fungi, archaea, nematodes, and viruses. Just 1g of soil can contain up to 50000 bacterial species along with millions of fungal hyphal cells (1). These intertwined species are pivotal for the health of the soil by providing nutrients to the plants that reside within it (2). The soil microbiome has many important functions, including the release and storage of the essential molecules of life: carbon and nitrogen (3).

Anthropogenic change is the effect and alterations that humans have on the environment, and it is a prevalent issue in society today (4). There is a constant stream of new studies exhibiting how human impact on the environment affects our climate (5), the air we breathe (6) (7), our water supply (8, 9) (10), the food we eat (11), and the biodiversity all around us from land (12) to sea (13) (14) (15). These aspects of our world can be altered by greenhouse gas emissions, over-fishing, industrial practices, and land use changes to name a few (15) (16).

Despite the clear negative impact anthropogenic stress is having on our natural environment, one relatively neglected question is how anthropogenic activities are affecting our soils, and particularly the microbes that inhabit our soils. Anthropogenic change can affect our soil microbial communities by altering composition, reducing diversity (17), and driving loss of key plant growth promoting bacteria (18). Anthropogenic activities, such as, soil pollution of toxic chemicals (19), the use of biofertilizers (20) and deforestation (21) have been shown to alter the composition and functioning of the soil microbiome.

This MSc project focused on investigating how two key anthropogenic stressors, temperature stress due to climate change and pesticide use, shape the properties of soil microbial communities over evolutionary timescales.

## 2. The impact of pesticides on soil communities - Abundance/Diversity

Pesticides are chemicals that deter the growth or proliferation of an organism. Types of pesticide can include fungicides, herbicides, and insecticides (22). Over 200 million tonnes of pesticide is used annually (23) and this ensures the security of the crop yields that are being grown each year (24). Fungicides can enter the soil microbiome *via* numerous

pathways. These include directly applying fungicides to plants, aerosolised fungicide being sprayed drifting to neighbouring environments, runoff into waterways, or by improper disposal of fungicides (25). Pesticides can have negative effects on non-target organisms, despite only being used to target their specific pests. For example, they can alter the diversity and abundance of the bacterial community within the soil (26) (27) (17). It has been shown that fungicides used in vineyards can reduce the growth and viability of some soil bacterial microbes such as *Rhodotorula rubra* and *Raphidocelis subcapitata* (28). Other fungicides such as iprodione can reduce overall bacterial biomass and shift the abundance of the bacterial community towards Proteobacteria (17). Similarly increase in Proteobacterial abundance can be seen in ginseng fields treated with fungicides (27). This is due to Proteobacteria's ability to overcome stressors due to adaptability genes commonly located on mobile genetic elements (29). Other pesticide classes show similar shifts in phyla dominance in exposed soil communities (30). Additionally, some broad spectrum fungicides have highlighted a decrease in abundance and composition of the soil bacterial communities (31) (32).

Of the minimal studies that have been published, previous literature predominantly focuses on the impact of pesticides on non-target organisms over relatively short periods of time. However, a notable gap exists in investigating the long-term effects of pesticides, particularly fungicides, have on non-target organisms (33). It is important to understand the long-term effects and responses to pesticidal stress as short term studies may only show a transient or plastic response to the stressor (34). However, long term exposure to the stressor may highlight selection within the community's structure, abundance and functioning which may have a more prolonged effect. Furthermore, if a stressor is exhibiting a particular response within a community for a short period of time, it does not necessarily guarantee that this response will continue and have the same effect in the long-term (35).

Despite this gap, some long-term studies have explored the consequences of herbicides, such as nicosulfuron. One notable investigation examined nicosulfuron's impact on a field adjacent to a production facility contaminated with the herbicide (36). Alpha diversity is the level of bacterial community richness within a single sample, whilst beta diversity is the difference of bacterial community richness between two samples (37). This study revealed a positive correlation between nicosulfuron contamination levels and alpha diversity by sampling different sections of the field that had varying levels of

contamination. The findings suggest that higher levels of nicosulfuron are associated with increased alpha diversity of the bacterial community, indicating a direct relationship between nicosulfuron concentration and biodiversity. Furthermore, an increase in bacteria from the phyla Proteobacteria and Bacteroides was observed (36), aligning with previous research findings (30). However, when compared to soil samples, analysis of water and mud revealed divergent outcomes, highlighting the necessity of examining bacterial communities directly within the soil when assessing fungicidal impacts (36), rather than assessing their impacts within different media.

### 3. The impact of increasing temperatures on soil communities - Abundance/Diversity

Temperature stress and warming climates are of growing concern. Due to the rise in human industrial activity over the past century, emissions of greenhouse gases such as carbon dioxide and methane have elevated global temperatures and are breaking temperature records year on year (38). This increase in temperature caused by anthropogenic impacts, has major repercussions on the soil community. The effect of warming tested on different soil layers, revealed that moving soil to areas of increased temperatures improves the alpha diversity of the soil community at soil depths of 20-30cm. However, this has no effect on alpha diversity at 0-10cm (39). Additional findings indicate that warmer temperatures primarily impact specific phyla, such as Firmicutes, while other phyla, including Proteobacteria and Actinobacteria, remain relatively unaffected (40). Similar results were shown under temperature stress of 50°C, where soil bacterial community diversity was reduced and was shifted towards more heat tolerant Actinobacteria (41).

A long-term study spanning 28 years has revealed that rising temperatures significantly impacts soil microbial biomass and diversity. Notably, bacterial diversity was reduced and shifted towards Gram-positive bacteria, predominantly Actinomycetes. Furthermore, the soil's carbon content, along with the ability of the soil microbiome to utilise carbon substrates, was also reduced (42). Another long term study spanning 7 years showed that increasing temperatures reduces all microbial diversity, including bacteria, fungi, and protists in grassland soil (43). Similar studies in grassland soil communities showed a reduction in microbial diversity, abundance, and structure during warming events and it had a more prominent impact during drought conditions (44). Furthermore, no major change in temperature needs to occur in order to create change within the soil community. Increasing

ambient temperature even by ~2°C has been shown to significantly affect the soil microbiome, especially during drought conditions (44). This highlights the influence that even minor fluctuations in temperature can exert on soil community diversity and abundance, thereby shaping soil microbial properties and functions.

## 4. Linking anthropogenic change to virulence of soil microbes

Exposure to fungicides and high temperatures can influence the virulence of the soil microbiome towards humans. There is a myriad of literature highlighting the effects fungicide has on the virulence of fungi (45) (46) (47), but there is very minimal research on how these fungicides affect the non-target soil bacterial community. For example, recent research has shown that triazole fungicides, such as tebuconazole, can reduce virulence in fungi such as *Cryptococcus gatti*, and *Cryptococcus neoformans* when tested on a murine model (48). While this paper does not focus on bacterial virulence, it is important to note that alterations in virulence may not be exclusive to the fungal community. This aspect still requires assessment and further investigation.

Literature has suggested that increasing global temperatures are affecting disease transmission (49) (50). This increase in temperatures may lead to expansion of ecological niches of pathogens, which could then lead to human infection (50). Higher temperatures have been correlated with an increase in virulence in some bacteria. The virulence of the fish pathogen *Flavobacterium columnare* towards zebrafish was evaluated, revealing an increase in virulence with rising temperatures. However, as the bacteria reached its upper limits of temperatures stress, its virulence was reduced (51). Similarly, the impact of rising temperatures on plant pathogens has been observed. Studies have shown that plant pathogens such as *Pseudomonas syringae*, can become more virulent when subjected to higher temperatures, specifically around 30°C. This increase in virulence is attributed to enhanced transport of effector proteins *via* the type 3 secretion system (T3SS), leading to a decrease in salicylic acid production (52). Conversely, temperatures of just 28°C, display reduced production of virulence proteins associated with the type 4 secretion system of the plant disease causing soil bacteria *Agrobacterium tumefaciens* and *Agrobacterium vitis* (53). These studies indicate that anthropogenic stressors can affect the virulence of a strain, and as suggested by Nnadi *et al* with further warming, microorganisms may broaden their niche to human hosts leading to additional diseases (50).

## 5. Linking anthropogenic change to antimicrobial resistance of soil microbes

Antimicrobial resistance (AMR) is when a microbe can withstand antimicrobial treatment which was previously effective. This can be a result of genetic mutations, permeability, or physiological changes, within the microbe population, leading it to adapted to the antimicrobial stress, inhibiting the antimicrobial effect (54). Adaptations in cell wall or membrane structure, the efficiency of efflux pumps, or the production of antimicrobial modifying enzymes are a few select antibiotic resistance mechanisms leading to AMR (55).

AMR is a growing concern and it is projected to cause 10 million deaths annually as a consequence by the year 2050 (56) (This report was completed before the Covid-19 pandemic, as a result, the figures presented may be affected). Anthropogenic change and activities have been shown to drive antimicrobial resistance. Pesticide use and increasing temperatures can influence AMR within the soil community. Fungicides can induce cross-resistance not only to other fungicides with different modes of action (57), but they can also induce cross-resistance to antimicrobial compounds in various fungi (58) (48). For example, azole based fungicides can induce antifungal resistance in *Aspergillus fumigatus* (59). Furthermore, both fungicides (60) and herbicides (61) have been shown to increase the frequency of transmission of antibiotic resistance related genes due to changes in cell wall permeability, allowing plasmids to be transferred more easily (60) (61). Additionally, herbicides have been shown to increase the Minimum Inhibitory Concentration (MIC) of clinically important pathogens such as *Salmonella* Typhimurium and *Escherichia coli*. However, this is dependent on the antibiotic and the herbicide used (62).

Rising temperatures can impact AMR within the soil community. This can affect ESKAPE pathogens, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp (63). A rise of 10°C can promote AMR in the ESKAPE organisms *Klebsiella pneumoniae* and *E. coli* (64). One long-term study has shown that evolution of *E. coli* under thermal stress can lead to *de novo* resistance to the antibiotic rifampicin, due to genetic mutations in *rpoB* gene after 2000 generations (65). A comparable study, which involved the evolution of *E. coli* over 2000 generations, also demonstrated a correlation between adaptation to high-temperature stress and antibiotic resistance. The findings revealed that culturing *E. coli* under higher temperatures (42°C) increases its resistance to levofloxacin, a fluoroquinolone antibiotic. However, this evolution rendered *E. coli* more sensitive to other

antibiotics, for example tetracycline, ampicillin, ciprofloxacin and clindamycin (66). This could be due to changes in protein expression under heat stress leading to antibiotic resistance, which results in susceptibility to antibiotics which mimic the cold shock response (67). Although this research is important in understanding the interactions and effects of fungicide and temperature stress on AMR within well-known and documented reference strains of bacteria in isolation, it is also important to conduct these types of tests on the soil bacterial community to see if similar interactions are upheld.

## 6. How anthropogenic change affects soil ecosystem functioning

Soil ecosystem functions are the processes provided by the soil microbiome which can influence the health of the soil. These functions can include the nitrogen cycling (68), carbon sequestration and release (69), promoting plant growth (70), and decomposition of organic matter (71). Typically, enzymatic activity (72), microbial diversity and abundance are key indicators of ecosystem functioning (73).

The use of fungicides and warming can also have effects on crop growth and health. As stated, the use of fungicides can lead to a shift and reduction in microbiome diversity and a reduction in ecosystem functioning (74) (26). Pesticide usage has been shown to shift functional groups or taxa altering soil functional potential (30). Moreover, fungicide stress has also been suggested to negatively affect plant growth promoting (PGP) activities of the soil bacteria. PGP activities can include nitrogen-fixation, solubilising minerals that the plant cannot utilise on its own (such as phosphate), producing phytohormones such as auxins (e.g. Indole Acetic Acid (IAA) and cytokinin), and bioremediation (75). Different pesticidal chemicals such as cypermethrin and chlorpyrifos can reduce nitrogen fixation activity (18) and other pesticides can reduce the nitrifying bacterial abundance (76). Pesticides, for instance, thiamethoxam can reduce phosphatase enzymatic activity in soil communities (76). The fungicides iprodione (17) and azoxystrobin (77) can reduce the activity of bacterial enzymes essential to the nitrogen cycle, including urease, phosphatase, and arylsulfatase, which are all important for soil health. This occurs due to the fungicide inserting itself into the active site of these enzymes (17). Other PGP activities reduced by pesticides include, indole acetic acid production (IAA; auxin) (78) and phosphate solubilisation (79). Not only can fungicides inhibit important enzyme and PGP activities, they reduce the overall abundance of the bacteria performing these activities (80). With the

inhibition of important enzymes and the reduction of the bacteria producing these enzymes, the health, quality, and functioning of the soil is reduced (81).

Similar to fungicide stress, temperature stress can alter soil microbiome functioning. Not only does temperature stress affects the diversity of important bacterial phyla (42), but temperature stress can also directly affect the enzymes of the soil bacterial community which have a role in PGP activities (82). It has been highlighted that soil bacteria can make use of nitrogenase enzymes in a wide range of temperatures (4-40°C), as demonstrated by soil samples taken in Australia (83). Temperatures between 20-40°C have been shown to increase phosphate solubilisation and auxin production. However, temperatures exceeding 50°C reduced this effect (84). Interestingly, it has been demonstrated that soil bacteria exposed to increased temperature may boost beneficial enzyme activity, that breaks down carbon sources, in the short term (85). However, prolonged exposure to increased temperature over the span of 2-10 years, can result in shifts in microbial diversity and reduce substrate affinity. Although, this does appear to be influenced by soil carbon availability (85). This is in accordance with other long term research showcasing prolonged temperature evolution affects the soil microbiome's ability to utilise carbon substrates (42), hence, affecting soil fertility.

## 7. Effects of single stressors *versus* multiple stressors on soil ecosystems

It is crucial to not only examine stressors that impact ecosystems individually but also to assess their combined effects. The world is in a state of constant stress. Many studies investigate the impact of a single stressor on an organism or ecosystem. However, this is not representative of what occurs in nature, as ecosystems are exposed to numerous stressors every day, be it physical, chemical, or biological (86). Therefore, analysing multiple stressors on an ecosystem will enhance our understanding of how they are affected by these stressors in real world settings and help us to understand and ameliorate the effect of these stressors (87).

Furthermore, it is essential to consider that single stressors may only reveal minimal effects as it may not paint the true picture of what is really occurring in an ecosystem (87). When the number of stressors increase, they could have additive or antagonistic effects on ecosystems. A study carried out by Rillig *et al* (88), showed that increasing the number of stressors on the soil ecosystem, negatively affected the functionality and ecosystem services provided by the microbiome to the soil. For example, carbon storage and crop

growth promotion were reduced. Moreover, the study also highlighted that temperature amplifies the negative effects of most other stressors. Which includes anthropogenic stressors, such as pesticide use. Since most ecosystems are under temperature stress due to climate change, the influences of other stressors may be more negative than previously stated (88). This highlights the importance of testing the effect that both rising temperature and fungicide use have on the soil community and ecosystem.

## 8. Introduction to the study system

### 8.1 Soil community evolution

A PhD student in our lab (Matthew Kelbrick) had previously evolved compost microbial communities in the presence and absence of two stressors (fungicide and increasing temperatures; imposed individually and in combination) (89). Briefly, 30g of potting soil (Verve John Innes No. 2) was placed in a glass microcosm and treated with one of four treatments:

1. Control (stress-free) (n=8)

2. Fungicide only (Fubol Gold; 5 mg/g w/w soil) (n=8)

3. Increasing temperatures (0.5°C increase weekly from 26°C up to 34°C) (n=8)

4. Fungicide (Fubol Gold; 5 mg/g w/w soil) and temperature (0.5°C increase weekly up to 34°C) (n=8)

Each treatment was replicated 8 times (8 communities), giving a total of 32 evolving microcosms. All 32 soil microbial communities (SMC's) were evolved for 6 months to facilitate evolutionary and ecological changes in response to stress (89) (**Table 1**).

**Table 1: Outline of evolved soil communities and the stressor in which they evolved under.**

| Evolved Soil Community | Number of Communities | Fungicide Stress | Temperature Stress |
|---|---|---|---|
| Control (C) | 8 | - | - |
| Fungicide (F) | 8 | + | - |
| Increasing Temperatures / Warming (W) | 8 | - | + |
| Increasing Temperatures + Fungicide (WF) | 8 | + | + |

(+) = Community underwent specified stress. (-) = Community did not undergo specified stress

Community composition and diversity for all 32 evolved plus ancestral communities was previously analysed by Kelbrick *et al* (89) using the 16S Swift Amplicon + Internal Transcribed Spacer (ITS) Panel (Swift Biosciences) and Illumina MiSeq platform for bacterial and fungal quantification. Unfortunately, fungal composition could not be determined due to technical errors with ITS quantification. Bacterial composition of evolved and ancestral communities (based on 16S rRNA similarity) reveals a shift in composition toward Enterobacteriaceae and Pseudomonadaceae in fungicide-evolved soil communities, irrespective of temperature treatment (89) (**Figure 1**).

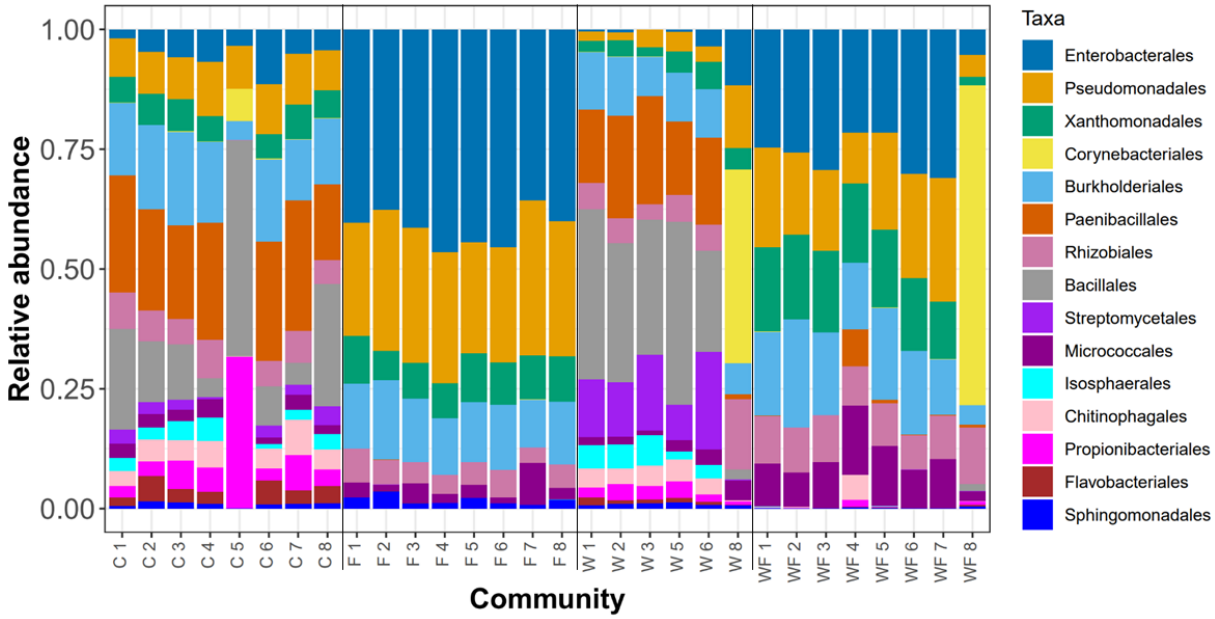


**Figure 1: Figure taken from Kelbrick et al (89), highlights the relative abundance of the microbial taxa across the 4 treatments.** Exposure to fungicide reduces microbial diversity and alters composition toward Enterobacteriaceae and Pseudomonadaceae. The communities are denoted as the treatment in which they evolved under, along with the replicate community number (C=Control, F=Fungicide stress, W=Warming, WF= Warming + Fungicide). Note: W7 relative abundance data is missing due to errors during sequencing.

Kelbrick *et al* (89) also found that along with this shift in soil community composition, there was a reduction in alpha and beta diversity in some evolved communities. Furthermore, the evolved communities had alterations in their metabolic profile as respiration was reduced and some communities had lost the ability to metabolise specific substrates (89). These shifts in diversity can affect the overall functioning of the soil community, because important PGP species could be removed in communities with lower diversity (90) (91) (92).

In this MSc project, further phenotypic analyses were carried out on a selection of the evolved communities described above. These include 3 randomly selected evolutionary

communities from each of the evolved four treatments: Control (C4, C5, C7), Fungicide (F1, F2, F5), Increasing Temperature (W1, W2, W7) and Increasing Temperature + Fungicide (WF1, WF4, WF5) (**Figure 1**). Only 3 evolutionary communities were used because if all communities were assessed, the scale of project would greatly increase, and it would not be feasibly to perform all experiments within the amounted time frame.

From the selected 12 communities, 6 isolates from each replicate community were used for phenotypic testing (4 Treatments X 3 Replicates X 6 isolates = 72 total isolates tested). All experiments performed were conducted under common garden conditions, meaning all isolates were grown and tested under identical conditions (in the absence of their evolved stressor). The ancestral community nor constituent taxa underwent any phenotypic testing except for virulence testing. This was due to the limited availability of the ancestral strains and that analysis with the ancestral strains would also increase the scale of the project, not making it feasibly to perform all experiments within the allotted timeframe. All communities were stored in 20% glycerol at -80°C by Matthew Kelbrick in 2023 (89).

### 8.2 Introduction to barley/aphid system

To test how evolved communities fare in their ability to promote plant growth, health, and defence, a barley aphid model system was employed, established by Sharon Zytynska at Liverpool. The barley plant (*Hordeum vulgare*) is a variety of cereal crop. It is a crucial crop for both human and animal food (93). Cereal aphids include species such as *Sitobain avenae* and *Metopolophium dirhodum*, are sap feeding herbivores. They feed off the phloem of the plant by injecting their mouthpiece, called a stylet into the plant (94) and different species of aphid have been shown to have different host specificities (95). Aphid herbivory can affect crop growth and health by infecting the crops they feed on with viruses and by limiting nutrient uptake (94) (96).

Although there has been a myriad of studies showing how aboveground herbivory can affect the soil microbiome, there are fewer studies showing the reverse interaction of how the soil microbiome can affect aboveground herbivory (97), especially towards aphids. One study inoculated the soil with an array of various communities. This had varying effects both positive and negative on the aphid population. Although it did not conclude an exact reason as to why aphid numbers are affected by the soil microbiome, it showed that the aphid population can be influenced by the soil community (98). Other studies have been conducted to determine the effect the soil microbial community has on aphid numbers and

feeding patterns. For example, Sanchez-Mahecha *et al* has shown that inoculating the soil with a known plant growth promoting rhizobacteria (PGPR), *Acidovorax radicis*, can reduce aphid numbers on barley plants. This reduction is dependent on how soon the inoculation occurred prior to aphid infestation with later inoculation having better effects (99). However, some PGP bacteria such as *Bacillus spp*., have been shown to reduce aphid suppression responses in cabbage plant fields. Furthermore, other non-pathogenic bacteria were shown to reduce aphid suppression due to attracting aphid predators (100). The variance in outcomes may stem from distinct plant species, aphid populations, and bacterial strains under examination, showcasing the case specificity of each plant-aphid interaction.

An alteration in aphid numbers and feeding caused by the soil microbiome could be due to several factors, but one of these can include the regulation of different biochemicals called phytohormones. Phytohormones such as salicylic acid, jasmonic acid, and ethylene do not only influence the plant in defence against pathogens but have also been shown to promote defence against herbivores, like aphids. These phytohormones have been shown to increase expression of genes and secondary metabolites which are related to plant defence against herbivores (95) (101). Although little is known, literature has suggested that the soil microbial community can affect the expression of genes related to phytohormone production. One study shows that soil inoculated with *Pseudomonas simiae* can promote jasmonic acid and ethylene production in the *Arabidopsis thaliana* which helps defence against leaf herbivory (102). Conversely, another study has suggested that the addition of the bacteria, *Pseudomonas fluorescens* to soil downregulates genes related to the salicylic acid response, thus inhibiting plant defence against the aphids (91).

**Thesis Objectives**

This thesis aims to understand how long-term evolution of soil microbial communities in the presence and absence of anthropogenic stress, shapes soil ecosystem functioning, virulence, and antimicrobial resistance of soil isolates along with the communities' ability to promote plant growth and defence, with interest in the barley plant (*Hordeum vulgare*) under common garden conditions.

Three key aims:

i) Examine whether fungicide and/or temperature treatment drives increased adaptation to fungicide and/or temperature stress in soil isolates.

ii) Investigating how fungicide and/or temperature treatment shapes clinically important phenotypes (antimicrobial resistance and virulence) of soil microbes.

iii) Testing how treatment affects ecosystem functioning of evolved communities (growth and health of a crop plant).

# Materials and Methods

1) **Examining individual taxa adaptation to stressors (increased temperatures and fungicide)**

**Species Isolation from Evolved Communities**

Soil isolates were selected from each of the 12 evolved communities described above (**Figure 1**). This was performed by inoculating 100µl of frozen evolved community stock into 10g of twice autoclaved soil. The inoculum was entered directly into the soil rather than growing in liquid media in order to give slow growing bacteria a chance to establish itself in the soil and to replicate the conditions the communities were cultivated in. The community was then allowed to re-establish itself for 7 days in the fresh soil. After 7 days, a soil wash was performed, whereby 10ml of sterile phosphate buffer saline (PBS;137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4; Sigma) was added to each soil microcosm along with 10 X 5mm sterile glass beads (Fischer Scientific). The soil microcosm was then vortexed for 45 seconds and left to sit for 30 minutes allowing it to settle. The soil wash was then extracted, and a serial dilution was performed on each community's soil wash using PBS in 96-well plates (CytoOne; Starlab). All dilutions up to $10^{-8}$ were plated out by pipetting 20µl on Lysogeny broth agar (LB; Fisher Scientific; 10g casein peptone, 5g yeast extract, 10g NACL, 15g agar/L), Reasoner's 2A agar (R2A; Fisher Scientific; 0.5g casein hydrolysate, 0.5g dextrose, 0.5g soluble starch, 0.5g yeast extract, 0.3g dipotassium phosphate, 0.3g sodium pyruvate, 0.25g sodium peptone, 0.25g meat peptone, 0.024g magnesium sulphate, 15g agar/L), and tryptic soy agar (TSA; Fisher Scientific; 15g pancreatic digest of casein, 5g papaic digest of soya bean, 5g NACL, 15g agar/L) plates, and they were spread using sterile 5mm glass beads. Each of the plates were incubated at 28°C for 7 days. A variety of agar types were used to isolate a wide range of bacteria from each of the evolved communities, rather than selecting for bacteria that are fast growing and only grow on one type of agar (**Table 2**).

After 7 days, single colonies were picked at random and streaked onto their own new LB plate using a sterile inoculation loop. When the colonies grew, they were passaged a further two times on LB agar plates to ensure the colony is the only species growing on the plate. After the third passage, a single colony was used to inoculate 5ml of LB broth. The culture was then incubated in a shaking incubator (Innova 42; Eppendorf) at 28°C at 180 revolutions per minute (rpm) for 18-24 hours. Frozen stocks were made from the overnight

LB cultures in 20% glycerol solution and stored at -80°C. Only 6 isolates taken from 3 replicate communities from each treatment (outlined above) were used for further phenotypic testing. The number of isolates from each agar type is not even (2 isolates from each agar type), as even at low dilutions some agar plates were overgrown with a variety of colonies ranging in different sizes with some containing fungal growth, rendering it unfeasible to accurately select a single colony. This resulted in an imbalanced selection of isolates across the agar media types. Note, that taxa were selected at random from each community and have not been identified using genomic analysis. Therefore, the same taxa may be selected multiple times from a single community. This is especially likely if the treatment, reduced community diversity for example, as was the case for treatments exposed to fungicide (**Figure 1**) (89).

**Table 2: Outline of the number of isolates taken from agar types (LB, TS, and R2A) across the 12 treatment communities.**

| Treatment | Community Replicate | Agar Type | | |
|---|---|---|---|---|
| | | LB | TS | R2A |
| Control | 4 | 1 | 3 | 2 |
| Control | 5 | 2 | 3 | 1 |
| Control | 7 | 1 | 3 | 2 |
| Fungicide | 1 | 1 | 2 | 3 |
| Fungicide | 2 | 2 | 2 | 2 |
| Fungicide | 5 | 2 | 2 | 2 |
| Temperature | 1 | 2 | 3 | 1 |
| Temperature | 2 | 2 | 3 | 1 |
| Temperature | 7 | 0 | 4 | 2 |
| Temperature + Fungicide | 1 | 3 | 1 | 2 |
| Temperature + Fungicide | 4 | 1 | 4 | 1 |
| Temperature + Fungicide | 5 | 2 | 4 | 0 |

LB = Lysogeny Broth Agar, TS= Tryptic Soy Agar, R2A = Reasoner's 2A Agar

## Testing whether isolates from different treatments differ in their Fubol Gold Minimum Inhibitory Concentration

It was predicted that soil isolates evolved in the presence of fungicide would display a greater minimum inhibitory concentration (MIC) for fungicide (Fubol gold) evolved isolates compared to isolates evolved in the absence of fungicide. To test this, soil isolates (6 isolates per community, from 3 community replicates of the 4 stressor treatments; 72 in total), were streaked individually, from a frozen stock, onto a LB agar plate. A single colony

was taken from each plate and added to 6ml of LB media in 30ml universal tubes (Fisher Scientific) before growing for 18 hours at 28°C, shaking at 180 rpm. The cultures were then standardised to 0.5 McFarland standard with PBS. This is ~1.5 X $10^8$ Colony Forming Units (CFU)/ml. 5mg/ml Fubol Gold solution was prepared by dissolving Fubol Gold granules in filter sterilised water. 100µl of Mueller Hinton (MH; Sigma) media was added to columns 1-11 in a 96 well plate. The 5mg/ml Fubol Gold solution was added to column 12. The Fubol Gold was 1:2 serially diluted until column 3. Columns 1 and 2 were used as a sterile control and a growth control respectively. 10µl of the standardised culture was added to columns 2-12. The plates were left to grow static at 28°C for 18 hours. The plates were then examined using a microplate reader (Epoch2; Biotek) at optical density (OD) 600nm to determine if growth occurred and to identify the MIC of Fubol Gold for each of the isolates. Three technical replicates for each isolate were performed for each of the two biological replicates carried out. *Escherichia coli* and a lab strain of *Pseudomonas aeruginosa* (PAO1) were used as internal controls to determine if the results were accurate between replicates.

**Testing whether isolates from different treatments differ in their temperature stress adaptation**

It was predicted that soil isolates evolving under warming conditions would grow better under high temperatures, compared to soil isolates evolving at 26°C. To test this, the 72 soil isolates (as before) were streaked individually, from a frozen stock, onto an LB agar plate. A single colony was taken from each plate and added to 6ml of LB media in a 30ml universal tube, before growing for 18 hours at 28°C, shaking at 180 rpm. The isolates were then standardised to 0.5 McFarland standard with PBS and 10µl of each of the 72 total overnights were added to their own well within a 96 well plate, containing 200µl of LB media. This was repeated a further two times in individual 96 well plates. The three plates were then placed into three different incubators set to 24°C, 28°C, and 34°C and were grown static. These three temperatures were chosen because 24°C is a common temperature for soil isolates to experience in nature (103), 28°C because this is generally the optimum temperature in which soil isolates proliferate (104), and 34°C was used as this was the max temperature in which the soil isolates from the increasing temperature treatment went under during their initial evolution by Kelbrick *et al* (89). The OD of the strains was recorded at the 0, 24, and 48 hour time points using a microplate reader at OD 600nm (Epoch2; Biotek). Three technical replicates for each isolate were performed for each of the two biological

replicates carried out. *E. coli* was used as an internal control. All isolates were grown under common garden conditions. Statistical analysis was carried out to test whether evolutionary history of soil isolates affected growth at increased temperatures, above optimum range.

2) **Investigating how fungicide and/or temperature treatment shapes clinically important phenotypes (virulence and antimicrobial resistance)**

***Galleria mellonella* – Evolved Community Virulence Assay**

Previous data indicated that different stressors (specifically fungicide application) drive community compositional shifts towards potentially opportunistic pathogens such as Pseudomonads or Enterobacteria (89). To test whether the treatments altered the ability of species within a community to establish an infection, a wax moth larval model host (*Galleria mellonella*) was used. *Galleria mellonella* were sourced from "Shauna's Pet Shop" (Capel Street, Dublin). All moths were in their final larval stage before pupation. The moths were weighed and those with a weight between 0.2-0.35g were used. They were kept at room temperature in wood shavings for 1 day before the experiment commenced.

To minimise the number of larval wax moths being used for this experiment, one whole evolved community from each of the treatments (i.e. not individual isolates) were analysed. A single ancestral community was also tested, to understand whether communities had overall increased, or decreased in virulence as a result of evolution.

In total, five communities were tested: One ancestral community, plus a single community from each of the four treatments described previously. The pathogen *Pseudomonas aeruginosa* (PAO1) was also included as a positive control, and PBS was used as a negative control. A set of un-injected wax moths was also used as a control for their health in the incubator at 37°C.

The five communities were grown for 24 hours at 28°C at 180 rpm, in 6ml of LB media using a scraping from frozen stock. The communities were standardised to 0.1 OD at 600nm using PBS which is ~1.5 X $10^8$ CFU/ml. The cultures were aspirated using a 10µl syringe (Hamilton) and were injected into the back left proleg of the larvae. Ten larvae were injected for each community. The syringe was sterilized between each injection by aspirating 70% ethanol three times and letting it sit for one minute each time. This was then followed by washing the syringe three times with PBS. The injected larvae were placed into an empty Petri dish and kept in the dark in an incubator set to 37°C. They were assessed for death at hourly intervals until all worms perished. Larvae were deemed as dead if they

did not respond to mechanical touch with a pipette tip. The total observation time lasted 24 hours.

**Table 3: Outline of the number of worms injected for each tested community standardised to a specified OD600nm value.**

| Treatment | Number of worms | OD 600nm |
|---|---|---|
| Ancestor | 10 | 0.1 |
| Control (C1) | 10 | 0.1 |
| Fungicide (F1) | 10 | 0.1 |
| Increasing Temperature (W1) | 10 | 0.1 |
| Temperature + Fungicide (WF1) | 10 | 0.1 |
| PAO1 (Positive Control) | 10 | 0.1 |
| PBS (Negative Control) | 10 | 0.1 |
| No Injection (Negative Control) | 10 | 0.1 |

**Antibiotic Disc Diffusion Assay**

The protocol used to perform the antibiotic disc diffusion assay was based on the protocol defined by the EUCAST guidelines (105). However, it was necessary to make some modifications from the EUCAST guidelines as they state the bacteria should be grown at 37°C. This is generally not within the optimum temperature range of soil bacteria (104). Therefore, isolates were grown at 28°C in order for them to undergo normal growth. 72 total taxa were tested from the 4 treatments (6 isolates per 3 replicate communities per each of 4 treatments). The isolates were streaked individually from frozen stocks onto a LB agar plate. A single colony was taken from each isolate and added to 6ml of LB media in 30ml universal tubes for 18 hours at 28°C at 180 rpm. The isolates were then standardised to 0.5 McFarland standard using PBS in 1.8ml microfuge tubes (Sigma), which is 0.08-0.1 OD600nm. A cotton swab was then dunked into the microfuge tube and used to lawn the Mueller Hinton agar plate. The antibiotic discs were placed onto the agar within 15 minutes of spreading, in accordance with the EUCAST guidelines (105). The antibiotic discs were placed on the surface of the MH2 plates using an antimicrobial susceptibility disc dispenser (Oxoid). The antibiotics tested were ciprofloxacin (5µg), chloramphenicol (30µg), tobramycin (10µg), gentamicin (30µg), piperacillin/tazobactam (36µg), and meropenem (10µg). These antibiotics were chosen as they are from several different antibiotic classes with varying modes of action. The plates were left to grow for 18 hours at 28°C. The diameter of the zones of inhibition were recorded using a ruler immediately after removing the plates from the incubator. *E. coli* was used as a control. Two technical replicates for

each isolate were performed in each of the two biological replicates carried out for this experiment.

### 3) Testing how treatment affects ecosystem functioning of evolved communities (growth and health of a crop plant)

**Barley Growth / Health Assays**

For this assay it was tested how whole evolved communities fared in their ability to promote plant growth and health. The effect of all 32 evolved communities (8 community replicates from the 4 treatments) were assessed (**Figure 1**).

Two different methods were used to assess the effect the evolved soil communities had on the growth and health of the barley plant (*Hordeum vulgare*). These methods were:

i) Direct Soil Inoculation
ii) Seed Inoculation

#### i) Direct Soil Inoculation Method

**Amplifying soil community stocks**

The evolved soil community stocks were amplified using the method derived from Hall *et al* (106). Firstly, 10g of soil was inserted into 30ml glass universal tubes (Fisher Scientific) and autoclaved twice at 121°C for 15 minutes. After autoclaving, the soil was left at room temperature for 5 days to allow for the reoxidation of toxic compounds, such as manganese, which can become soluble when exposed to high temperatures (107). After 5 days, 100µl of each community frozen stock was added to individual 30ml glass universal containing the 10g of soil. After inoculation the communities were allowed to re-establish in the soil for 7 days. A soil wash was performed after 7 days by adding 10ml of water with 10 X 5mm sterile glass beads to the soil. The tubes were vortexed for 60 seconds and then allowed to settle for 30 minutes. From this, 300µl was taken and added to another beaker containing 30g of autoclaved soil and was allowed to establish itself for 7 days. This step was necessary to ensure we had sufficient frozen soil community stocks to enable our experiment to be repeated if needed. All evolved soil communities were prepared under the same conditions (common garden conditions), as similar conditions would be used during plant growth experiments.

**Soil preparation for plant experiments**

To test how communities fared in promoting plant growth and health, soil inoculated with different evolved communities were established in individual pots. For each pot used in the experiment, 30g of soil was added to 30ml universals and steamed sterilised twice at 121°C for 15 minutes and left to 5 days to allow for the reoxidation of toxic compounds (107).

After 5 days, 300µl soil wash of each of the 32 evolved soil communities prepared previously (8 community replicates within the 4 treatments) were added to individual microcosms. 3 technical replicates of each community were prepared. The universals were then vortexed for 45 seconds and then incubated statically at 26°C at 60% humidity for 7 days to allow the bacteria to proliferate throughout the soil. After 3 days, the microcosms were vortexed again for 45 seconds and placed back into the incubator to complete their incubation time. This allows the community to fully proliferate throughout the soil microcosm. After 7 days, the full contents of each microcosm were added to their own plant pot (20oz cup, 12cm high, 6.5cm bottom diameter, 9.5cm top diameter). 96 plant pots were prepared consisting of 8 communities from each of the 4 treatments, with 3 replicates for each community (4 Treatments X 8 communities per treatment X 3 replicates = 96 pots) (**Figure 2**).

**Seed Preparation**

The variety of spring barley seed (*Hordeum vulgare*) used was "Curtis" (KWS UK LTD). The barley seeds were sterilised in 10% bleach for 5 minutes and then washed with tap water for 5 minutes before planting in each pot.

**Aphid Preparation**

To test whether the soil community's treatment affected the ability of plants to defend against pests, aphids were included in all the plant pots. The aphids (*Sitobian avenae*) were acclimatised for 7 days in a growth chamber set to 26°C to accustom them to the high temperatures. This ensures they are not stressed for the duration of the experiment and undergo standard behaviour. Mesh was glued on top of pot lids that had a 2.5cm hole on top to prevent aphids from escaping and contaminating other pots (Inn Supplies LTD) during the experiment.

**Planting Preparation**

A single sterilised seed was planted into each of the 96 pots prepared with soil communities described above. Using a sterile forceps, the seeds were sowed 1cm down into their pots and covered in soil. The forceps were sterilised with 70% ethanol after each seed was planted. 3ml of water was added to each pot prior to placing them in a growth chamber set at 26°C with 60% humidity. The growth chamber (MLR-352; PHCBI) was calibrated to a light cycle of 600 lumens for 16 hours and 0 lumens for 8 hours a day, to simulate the day-night cycle. All 96 pots were assigned a unique identification (ID) and were placed at random locations into the growth chamber. However, each replicate pot was placed onto a different shelf in the growth chamber. This was done to mitigate bias in growth rate, based on the seed's location within the growth chamber. The seeds were watered approximately every 3 days with 3-5ml of tap water over the course of the experiment.

**Growing Stage**

The planted seeds were allowed to grow for 6 days. On day 6 of the experiment, the height of the plant was measured and 2 fourth instar aphids were then added. On day 12, the height of the plant was measured, and the number of adult, offspring, and winged aphids were counted. These parameters were again measured on day 16. On day 20, these parameters were measured again, along with root length. The shoots and roots were also cut, washed with tap water, inserted into a paper bag, and placed in an incubator set to 50°C. and they were then left to dry for 5 days. The dried roots and shoots were then weighed to calculate dry biomass. The growing stage lasted 20 days with 14 of these days with the presence of aphids (**Table 4**).

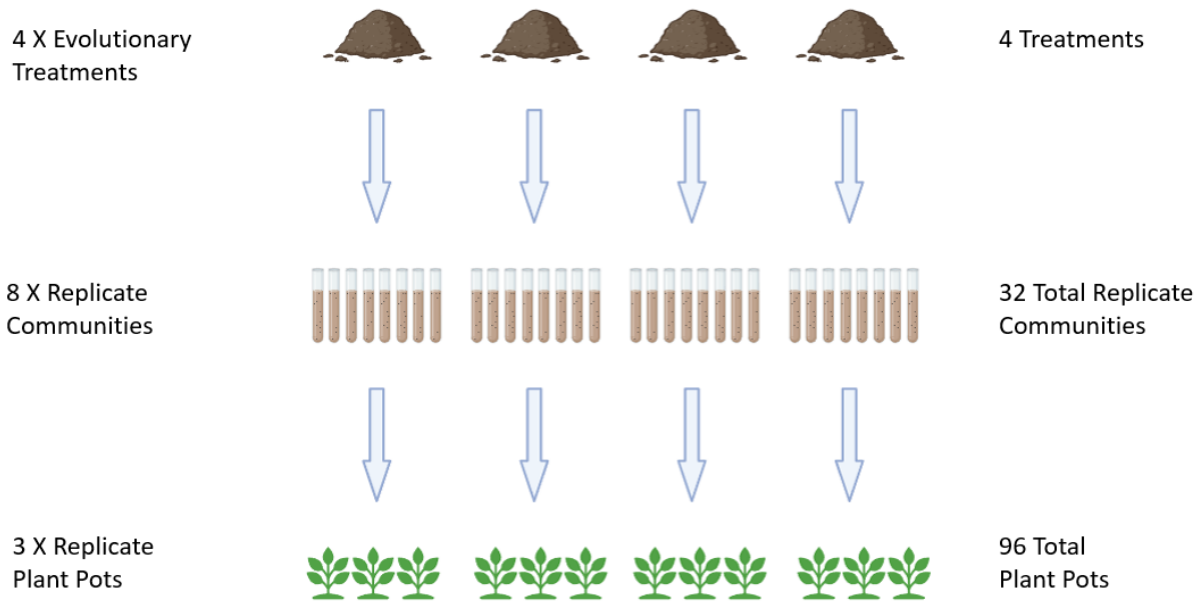


**Figure 2: Outline of the experimental design to test how treatment shapes plant growth and health**. Eight replicate communities from 4 treatments were inoculated into individual plant pots. Each community was replicated 3 times, giving a total of 96 pots. Image created in BioRender.

**Table 4: Timeline of direct soil inoculation experiment with activities performed on each day.**

| Day 0 | Day 6 | Day 12 | Day 16 | Day 20 |
|---|---|---|---|---|
| Plant seeds into soil inoculated with evolved communities | Measure plant height + add 2 fourth instar aphids | Measure plant height + count adult, offspring, and winged aphids | Measure plant height + count adult, offspring, and winged aphids | Measure plant height + count aphids + measure root length + dry shoots and roots for dry biomass |

### ii) Seed Inoculation Method

This experiment is similar to the direct soil inoculation experiment. However, instead of inoculating the bulk soil with the evolved soil communities, the seeds were instead inoculated with the evolved communities before planting into sterilised soil. This experiment was performed to determine if only the seeds need to be inoculated with the communities tested rather than the soil to have an effect on the growth of the barley plant.

The barley seeds were sterilised with 10% bleach for 5 minutes and then washed for a further 5 minutes in tap water. Each bacterial community was diluted to a 1/10 dilution with 500µl into 4.5ml PBS into a 50ml Falcon tube. 3 barley seeds were then placed into each

evolved soil community solution and left to soak for 2 hours. After 2 hours, the 3 seeds were placed with a sterile forceps directly into a single pot (9cm tall, 6.5cm bottom diameter, 9.5cm top diameter; Modiform) containing 150g of sterile John Innes No. 2 soil. 3 seeds instead of 1 seed were used to ensure some results were obtained as there were time constraints for the equipment used. The forceps were sterilised with 70% ethanol after each set of seeds. After the seeds were planted, 45ml of tap water was added to the pots and a further 45ml was added approximately every 3 days after that. The seeds were left to grow for 20 days in a growth chamber (MLR-352; PHCBI) set at 26°C at 60% humidity with a day-night cycle of 15 hours at 600 lumens and 9 hours at 0 lumens. Unlike the direct soil inoculation experiment, no aphids were added during this experiment, due to time constraints. The height of the plants was measured after 6, 12, 17, and 20 days. After day 20 the shoots were cut, washed with tap water, put into paper bags, and placed within an incubator at 50°C for 5 days to dry. The dried shoots were then weighed to calculate dry biomass **(Table 5)**.

**Table 5: Timeline of seed inoculation experiment with activities performed on each day.**

| Day 0 | Day 6 | Day 12 | Day 17 | Day 20 |
|---|---|---|---|---|
| Plant seeds into inoculated soil | Measure plant height | Measure plant height | Measure plant height | Measure plant height + dry shoots and roots for dry biomass |

**Statistical Analysis**

R version 2023.12.1 (108) was used for all statistical analyses associated with the project. To examine the impact of the soil treatments on the MIC of Fubol Gold and antibiotic susceptibility, we employed Kruskal-Wallis rank sum tests for each antibiotic. Subsequently, we conducted Dunn's tests with Bonferroni adjustment to evaluate differences between treatments. These analyses were performed using the 'dunn.test' package (109). Additionally, to assess the influence of varying temperature on the evolved soil communities, we conducted ANOVA analyses followed by post hoc Tukey tests. In these tests, we used the average OD as our response variable, with temperature and treatment serving as factors.

Moreover, a similar approach was employed for the soil inoculation test, where an ANOVA test was performed followed by a Tukey test, using shoot height on each day as a response variable and the treatments as factors. Similarly, ANOVA tests were conducted to assess

shoot biomass, root biomass, and root length. Additionally, ANOVA tests were utilised to test overall aphid numbers for each treatment as well as aphid numbers on each day. Furthermore, a Spearman's rank correlation test was carried out to examine the correlation between plant height as well as aphid numbers on day 20.

Quality control measures were taken to ensure the reliability of results. Various tests including plotting the residual *versus* fitted data and normal Q-Q tests were conducted to assess the data for normality. All graphs were designed within R-studio utilising the R packages "ggplot2" (110) and "gridExtra" (111).

# Results

## 1) Examining individual taxa adaptation to stressors (increased temperatures and fungicide)

The first objective was to evaluate how each of the isolates taken from the evolved soil communities has adapted to the anthropogenic stressors they were exposed to in the evolution experiment (fungicide and warming/increasing temperatures) (89). Additionally, we aimed to determine whether 6 months of exposure to these stressors is sufficient time to cause evolved changes in the phenotype of constituent taxa. To achieve this, 72 soil isolates (6 isolates from each 3 communities, taken from 4 treatments) were measured on their capacity to grow in the presence of fungicide (Fubol Gold), and to proliferate whilst experiencing varying temperature environments (24°C, 28°C, 34°C) under common garden conditions. Note that individual isolates were used in this experiment as assaying entire communities, a) only gives an "average" value across all community members, which could be misleading, if for example division of labour is occurring in communities, and b) ecological filtering during the community cultivation process in LB media could miss-represent community dynamics in soil.

### Testing whether isolates from different treatments differ in their Fubol Gold Minimum Inhibitory Concentration

In this study, the MIC of Fubol Gold was evaluated for all 72 soil isolates. In some technical replicates for certain isolates, the positive control did not grow. This removed 25 datapoints across 10 isolates. In some cases, the positive control of all replicates for a given isolate failed to grow (e.g. in the community C5). However, in other cases, only one or some of the technical replicates failed to grow. Isolates were omitted from analyses when they did not grow in any technical or biological replicate.

It was found that the MIC of soil isolates differed depending on the treatment from which they originated (Kruskal Wallis rank sum test; $X^2= 16.341$, df= 3, p= 0.0009652). Post hoc analysis revealed this difference was largely driven by increased MIC in communities evolving under fungicide conditions (Dunn test; control *versus* fungicide; $p<0.01$) (Dunn test; control *versus* fungicide + temperature; $p<0.01$) compared to the control.

However, isolates from the increasing temperature/warming treatment did not have any significant difference relative to the stress-free control isolates (Dunn test; control *versus* temperature; $p<0.01$). (**Figure 3, Table 6**).

**Table 6: Dunn test results for fungicide stress adaptation of treatments compared to the stress-free control treatment**.

| Comparison | P-Value | Adjusted P-Value | Significance |
|---|---|---|---|
| Control *Versus* Fungicide | 0.003273112 | 0.0196 | ** |
| Control *Versus* Temperature | 0.497913205 | 1.00 | Ns |
| Control *Versus* Temperature + Fungicide | 0.001633669 | 0.00980 | ** |

Asterisks signify significant differences between treatments (* = p<0.05, ** = p<0.01, Ns = Not Significant).

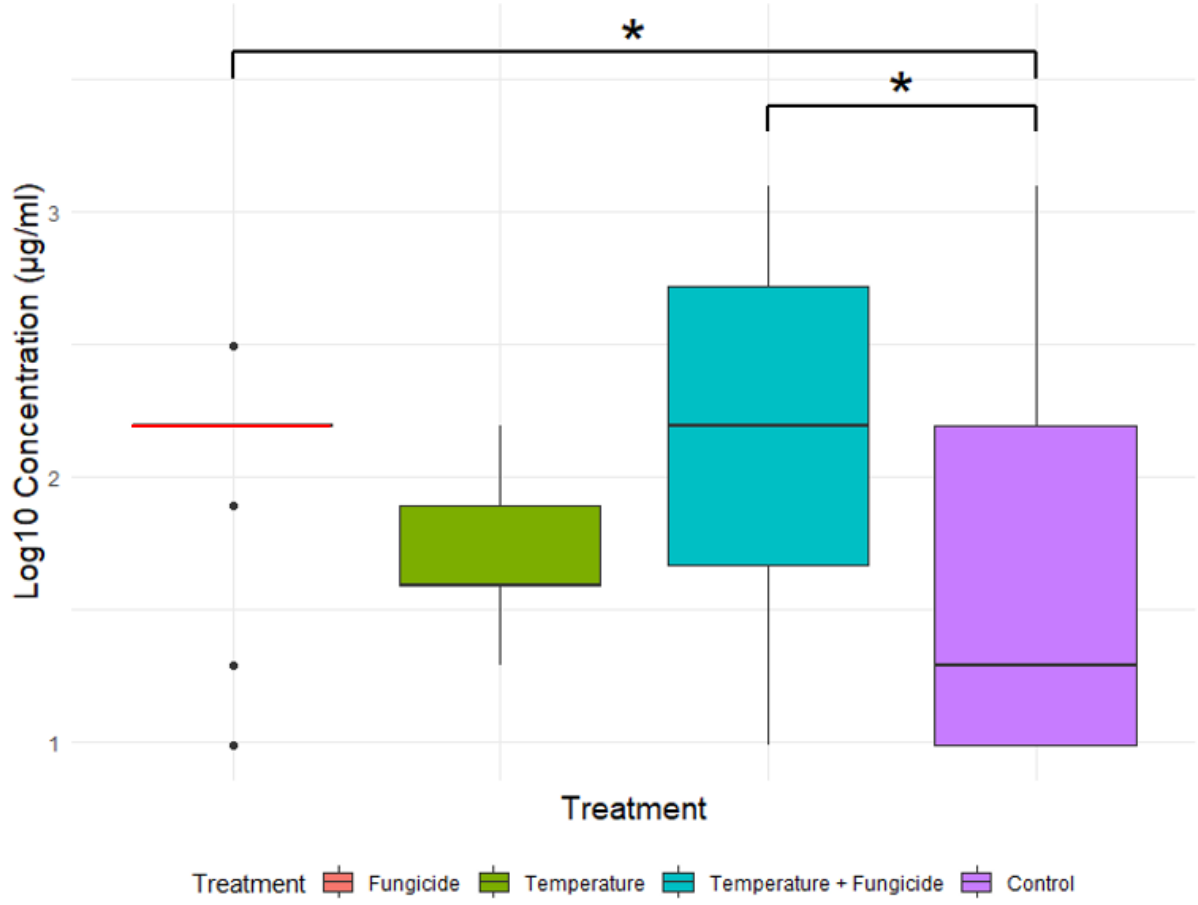


**Figure 3: Log10 MIC of Fubol Gold (µg/ml) performed on isolates from each of the evolved soil treatments.** 18 isolates were tested for each treatment (6 isolates from 3 different communities). Both the fungicide and the combination treatment (increasing temperature + fungicide) had a greater MIC value compared to the control. This indicates that fungicide exposure increases fungicidal resistance. Asterisks signify differences between treatments (* = p<0.05).

The MIC values of the six isolates from the same community tend to exhibit large variation. The non-transformed MIC values for the isolates used to create Figure 3 are highlighted below **(Table 7 + 8)**. Table 7 highlights the MIC for the total number isolates within each treatment, whilst Table 8 divides the information further into the MIC value of the isolates

within each replicate community. For example, the control community contains isolates with MIC values ranging from 9.7µg/ml to 1250µg/ml (**Table 7**). However, it is displayed that this only occurs in the community replicate C7 (warming + fungicide treatment) (**Table 8**).

It is important to note that the isolates have not been sequenced and they may belong to the same or different species, genera, or families of bacteria. The goal here was not to identify species that increase MIC against Fubol Gold, but rather to determine whether we see broad shifts towards increased fungicide tolerance in fungicide-evolved populations. These variations may be occurring *via* ecological filtering for already less susceptible taxa, or evolution within taxa towards enhanced tolerance to fungicide.

**Table 7: The 18 isolates from each treatment were categorized according to their mean Fubol Gold Minimum Inhibitory Concentration (MIC).**

| Treatment | Number of Isolates | Concentration µg/ml | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 9.7 | 19.5 | 39 | 78 | 156 | 312 | 625 | 1250 |
| **C** | **17** | 8 | 1 | 1 | 2 | 2 | 2 | - | 1 |
| **F** | **18** | 1 | 1 | - | 2 | 11 | 2 | - | - |
| **W** | **18** | - | 3 | 9 | 5 | 1 | - | - | - |
| **WF** | **18** | 2 | - | 3 | 1 | 4 | 3 | 4 | 1 |

The values in the table denote the number of isolates (out of a maximum 18) within the evolved community exhibiting that MIC at the specified concentration. Note the control treatment has 17 isolates since one of the isolates did not grow for any technical replicate performed. (C=Control, F=Fungicide stress, W=Warming, WF= Warming + Fungicide)

**Table 8: 6 isolates from each of the 3 communities per treatment were categorized according to their mean Fubol Gold Minimum Inhibitory Concentration (MIC).**

| Treatment | Number of Isolates | Community | Concentration µg/ml | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 9.7 | 19.5 | 39 | 78 | 156 | 312 | 625 | 1250 |
| Control | 6 | C4 | 4 | - | 1 | - | 1 | - | - | - |
| | 5 | C5 | 3 | - | - | - | 1 | 1 | - | - |
| | 6 | C7 | 1 | 1 | - | 2 | - | 1 | - | 1 |
| Fungicide | 6 | F1 | 1 | 1 | - | - | 2 | 2 | - | - |
| | 6 | F2 | - | - | - | 1 | 4 | 1 | - | - |
| | 6 | F5 | - | - | - | 1 | 5 | - | - | - |
| Increasing Temperatures | 6 | W1 | - | 1 | 1 | 3 | 1 | - | - | - |
| | 6 | W2 | - | 1 | 4 | 1 | - | - | - | - |
| | 6 | W7 | - | 1 | 4 | 1 | - | - | - | - |
| Increasing Temperatures + Fungicide | 6 | WF1 | - | - | - | - | 1 | 3 | 2 | - |
| | 6 | WF4 | - | - | 3 | 1 | - | - | 1 | 1 |
| | 6 | WF5 | 2 | - | - | - | 3 | - | 1 | - |

The values in the table denote the number of isolates within the evolved community (maximum 6) exhibiting that MIC at the specified concentration. Note: The community C5 only has 5 isolates since one of the isolates did not grow for any technical replicate performed. (C=Control, F=Fungicide stress, W=Warming, WF= Warming + Fungicide)

## Testing whether isolates from different treatments differ in their temperature stress adaptation

The ability of the evolved soil isolates to grow under varying temperature conditions was investigated next. All 72 isolates were cultivated under three different temperature conditions: 24°C, 28°C, and 34°C, with cell density (OD600nm) measured after 0, 24, and 48 hours (**Figure 4**).

At 24 hours, it was found that the temperature condition (24°C, 28°C, 34°C) at which the isolates grew under significantly affected their growth, irrespective of treatment (2-way ANOVA; non-significant treatment *versus* temperature conditions ($F= 6.9423$, $df= 2$, $p= 0.0012$). From this a Tukey test was performed, and it was established that higher temperatures, 28°C (Tukey test; 24°C *versus* 28°C; $p<0.02$) and 34°C (Tukey test; 24°C *versus* 34°C; $p<0.001$), resulted in higher isolate growth than 24°C (2-way ANOVA; Temperature conditions; $F= 6.989$ $df= 2$ $p= 0.0015$). There was no significant difference between 28°C and 34°C (Tukey; 28°C *versus* 34°C; $p>0.05$)

Despite the fact that the temperature conditions under which the isolates grew affected their growth and was not influenced by treatment, significant differences were found between treatments when temperature conditions were disregarded. Specifically, only the isolates from the fungicide evolved treatment grew significantly better than all other evolved

treatments (**Table 9**) including the stress-free control (Tukey test; fungicide *versus* control; $p<0.0001$).

However, after 48 hours, the same effect of temperature conditions was not observed. Irrespective of treatment, there was no significance between treatments at any temperature (2-way ANOVA; ($F= 2.3442$, $df= 2$, $p= 0.0985$). Similarly, significant differences were found between treatments when temperature conditions were disregarded. Again, the fungicide treatment grew better than all other treatments including the stress-free control. Furthermore, the increasing temperature (Tukey test; fungicide *versus* control; $p<0.0001$) and dual stressor treatment (Tukey test; fungicide *versus* control; $p<0.05$) had significantly more growth relative to the control treatment.

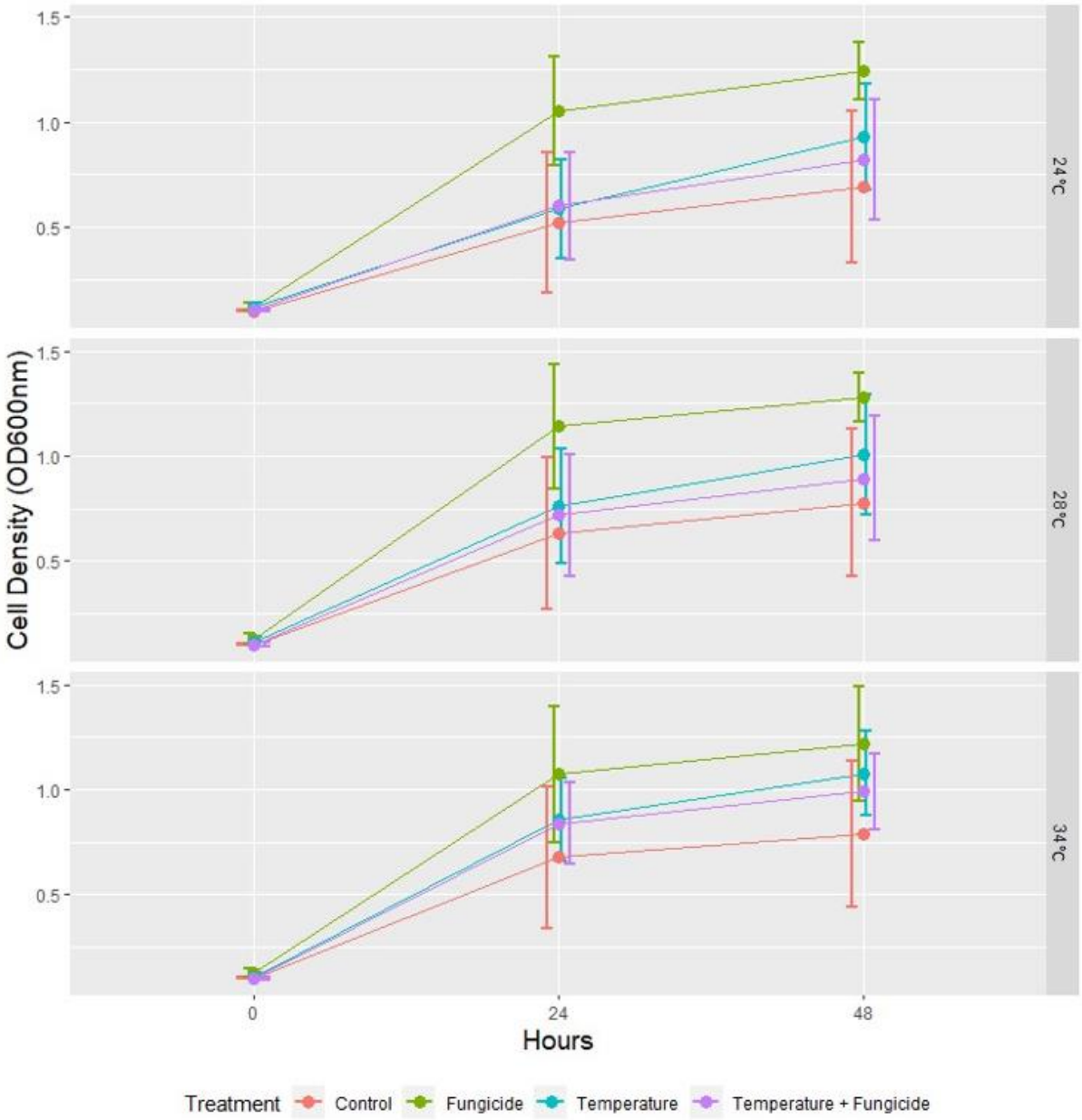


**Figure 4: Cell density of isolates from the evolved soil communities grown at 24°C, 28°C and 34°C.** The isolates were grown statistically in 96 well plates and their cell density was measured after 0, 24 and 48 hours at OD 600nm. The error bars indicate ± standard deviation.

**Table 9: Tukey test results highlighting interactions between treatments irrespective of growth temperature conditions**.

| Hours | Comparison | P-Value | Significance |
|---|---|---|---|
| 24 | Control *Versus* Fungicide | 0.00001 | *** |
| 24 | Control *Versus* Temperature | 0.098 | Ns |
| 24 | Control *Versus* Temperature + Fungicide | 0.187 | Ns |
| 24 | Fungicide *Versus* Temperature | 0.00001 | *** |
| 24 | Fungicide *Versus* Temperature + Fungicide | 0.00001 | *** |
| 24 | Warming *Versus* Temperature + Fungicide | 0.990 | Ns |
| 48 | Control *Versus* Fungicide | 0.00001 | *** |
| 48 | Control *Versus* Temperature | 0.00001 | *** |
| 48 | Control *Versus* Temperature + Fungicide | 0.022 | * |
| 48 | Fungicide *Versus* Temperature | 0.0001 | *** |
| 48 | Fungicide *Versus* Temperature + Fungicide | 0.00001 | *** |
| 48 | Warming *Versus* Temperature + Fungicide | 0.170 | Ns |

(* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, Ns = not significant)

## 2) Investigating how treatment shapes clinically important phenotypes (antimicrobial resistance and virulence)

The next aim assessed whether long-term exposure to the stressors of fungicide and increasing temperature stress affected community virulence and resistance to antibiotics. This was determined by (i) assessing the ability of whole evolved communities to establish an infection in a wax moth larval host and (ii) characterising antibiotic susceptibility for individual isolates from evolved communities. Note, the former determines whether the treatment selects for opportunistic pathogens in soil, which may be mediated through both evolutionary changes and compositional shifts.

### *Galleria mellonella* virulence assay

This experiment aimed to investigate the impact that evolution under fungicide and temperature stress has on the potential for a community to contain virulent species that may infect a wax moth larval host **(Figure 5).**

Due to the intensive nature of this experiment, only one replicate community could be tested per treatment, hence it was not possible to statistically test whether the treatments shaped survival. However, these preliminary results suggest that survival tends to split into

two groups depending on the treatment. The ancestral, the controls, and warming temperature communities killed all 10 of their hosts between 7 - 12 hours. Conversely, it took 19 hours until both fungicide and the dual stressor warming + fungicide communities killed all 10 wax moth hosts. This suggests that fungicide-mediated shifts in species composition and/or evolution within constituent species, reduces the likelihood of opportunistic pathogens establishing infections from these communities. However, PBS injection did cause mortality of the larvae (10/10 larvae died after 21 hours), which likely occurred either because the syringe was not adequately sterilized between injections, the injection method harmed the larvae, or that the PBS was contaminated. As a result, these findings point to some interesting trends, but they have major caveats and experiments should be repeated.

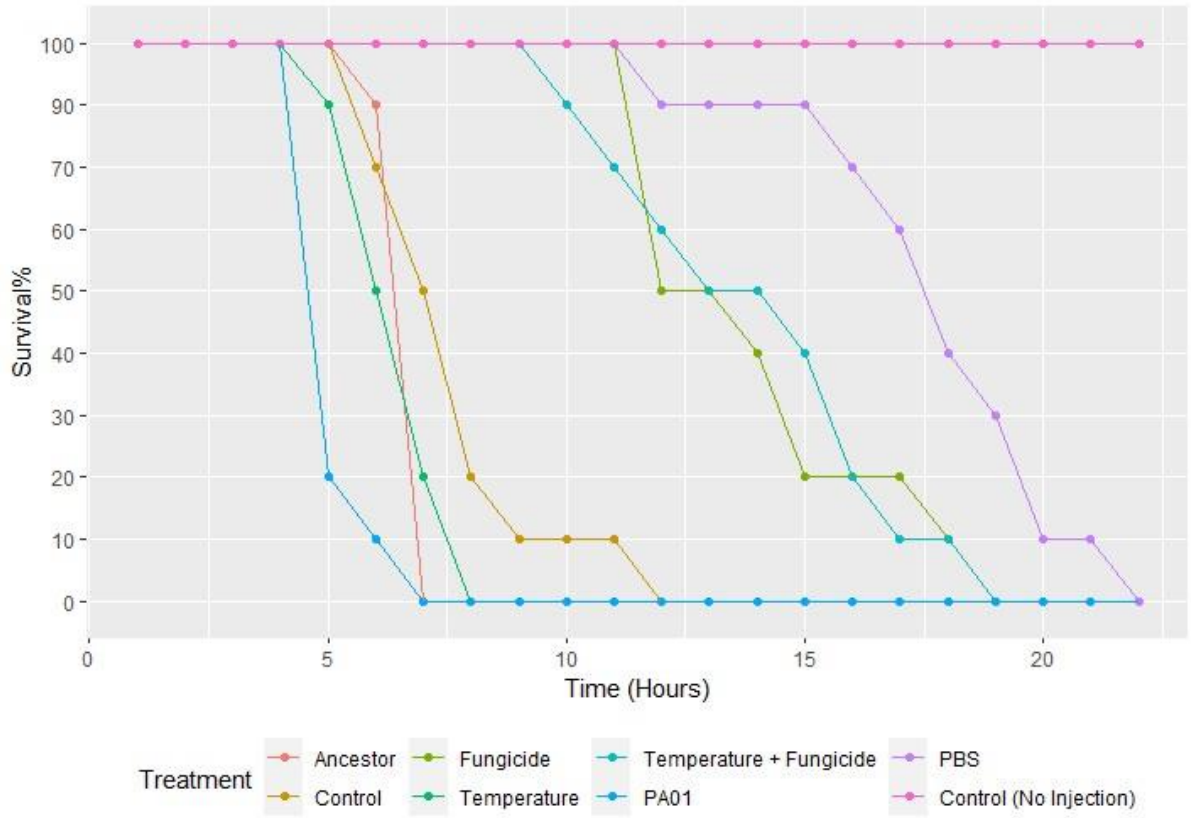


**Figure 5: Survival of wax moths (Galleria mellonella) after infection with five different soil communities (one ancestral and four evolved).** PAO1 and no injection were used as a positive and negative control respectively. Only 1 biological replicate was performed (n=1)

### Antimicrobial Resistance

Antimicrobial resistance was assessed using the antibiotic disc diffusion assay across all 72 soil isolates. The antibiotics chloramphenicol (30µg), tobramycin (10µg), gentamicin (30µg), piperacillin/tazobactam (36µg), meropenem (10µg) and ciprofloxacin (5µg), were

tested for their ability to inhibit a lawn of bacteria established from each individual isolate. Zones of inhibition varied greatly among isolates, ranging from 0mm to 40mm for all antibiotics tested. Note that when zone of inhibition = 40, the result is conservative, since extremely large zones of inhibition from different antibiotics merge together on the same testing plate.

For chloramphenicol, it was found that zones of inhibition were significantly affected by the treatment (Kruskal-Wallis rank-sum test, $X^2 = 26.695$, df= 3, $p<0.0001$) (**Figure 6**). Specifically, isolates from the increasing temperature treatment were more susceptible to chloramphenicol than isolates exposed to the dual stressor (W *versus* WF; Dunn test, p= 0.00235), fungicide-only (W *versus* F; Dunn test, p= 0.0000929), and the stress-free control (W *versus* C; Dunn test, p= 0.0000269) treatments. Hence, warming either increased or maintained susceptibility to chloramphenicol. Note, it is not possible to elucidate whether susceptibility increased or is maintained because isolates from the ancestral community were not assayed here. However, 54% of the isolates were completely resistant (zone of inhibition = 0) to chloramphenicol.

For gentamicin, it was found that zones of inhibition were significantly affected by treatment (Kruskal-Wallis rank-sum test, $X^2 = 20.067$, df= 3, $p<0.000164$). Similarly, isolates from the increasing temperature treatment were more sensitive to gentamicin than isolates exposed to warming + fungicide (W *versus* WF; Dunn test, p= 0.0058), fungicide-only (W *versus* F; Dunn test, p= 0.0003), and the stress-free control (W *versus* C; Dunn test, p= 0.0004). Thus, increasing temperature stress either increased or maintained susceptibility to gentamicin.

The zones of inhibition for piperacillin/tazobactam exhibited significant variation in response to the treatment (Kruskal-Wallis rank-sum test, $X^2 = 9.8825$, df= 3, $p<0.01959$). Isolates from the increasing temperature treatment were significantly more sensitive to gentamicin that isolates from the stress-free control only (W *versus* C; Dunn test, p= 0.0123). Therefore, increasing temperature stress either increased or maintained susceptibility to piperacillin/tazobactam.

The treatment used had a notable impact on the zones of inhibition for meropenem (Kruskal-Wallis rank-sum test, $X^2 = 10.55$, df= 3, $p<0.01443$). There was no significance observed between any of the stress evolved isolates compared to the stress-free control. However, isolates from the increasing temperature treatment were significantly more

sensitive to meropenem than that of isolates from the fungicide-only treatment (W *versus* F; Dunn test, p= 0.0061). There was no significant difference between any other treatment.

No significant differences were observed among any of the treatments for the antibiotics tobramycin (Kruskal-Wallis rank-sum test, $X^2$ = 0.31058, df= 3, P=0.958) and ciprofloxacin (Kruskal-Wallis rank-sum test, $X^2$ = 1.033, df= 3, p=0.7933)

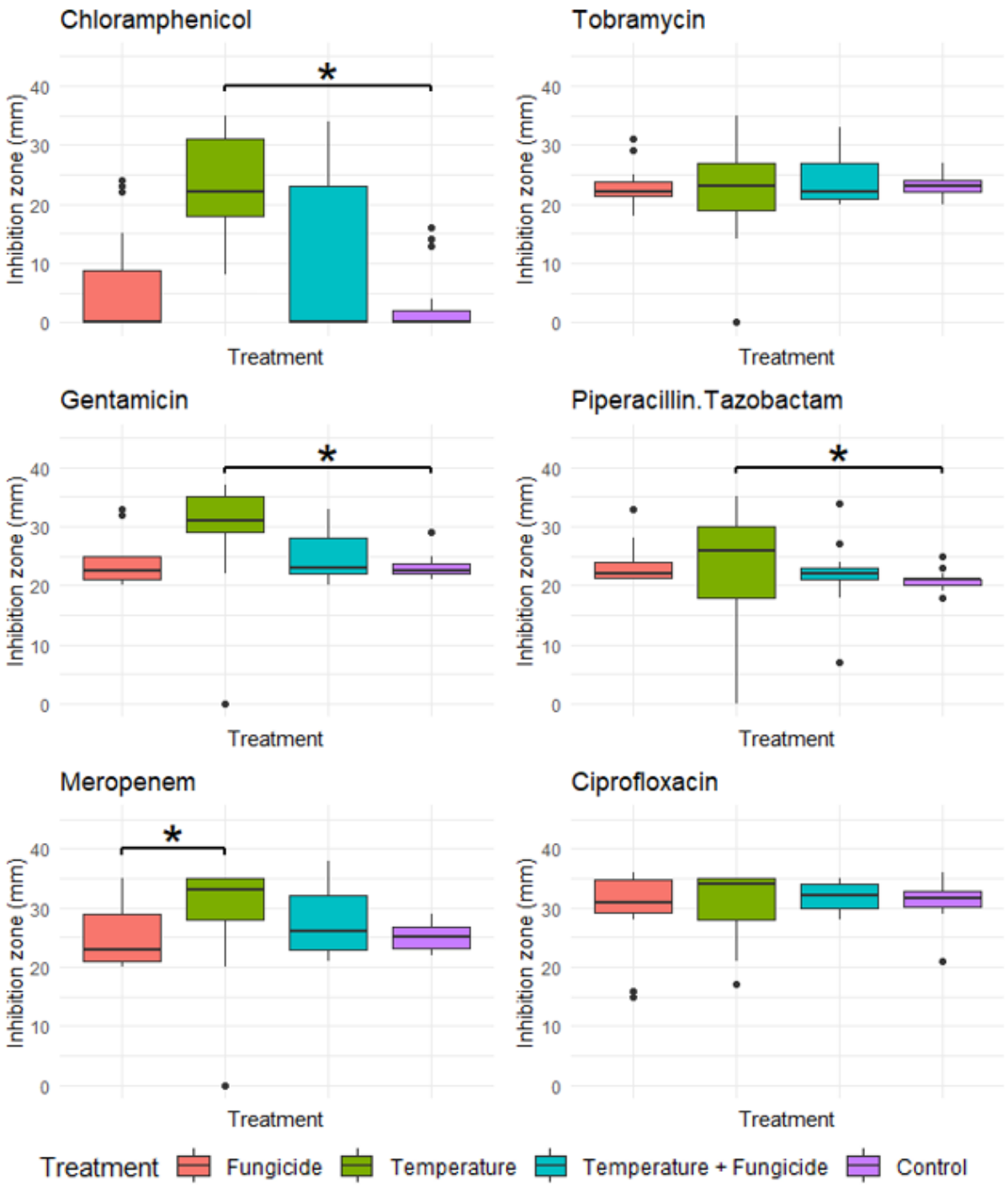


**Figure 6: Antibiotic susceptibility testing using the disc diffusion method on isolates from different treatments.** Data represents the zone of inhibition (mm) for each treatment. The disc content for each antibiotic consisted of ciprofloxacin (5µg), chloramphenicol (30µg), tobramycin (10µg), gentamicin (30µg), piperacillin/tazobactam (36µg), and meropenem (10µg). Significant stars signify a statistical difference between treatments, based on Dunn test results. Asterisks signify significant differences between treatments (* = $p<0.05$).

**Table 10: Dunn Test results, highlighting the interactions between treatments compared to the stress-free control treatment for each antibiotic.**

| Antibiotic | Comparison | Test Statistic | Adjusted P-Value | Significance |
|---|---|---|---|---|
| Chloramphenicol | Control *Versus* Fungicide | 0.387 | 1 | Ns |
| Chloramphenicol | Control *Versus* Temperature | 0.000002 | 0.000012 | * |
| Chloramphenicol | Control *Versus* Fungicide + Temperature | 0.117 | 0.706 | Ns |
| Tobramycin | Control *Versus* Fungicide | 0.305 | 1 | Ns |
| Tobramycin | Control *Versus* Temperature | 0.34 | 1 | Ns |
| Tobramycin | Control *Versus* Fungicide + Temperature | 0.338 | 1 | Ns |
| Gentamicin | Control *Versus* Fungicide | 0.455 | 1 | Ns |
| Gentamicin | Control *Versus* Temperature | 0.0000715 | 0.0004 | * |
| Gentamicin | Control *Versus* Fungicide + Temperature | 0.254 | 1 | Ns |
| Piperacillin/Tazobactam | Control *Versus* Fungicide | 0.00586 | 0.0352 | Ns |
| Piperacillin/Tazobactam | Control *Versus* Temperature | 0.00204 | 0.0123 | * |
| Piperacillin/Tazobactam | Control *Versus* Fungicide + Temperature | 0.0286 | 0.171 | Ns |
| Meropenem | Control *Versus* Fungicide | 0.233 | 1 | Ns |
| Meropenem | Control *Versus* Temperature | 0.00888 | 0.053 | Ns |
| Meropenem | Control *Versus* Fungicide + Temperature | 0.191 | 1 | Ns |
| Ciprofloxacin | Control *Versus* Fungicide | 0.381 | 1 | Ns |
| Ciprofloxacin | Control *Versus* Temperature | 0.163 | 0.978 | Ns |
| Ciprofloxacin | Control *Versus* Fungicide + Temperature | 0.292 | 1 | Ns |

Note: For the Dunn test $p \leq 0.025$ is needed to reject $H_0$. Asterisks signify significant differences between treatments (* = $p < 0.025$, NS = Not Significant).

### 3) Testing how treatment affects ecosystem functioning of evolved communities (growth and health of a crop plant)

Finally, the whole evolved soil communities from each of the treatments were evaluated on their ability to promote crop growth and crop defence of the barley plant (*Hordeum vulgare*). This involved a 20-day cultivation period, during which various growth and health metrics were recorded. These metrics were indicators of crop growth and health and included shoot height, shoot biomass, root length, root biomass, and their ability to combat aphids.

#### i) Crop Growth

The ability to promote crop growth and health is an important function of the soil microbiome. This experiment investigated whether prolonged exposure of the soil community to the stressors, fungicide and increasing temperatures, alters the community's function at promoting crop health. The assessment focused on determining the effectiveness of evolved soil communities in promoting crop growth, utilizing shoot height, shoot biomass, root length, and root biomass as key indicators.

**Shoot height:** Shoot height did not differ between treatments on day 6 (Day 6 ANOVA: $F= 1.7896$, $df= 3$, $p>0.05$) or day 12 (Day 12 ANOVA: $F= 1.8689$, $df= 3$, $p>0.05$). However, at day 16 and 20, shoot height significantly differed between treatments (Day 16 ANOVA: $F= 2.6046$, $df= 3$, $p= 0.0634$), (Day 20 ANOVA: $F= 4.0755$, $df= 3$, $p= 0.0121$). At day 16, these differences were specifically driven by the fungicide-only treatment compared to the control (Tukey Post-hoc test; Fungicide *versus* control; $p<0.05$). At day 20, both the fungicide-only treatment and the fungicide + temperature treatment both had significantly increased shoot height relative to controls (Tukey post hoc test, control *versus* fungicide, $p<0.01$; control *versus* fungicide + temperature, $p<0.05$) **(Figure 7)**.

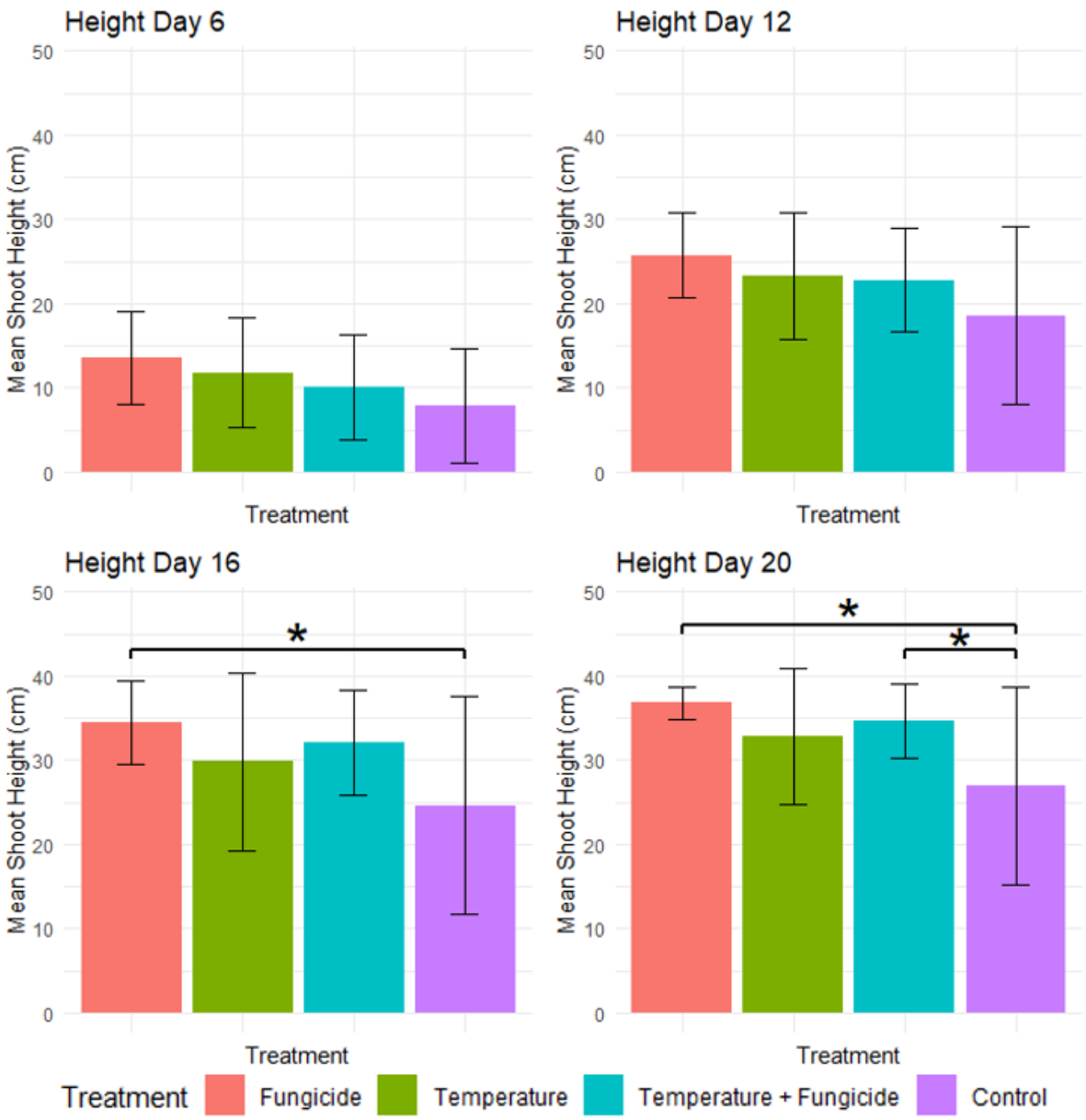


**Figure 7: Shoot height of barley plants where the soil was directly inoculated with evolved soil communities from four different treatments.** The plants were left to grow for 20 days, and their shoot height was recorded after 6, 12, 16 and 20 days. Error bars signify the ± standard deviation of shoot height. Significant differences between treatments are indicated by asterisks (* = $p<0.05$).

**Shoot biomass:** Shoot dry biomass at day 20 differed between treatments (($df= 3$, F-value=2.829, $p<0.05$). Specifically, communities that had evolved in the presence of fungicide-only, caused plants to have increased shoot biomass compared to the control communities (Tukey test, $p<0.05$). However, unlike shoot length data (above) there was no difference between fungicide + temperature treatment and the control (Tukey test, control *versus* fungicide + temperature, $p>0.05$). This reinforces that soil inoculated with microbes from fungicide-evolved communities promote crop growth, although this effect may be reduced or lost when fungicide is imposed alongside increasing temperatures (**Figure 8**).

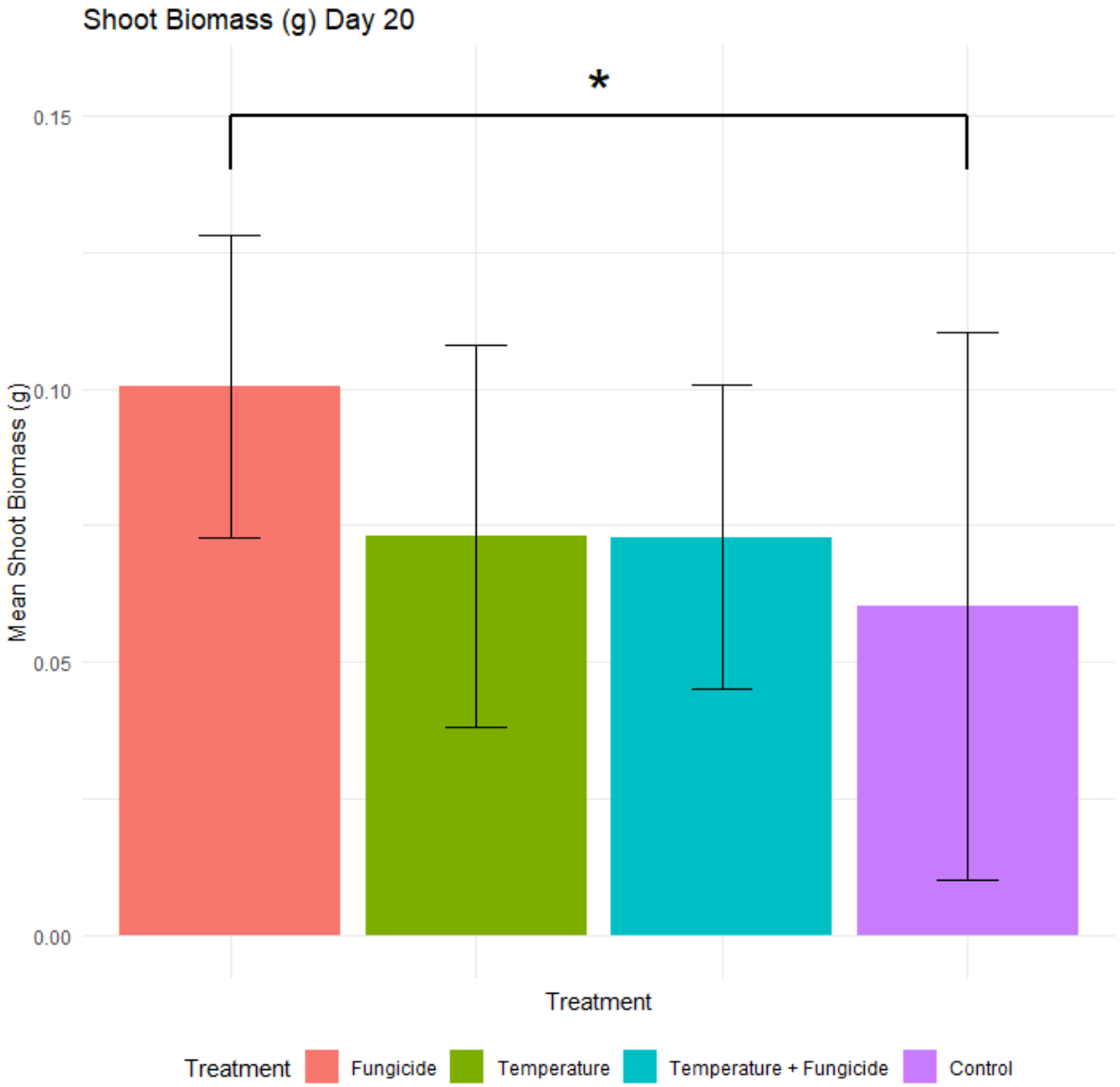


**Figure 8: Shoot biomass of the barley plants exposed to different evolved soil communities.** Fungicide-evolved communities conferred increased shoot biomass control communities. Error bars signify the ± standard deviation of plant height. Asterisks signify significant differences between treatments (* = $p < 0.05$).

**Root length and biomass:** Root length and biomass were additional factors considered to assess plant health **(Figure 9).** On day 20, root length was measured to evaluate the impact of evolved soil communities on plant roots. However, no significant differences were found among treatments for root biomass (ANOVA; df= 3, F-value=1.8403, $p>0.05$) or root length (ANOVA; df= 3, F-value= 51.332, $p>0.05$).

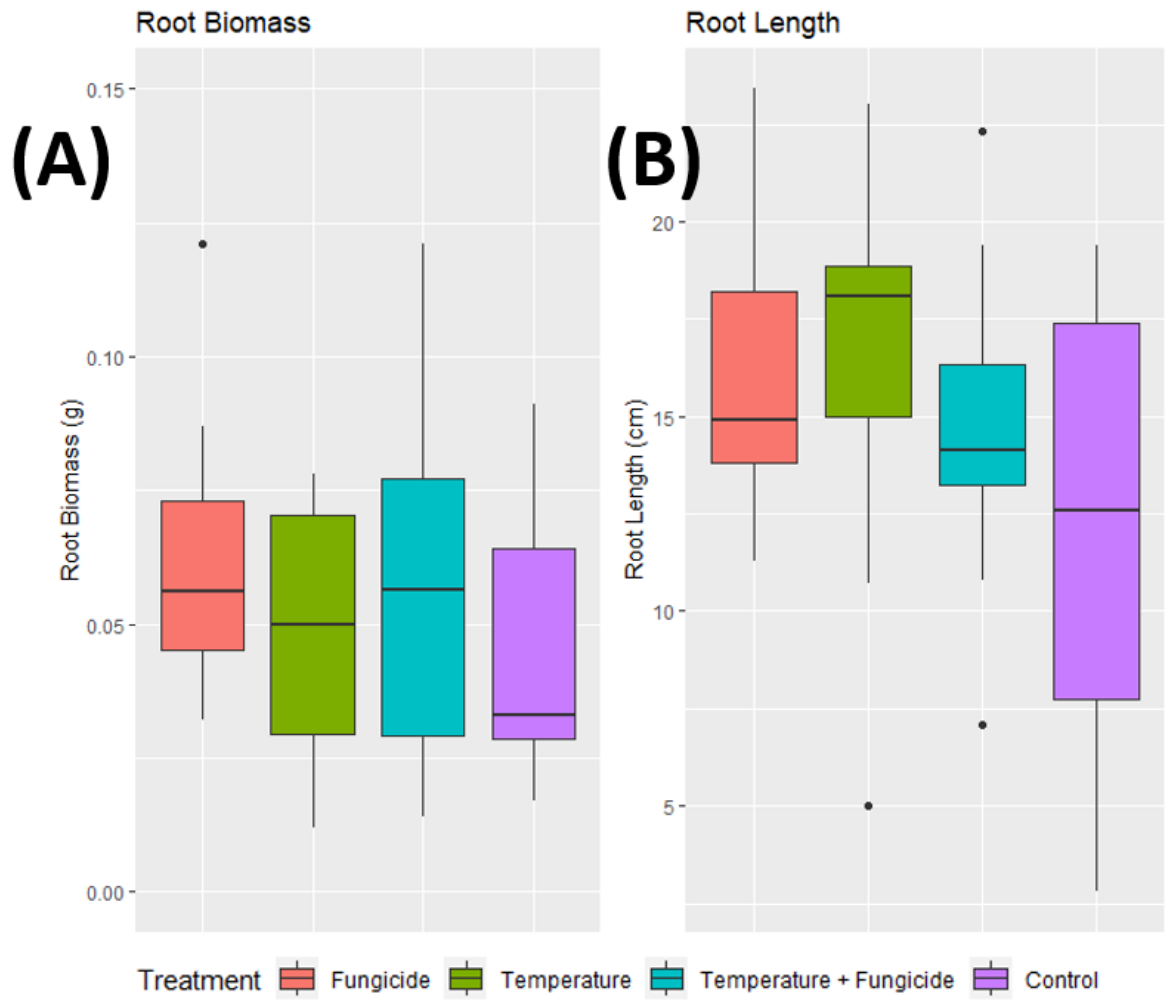


**Figure 9: Root biomass (g) (A) and root length (cm) (B) of barley plants inoculated with microbial communities that had evolved under 4 different treatment conditions.** Root length and biomass was collected on day 20. No significant differences are observed for either root biomass or length between treatments.

**Seed Wash**

After completing the initial experiment, we conducted a seed wash assay to explore whether subjecting the evolved soil communities exclusively to the seeds before planting would produce comparable effects on shoot height, like those observed with direct soil inoculation. In contrast to the outcomes observed with direct soil inoculation, this method did not yield any statistically significant effects across the different treatments on any day, even on day 20 (ANOVA, day 20, df= 3, F-value= 1.4487, $p>0.05$) (**Figure 10**). It is important to note that seeds inoculated with identical community replicates were planted into pots containing 150g of soil, as opposed to the 30g of soil used in the direct soil inoculation experiment. This discrepancy in soil volume may have influenced the results, introducing varied conditions that could have potentially impacted the results.

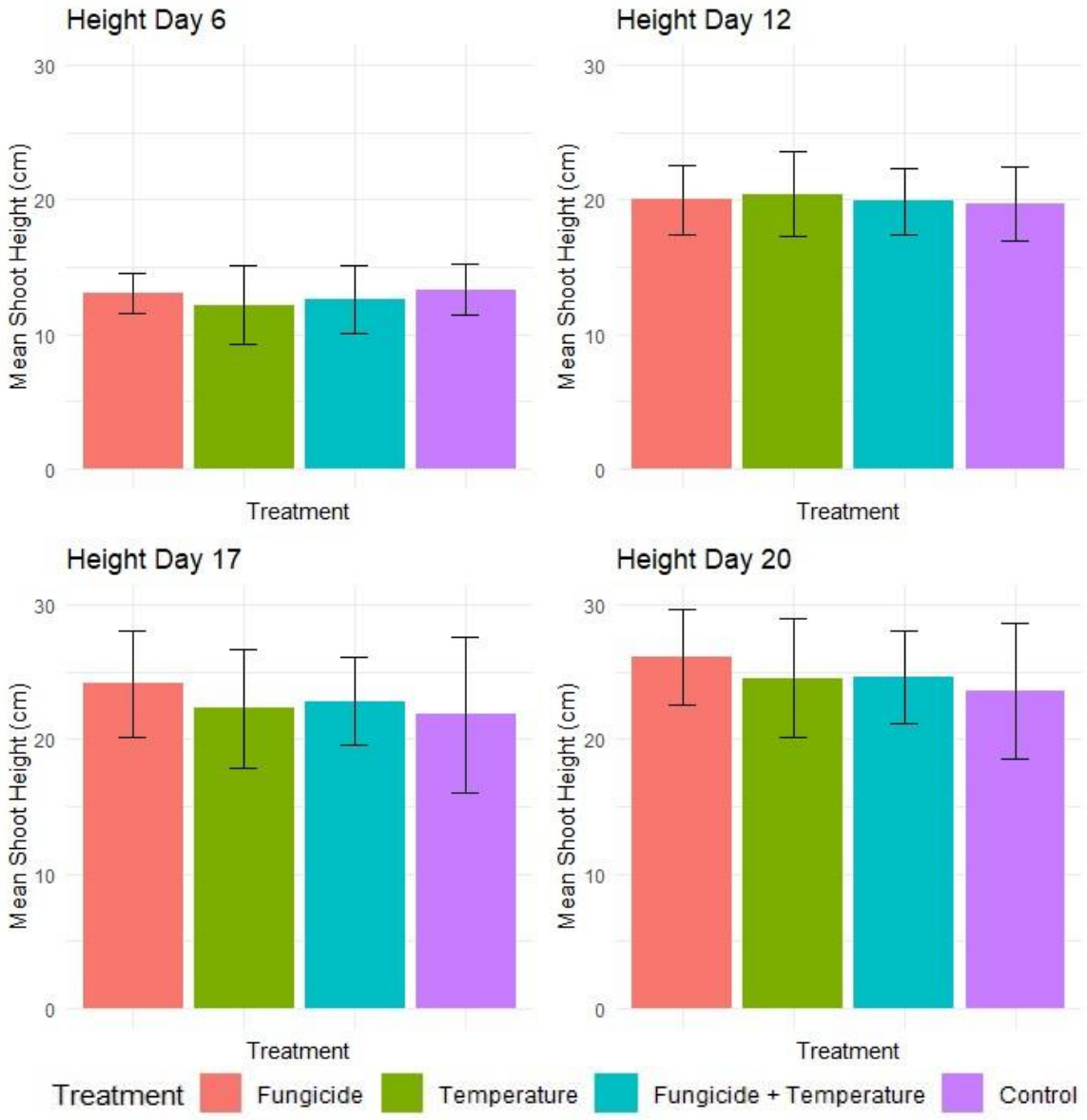


**Figure 10: Barley seeds were inoculated with evolved soil communities and allowed to grow for 20 days in sterile soil.** Shoot height measurements were taken at 6, 12, 17, and 20 days. Statistical analysis revealed no significant differences between treatments at any time point throughout the. Error bars signify the ± standard deviation of plant height.

### ii) Crop Defence

Finally, we wanted to determine if exposing the soil in which the crops grow, to the evolved communities influenced crop health, in particular defence against herbivores. Our focus was on aphids (*Sitobian avenae*), which are sap feeding herbivores. We wanted to assess if soil microbial communities differed in their ability to protect the plant from aphids (*i.e.* more aphid-suppressive communities should have fewer aphid numbers). Two aphids in

their fourth instar stage were added to each barley plant on day 6 of the barley growing experiment and were allowed to proliferate freely. The aphids were counted on days 12, 16 and 20 of the experiment (**Figure 11**). There was no effect of community treatment on aphid numbers on day 12 (day 12 ANOVA; $F = 1.4752$, $df= 3$, $p= 0.234$), day 16 (day 16, ANOVA; $F = 0.3366$, $df= 3$, $p= 0.7989$) or day 20 (day 20 ANOVA; $F = 1.2609$, $df= 3$, $p= 0.2991$) of the experiment (**Table 11**).

There was a positive correlation between shoot height and aphid numbers **(Figure 12)** (Spearman's rank correlation test, $S=12606$, $p<0.05$, $R=0.49$). Hence, it is likely that as plant height increases, so does the available habitat for aphids. Therefore, the above analysis was repeated but using the number of aphids per centimetre of shoot height and per gram of shoot biomass. This analysis again reveals that there is no significant difference between the number of aphid present and shoot height (Aphid numbers per centimetre of shoot height ANOVA; $F= 0.851$, $df= 3$, $p>0.05$), or shoot biomass (Aphid numbers per gram of shoot biomass ANOVA; $F= 1.8193$, $df= 3$, $p>0.05$).

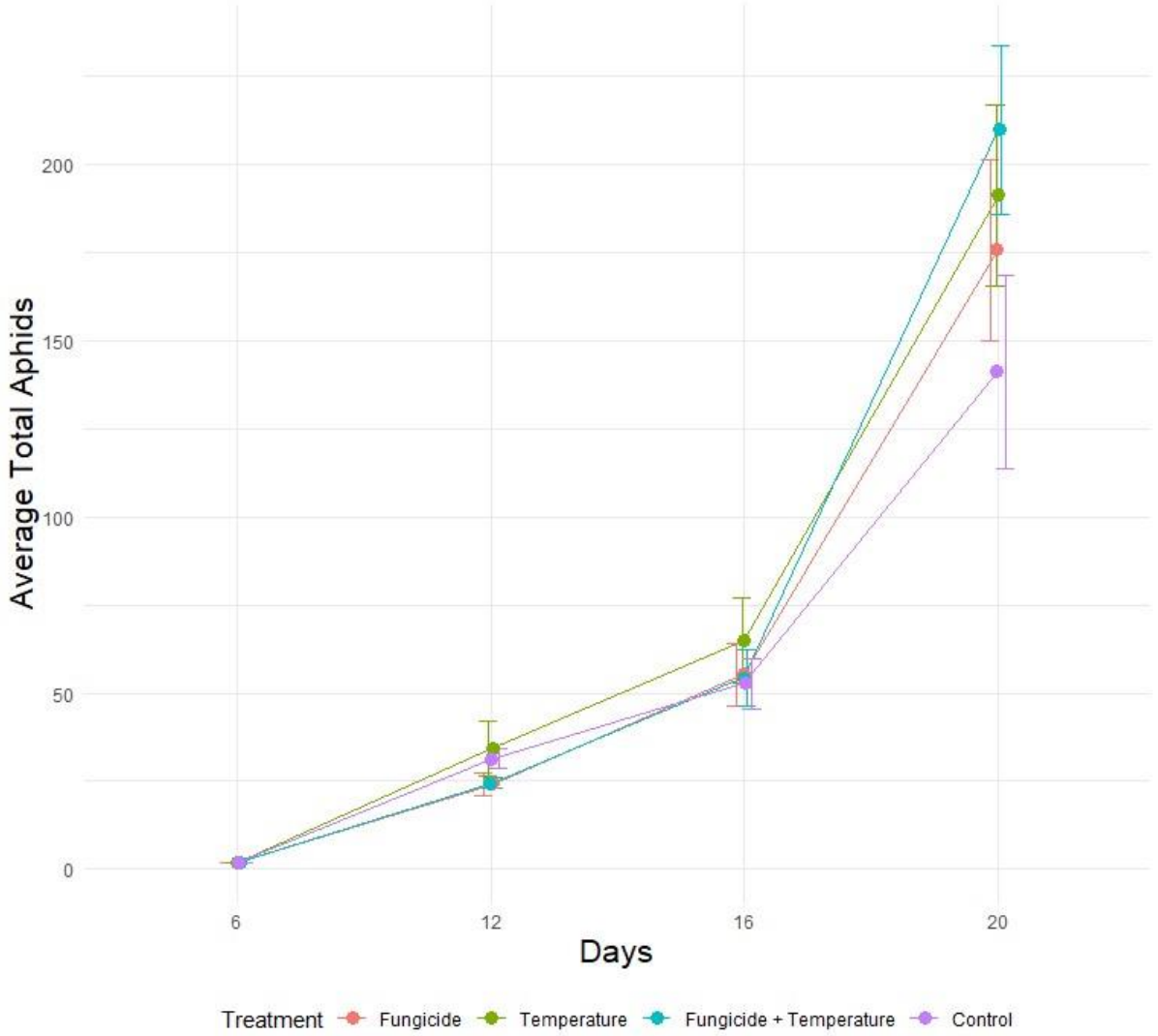


**Figure 11: The total number of aphids on barley plants inoculated with microbial communities that had evolved under 4 different treatments.** 2 aphids were added on day 6 and counted and the total number of aphids was counted on day 12, 16 and 20. No significant differences of aphid numbers was observed in days 12, 16, or 20 between any treatment. The error bars indicate the ± SEM.

**Table 11: ANOVA analysis of the number of aphids affected by treatment on each day.**

| Day | Degrees of Freedom | F-Value | P-Value |
|---|---|---|---|
| Day 12 | 3 | 1.4752 | 0.234 |
| Day 16 | 3 | 0.3366 | 0.798 |
| Day 20 | 3 | 1.2609 | 0.299 |

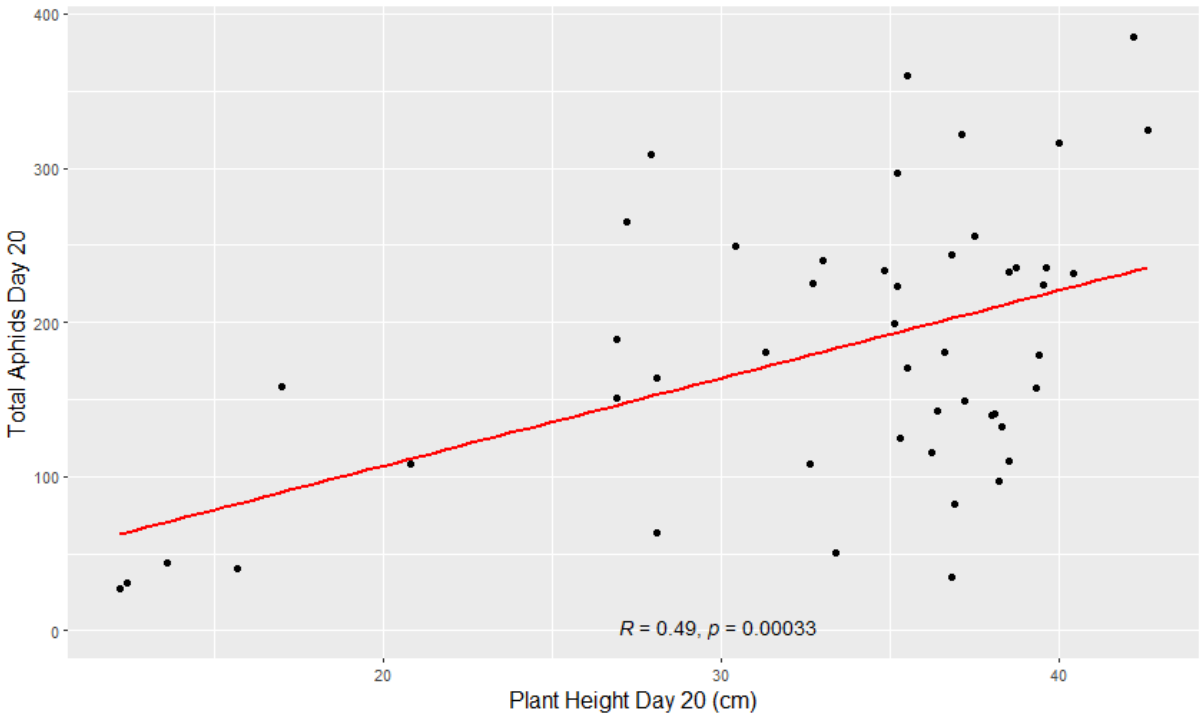


**Figure 12: The height on day 20 of the plant *versus* the total number of aphids on day 20**. There is a weak positive correlation (R= 0.5) between height and the number of aphids present of the plant ($p<0.001$). Analysis was performed using the Spearman rank correlation method.

# Discussion

This study investigated how anthropogenic stressors (pesticide and warming), imposed both individually and in combination, affect stress adaptation, virulence, and ecosystem functioning of soil communities over evolutionary timescales. My results revealed that for some measurements, soil isolates from specific treatments displayed distinct phenotypes *versus* soil isolates from stress-free controls. Moreover, whole community analyses (virulence and ecosystem functioning measurements) revealed that fungicide-evolved soil communities are less virulent towards insect larval hosts and confer enhanced plant growth when used to inoculate barley. Together, this shows that anthropogenic disturbances could have important consequences for both the functioning of soil communities, and for virulence potential of opportunistic pathogens residing within soil.

**Examining adaptation to stressors (increased temperatures and fungicide) for soil isolates**

The goal of this section was to understand whether soil isolates evolving under a particular treatment (*i.e.* pesticide/warming) displayed enhanced tolerance to that treatment. It was also tested whether a dual stress condition would impede or promote adaptation to constituent stressors. For fungicide adaptation, my results revealed that as predicted, the soil communities that were exposed to fungicide stress (fungicide-only and dual stressor treatment) had isolates with greater resistance to the fungicide, thus yielding a higher MIC value *in vitro* (**Figure 3**). While research exploring the impact of fungicides or other pesticides on non-target bacterial communities remains limited, existing studies have indicated that fungicide exposure can induce resistance in fungi (112). These findings partially corroborate the outcomes observed in this study.

It was also assessed whether the treatments affected the ability of soil isolates to grow under different temperature regimes (24°C, 32°C and 34°C) (**Figure 4**). It was found that all isolates grew better as their temperature conditions increased, indicating that 34°C temperature conditions were not actually stressful for the selected isolates. This is in contrast with Kelbrick *et al* (89), who found that *Pseudomonas fluorescens* grown at 34°C, had significantly reduced growth. However, the *P. fluorescens* strain used by Kelbrick was a lab adapted SBW25, with an optimum temperature of 26°C (89), while compost isolates display a wide variation in optimum temperatures (113) (114).

Regarding the temperature evolved isolates growing at a faster rate than the control isolates, contrasting results were shown in previous studies where *E. coli* that evolved under thermal stress was less effective at growing at various temperatures from 12-50°C compared to the ancestral strains (66). Similar to my results, a wide body of literature shows that mild temperature increases actually stimulate growth and respiration rate of soil communities (115) (116).

The isolates from the fungicide alone evolved treatment had the greatest growth compared to all other treatments. There is currently an absence of research regarding how fungicides affect the bacterial community's response to temperature. However, it could be due to genetic mutation, allowing the isolates from the fungicide treatment to grow at a faster rate (117). This could be confirmed by sequencing the isolates. Although the fungicide treatment has a greater cell density compared to the other treatments, it does not determine how viable or how fit the bacterial cells are compared to other treatments. Some studies suggest bacteria evolved at higher temperatures can be more fit compared to the ancestor after being returned to normal temperatures (118). Also, it is important to note that it is possible that isolates selected were not sufficient to capture a true representation of the community's ability to respond to varying temperatures.

**Investigating how treatment shapes clinically important phenotypes (antimicrobial resistance and virulence)**

The results of antimicrobial and virulence assays revealed that the treatment used is an important predictor of virulence and for clinically important traits in soil isolates. First, it was assessed how the soil evolved communities differ in virulence in a wax moth larval host (*Galleria mellonella*). Initially, we hypothesised that communities evolved in the presence of fungicide would display increased virulence *versus* increasing temperature and control communities, due to compositional shifts towards potentially opportunistic pathogens, for example *Pseudomonas aeruginosa* or *E. coli* in fungicide-evolved communities (**Figure 5**) (89). Instead, the results revealed that the soil communities evolved under fungicide stress potentially exhibited lower virulence compared to the other evolved communities. This was observed in communities which were evolved with fungicide alone or under the dual stressor treatment. Importantly, however, this experiment was conducted only once, so the results presented are preliminary, and future replicates are needed to determine statistical significance.

Despite the caveat associated with the results, there is some previous work suggesting that fungicides can reduce the virulence of pathogens. One study (48) reported that the virulence of fungal pathogens, *C. gatti* and *C. neoformans*, was reduced in a murine model when exposed to a fungicide for several weeks. However, this study looks at fungal pathogens specifically (48). In my project, it is possible that there are fungal pathogens in the community that are removed during exposure to fungicide. This could be what is reducing virulence in wax moths, and corroborates findings discussed below that fungicide evolved communities confer improved plant growth. However, it was not possible to assess fungal community composition in this study, and future work on these communities will determine whether fungal community composition differs between treatments.

The isolates from the evolved communities underwent additional testing to assess their resistance to antibiotics. Intriguingly, the most significant changes occurred in the isolates that were evolved under increasing temperature stress. In comparison to the control, three out of the six antibiotics from various classes, had increased sensitivity in the evolved isolates from the warming treatment, while those from the dual stressor treatment or the fungicide only treatment had no significant difference observed between any antibiotics relative to the control (**Figure 6**).

Communities that evolved under increasing temperature stress have induced phenotypic changes in the isolates, rendering them more susceptible to the antibiotics chloramphenicol, gentamicin, and piperacillin/tazobactam. Hence, there seems to be a genetic trade-off in being able to grow under temperature stress, which in turn, increases sensitivity to certain antibiotics. High temperature stress has also been shown to increase susceptibility to antibiotics that imitate the cold-stress response, for example, chloramphenicol (67). Previous research has indicated that *E. coli* has increased sensitivity to ciprofloxacin and decreased sensitivity to gentamicin when evolved under temperature stress conditions (66), which does not align with the results of this study. One possible reason this could be is that the antibiotics were tested in liquid media whilst this study performed antibiotic susceptibility testing in solid agar media.

Previous literature has indicated that prolonged exposure to a combination of fungicides can enhance the resistance of *E. coli* to streptomycin, an aminoglycoside antibiotic (119). However, my findings highlight that the treatments exposed to fungicide stress had no effect on aminoglycoside antibiotics (gentamicin and tobramycin). The absence of change

in the resistance to antibiotics within the soil communities exposed to fungicides contradicts prior studies suggesting that fungicide exposure promotes resistance to antibiotics to bacterial populations, as it enhances the transmission of plasmids containing antimicrobial resistance genes, such as *tetA*, *via* horizontal gene transfer (120) (60). Moreover, mancozeb, a primary component of Fubol Gold, has also been demonstrated to promote horizontal gene transfer of genes related to antimicrobial resistance (121). Pesticides have also been shown to induce cross-resistance to antibiotics, including tetracycline and kanamycin, increasing their MIC (62), although, this was performed on a selection of herbicides, not fungicides. Regardless, it is interesting that similar outcomes were not replicated in my experiment. Further contrasting my results, Kelbrick *et al* (89), who conducted the evolution of the soil communities that underwent testing, reported an increase in resistance to the antibiotics nalidixic acid (quinolone) and sulphatriad (sulphonamides).

Additionally, the isolates that underwent phenotypic testing are unknown, so it cannot be determined if antibiotic resistance was affected in biologically important microbes such as ESKAPE organisms, for example, *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*. Furthermore, a limitation of all the performed assessments is that the soil communities and isolates examined were all evolved in soil microcosms by Kelbrick *et al* (89). Therefore, testing of phenotypic traits in broth and agar may not accurately reflect their behaviour in soil. This could be amended by testing these phenotypic traits in a soil microcosm.

**Testing how treatment affects ecosystem functioning of evolved communities (growth and health of a crop plant)**

While extensive research has investigated the effects of temperature and fungicides on plant health and defence, there remains a notable gap in understanding these stressors' long-term impact on the soil community which in turn affects the plant. Consequently, comparisons with existing literature are limited. Nonetheless, the soil wash variation of the crop growth experiment produced significant results, indicating that the soil communities evolved under fungicide stress promoted barley shoot growth and biomass, compared to the control (**Figure 7**). I hypothesise this is due to the lack of harmful pathogenic fungi within the community which hinders the growth of the barley plant.

This finding is noteworthy, as previous studies suggest that various pesticides, including fungicides, can affect key enzymatic activities within the soil community. For example,

they can disrupt enzymes related to the nitrogen cycle (122). Fungicidal chemicals including mancozeb (80) and metalaxyl (123), the two key components of Fubol Gold, can also inhibit indole acetic acid and salicylic acid production, which are both important for crop growth and health (123). Since the literature suggests PGP activities are inhibited by fungicide exposure, it is of interest that the soil communities evolved under fungicide stress were the only treatments that significantly increased plant growth. Nevertheless, as indicated by Kelbrick *et al* (89), there is a stark reduction of microbial diversity within the fungicide treatments (**Figure 1**). Research has shown that a reduction in rare species can lead to increased plant growth. It is yet to be determined if this is attributed to the rare species themselves or shifts in microbial abundance and diversity due to the loss of these rare species. Nonetheless, this study shows the impact rare, low abundant species have on the functionality of a soil community (124). This could be a possible reason for the increased growth within the fungicide treated communities. It is noteworthy that the temperature evolved soil communities did not significantly affect crop growth, despite previous studies suggesting that higher temperatures promote PGP activities (84) (85).

The seed wash variation experiment did not show a significant effect of fungicide treatment on shoot height. Previous studies have shown that inoculating the seeds prior to planting improves growth (125) (126). My experimental design may have also affected the results; 500µl of the evolved soil communities were diluted into 4.5ml of sterile water and were left to grow for only 3 hours at room temperature before the seeds were added for a further 2 hours. This was done to eliminate the risk of losing rare species due to potential competition in an overnight culture, as it has been shown that this loss can influence plant biomass (91) (124). As a result, starting communities may have had a very low cell density.

No significant differences were observed on root length and biomass after the 20 days of growth for any of the plants where the soil was inoculated with the evolved communities (**Figure 9**). This is unexpected as fungicides can disrupt PGP activities (80, 123), while higher temperatures, especially between 20°C and 40°C, can improve PGP activities which can promote root growth (84). It must be noted that the plants were growing in pots with no holes in the bottom, and with limited soil (30g), which could influence the physiology of the plant and impact the results. The outcomes might vary if this experiment were repeated with additional space or soil provided for plant root growth.

The barley plant's defence response was determined by its capacity to deter aphids. The results highlight that there was no significant difference in the number of aphids between plants growing in any evolved soil community on any day (**Figure 11**). Although minimal comparative studies could be found, it has been shown that inoculating the plant roots with PGPB reduces aphid numbers, especially when the inoculation happened closer to aphid infestation period (99). These soil communities may lack the ability to deter aphids. Predictably, as plant height increases, aphid numbers increase (**Figure 12**). This is due to the increased space and resources for aphids. There was no significant difference in aphid numbers between any treatment group, even when the total aphid numbers were calculated per centimetre and gram of plant. This reinforces that the soil communities do not affect the barley plant's ability to deter aphids.

It is imperative to acknowledge that this experiment only shows the effect the evolved soil communities had on the barley plants after 20 days. This does not necessarily guarantee that the evolved soil communities will continue to have the same effect on the barley plant throughout its entire development.

**Future Work**

There are myriad of future studies that can be performed. This includes replicating all experiments with the ancestor community alongside and its associated isolates, this would allow us to compare and evaluate how the evolved soil communities differ from the ancestor community. All isolates should undergo genomic sequencing in order to understand the effects of prolonged fungicide and/or temperature stress affect the genotypic profiles of different species. Genes related to efflux pumps or auxin production could be analysed to understand how the different treatments effect antibiotic resistance and plant height.

Other aspects to be explored include determining how fungicide treated soil communities foster plant growth. Various PGP activities could be investigated in all evolved communities, such as auxin production, phosphate solubilisation, and nitrogen fixing. Finally, extending the duration of the barley growth experiment beyond the initial 20 days could provide valuable insights. This extended observation period would ascertain whether the plant growth promoting effects persist in barley cultivated with fungicide evolved soil communities. Moreover, it would allow us to determine whether other treatments induce plant growth or aphid defence later in the barley's development.

These findings bear significant implications for agricultural practices, particularly in the context of a warming climate. While many of the observed outcomes, such as enhanced plant growth, reduced virulence and antimicrobial resistance, do not directly undermine farming practices or human health, this experiment offers only a snapshot of a semi-natural ecosystem, and its real-world applicability may vary. Notably, this study only examined two stressors, yet emerging research suggests that the exposure to multiple stressors may have increasingly detrimental effects on soil microbiome dynamics and functioning.

Rillig *et al* (88) has highlighted the limitations of studying ecosystems under singular stressors, emphasizing that this approach may fail to capture the complex interactions within natural systems. Only through the comprehensive examination of ecosystems under multiple stressors can we grasp the true magnitude of their impact. Thus, one must be cautious when linking these findings to real-world agricultural scenarios. Nonetheless, conducting research in semi-natural systems remains invaluable, offering insights that bridge the gap between short-term microbial responses to stress and the dynamics observed in natural systems.

# References


1. Ossowicki A, Raaijmakers JM, Garbeva P. 2021. Disentangling soil microbiome functions by perturbation. Environmental Microbiology Reports 13:582-590.
2. Van Der Heijden MG, Bardgett RD, Van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology letters 11:296-310.
3. Banerjee S, Van Der Heijden MG. 2023. Soil microbiomes and one health. Nature Reviews Microbiology 21:6-20.
4. Gottdenker NL, Streicker DG, Faust CL, Carroll C. 2014. Anthropogenic land use change and infectious diseases: a review of the evidence. EcoHealth 11:619-632.
5. Stern DI, Kaufmann RK. 2014. Anthropogenic and natural causes of climate change. Climatic change 122:257-269.
6. Deabji N, Fomba KW, El Hajjaji S, Mellouki A, Poulain L, Zeppenfeld S, Herrmann H. 2021. First insights into northern Africa high-altitude background aerosol chemical composition and source influences. Atmospheric Chemistry and Physics 21:18147-18174.
7. Kampa M, Castanas E. 2008. Human health effects of air pollution. Environmental pollution 151:362-367.
8. Akhtar N, Syakir Ishak MI, Bhawani SA, Umar K. 2021. Various natural and anthropogenic factors responsible for water quality degradation: A review. Water 13:2660.
9. Battaglin WA, Sandstrom MW, Kuivila KM, Kolpin DW, Meyer MT. 2011. Occurrence of azoxystrobin, propiconazole, and selected other fungicides in US streams, 2005–2006. Water, Air, & Soil Pollution 218:307-322.
10. Khatri N, Tyagi S. 2015. Influences of natural and anthropogenic factors on surface and groundwater quality in rural and urban areas. Frontiers in life science 8:23-39.
11. Zandalinas SI, Fritschi FB, Mittler R. 2021. Global warming, climate change, and environmental pollution: recipe for a multifactorial stress combination disaster. Trends in Plant Science 26:588-599.
12. Sigwart JD, Bennett K, Edie SM, Mander L, Okamura B, Padian K, Wheeler Q, Winston JE, Yeung NW. 2018. Measuring biodiversity and extinction—present and past. Integrative and comparative biology 58:1111-1117.

13. Halpern BS, Selkoe KA, Micheli F, Kappel CV. 2007. Evaluating and ranking the vulnerability of global marine ecosystems to anthropogenic threats. Conservation biology 21:1301-1315.
14. Hoegh-Guldberg O, Poloczanska ES, Skirving W, Dove S. 2017. Coral reef ecosystems under climate change and ocean acidification. Frontiers in Marine Science 4:158.
15. He Q, Silliman BR. 2019. Climate change, human impacts, and coastal ecosystems in the Anthropocene. Current Biology 29:R1021-R1035.
16. Fang Z, Ding T, Chen J, Xue S, Zhou Q, Wang Y, Wang Y, Huang Z, Yang S. 2022. Impacts of land use/land cover changes on ecosystem services in ecologically fragile regions. Science of the Total Environment 831:154967.
17. Zhang M, Wang W, Zhang Y, Teng Y, Xu Z. 2017. Effects of fungicide iprodione and nitrification inhibitor 3, 4-dimethylpyrazole phosphate on soil enzyme and bacterial properties. Science of the total environment 599:254-263.
18. Singh S, Gupta R, Kumari M, Sharma S. 2015. Nontarget effects of chemical pesticides and biological pesticide on rhizospheric microbial community structure and function in *Vigna radiata*. Environmental Science and Pollution Research 22:11290-11300.
19. Sazykin I, Khmelevtsova L, Azhogina T, Sazykina M. 2023. Heavy metals influence on the bacterial community of soils: A review. Agriculture 13:653.
20. Pierart A, Dumat C, Maes AQ, Roux C, Sejalon-Delmas N. 2018. Opportunities and risks of biofertilization for leek production in urban areas: Influence on both fungal diversity and human bioaccessibility of inorganic pollutants. Science of the total environment 624:1140-1151.
21. Navarrete AA, Tsai SM, Mendes LW, Faust K, de Hollander M, Cassman NA, Raes J, van Veen JA, Kuramae EE. 2015. Soil microbiome responses to the short-term effects of Amazonian deforestation. Molecular ecology 24:2433-2448.
22. Tudi M, Daniel Ruan H, Wang L, Lyu J, Sadler R, Connell D, Chu C, Phung DT. 2021. Agriculture development, pesticide application and its impact on the environment. International journal of environmental research and public health 18:1112.
23. Sharma A, Kumar V, Shahzad B, Tanveer M, Sidhu GPS, Handa N, Kohli SK, Yadav P, Bali AS, Parihar RD. 2019. Worldwide pesticide usage and its impacts on ecosystem. SN Applied Sciences 1:1-16.

24. Ahvo A, Heino M, Sandström V, Chrisendo D, Jalava M, Kummu M. 2023. Agricultural input shocks affect crop yields more in the high-yielding areas of the world. Nature food 4:1037-1046.
25. Shahid M, Khan MS. 2022. Ecotoxicological implications of residual pesticides to beneficial soil bacteria: A review. Pesticide Biochemistry and Physiology:105272.
26. Zhang H, Song J, Zhang Z, Zhang Q, Chen S, Mei J, Yu Y, Fang H. 2021. Exposure to fungicide difenoconazole reduces the soil bacterial community diversity and the co-occurrence network complexity. Journal of Hazardous Materials 405:124208.
27. Ma G, Gao X, Nan J, Zhang T, Xie X, Cai Q. 2021. Fungicides alter the distribution and diversity of bacterial and fungal communities in ginseng fields. Bioengineered 12:8043-8056.
28. Marinho MdC, Diogo BS, Lage OM, Antunes SC. 2020. Ecotoxicological evaluation of fungicides used in viticulture in non-target organisms. Environmental Science and Pollution Research 27:43958-43969.
29. Ettema TJ, Andersson SG. 2009. The α-proteobacteria: the Darwin finches of the bacterial world. Biology letters 5:429-432.
30. Ma Q, Li Q, Wang J, Parales RE, Li L, Ruan Z. 2023. Exposure to three herbicide mixtures influenced maize root-associated microbial community structure, function and the network complexity. Environmental Pollution 336:122393.
31. Verdenelli RA, Dominchin MF, Barbero FM, Pérez-Brandán C, Aoki A, Gil SV, Meriles JM. 2023. Effect of two broad-spectrum fungicides on the microbial communities of a soil subjected to different degrees of water erosion. Applied Soil Ecology 190:104984.
32. Han L, Kong X, Xu M, Nie J. 2021. Repeated exposure to fungicide tebuconazole alters the degradation characteristics, soil microbial community and functional profiles. Environmental Pollution 287:117660.
33. Imfeld G, Vuilleumier S. 2012. Measuring the effects of pesticides on bacterial communities in soil: a critical review. European journal of soil biology 49:22-30.
34. Price TD, Qvarnström A, Irwin DE. 2003. The role of phenotypic plasticity in driving genetic evolution. Proceedings of the Royal Society of London Series B: Biological Sciences 270:1433-1440.
35. Kissling M, Hegetschweiler KT, Rusterholz H-P, Baur B. 2009. Short-term and long-term effects of human trampling on above-ground vegetation, soil density, soil

organic matter and soil microbial processes in suburban beech forests. Applied soil ecology 42:303-314.

36. Ma Q, Tan H, Song J, Li M, Wang Z, Parales RE, Li L, Ruan Z. 2022. Effects of long-term exposure to the herbicide nicosulfuron on the bacterial community structure in a factory field. Environmental Pollution 307:119477.
37. Walters KE, Martiny JB. 2020. Alpha-, beta-, and gamma-diversity of bacteria varies across habitats. PLoS One 15:e0233872.
38. Laimighofer J, Formayer H. 2024. Climate change contribution to the 2023 autumn temperature records in Vienna. Scientific Reports 14:4213.
39. Fu F, Li J. 2023. Simulating the effect of climate change on soil microbial community in an Abies georgei var. smithii forest. Frontiers in Microbiology 14:1189859.
40. Ma L, Liu L, Lu Y, Chen L, Zhang Z, Zhang H, Wang X, Shu L, Yang Q, Song Q. 2022. When microclimates meet soil microbes: Temperature controls soil microbial diversity along an elevational gradient in subtropical forests. Soil Biology and Biochemistry 166:108566.
41. Riah-Anglet W, Trinsoutrot-Gattin I, Martin-Laurent F, Laroche-Ajzenberg E, Norini M-P, Latour X, Laval K. 2015. Soil microbial community structure and function relationships: a heat stress experiment. Applied Soil Ecology 86:121-130.
42. Melillo JM, Frey SD, DeAngelis KM, Werner WJ, Bernard MJ, Bowles FP, Pold G, Knorr MA, Grandy AS. 2017. Long-term pattern and magnitude of soil carbon feedback to the climate system in a warming world. Science 358:101-105.
43. Wu L, Zhang Y, Guo X, Ning D, Zhou X, Feng J, Yuan MM, Liu S, Guo J, Gao Z. 2022. Reduction of microbial diversity in grassland soil is driven by long-term climate warming. Nature Microbiology 7:1054-1062.
44. Sheik CS, Beasley WH, Elshahed MS, Zhou X, Luo Y, Krumholz LR. 2011. Effect of warming and drought on grassland microbial communities. The ISME journal 5:1692-1700.
45. de Ramón-Carbonell M, Sánchez-Torres P. 2017. Involvement of *Penicillium digitatum* PdSUT1 in fungicide sensitivity and virulence during citrus fruit infection. Microbiological research 203:57-67.
46. Bastos RW, Rossato L, Goldman GH, Santos DA. 2021. Fungicide effects on human fungal pathogens: cross-resistance to medical drugs and beyond. PLoS Pathogens 17:e1010073.

47. Stepanov AA, Poshvina DV, Vasilchenko AS. 2022. 2, 4-Diacetylphloroglucinol modulates *Candida albicans* virulence. Journal of Fungi 8:1018.
48. Bastos RW, Carneiro HCS, Oliveira LVN, Rocha KM, Freitas GJC, Costa MC, Magalhães TFF, Carvalho VSD, Rocha CE, Ferreira GF. 2018. Environmental triazole induces cross-resistance to clinical drugs and affects morphophysiology and virulence of *Cryptococcus gattii* and *C. neoformans*. Antimicrobial agents and chemotherapy 62:10.1128/aac. 01179-17.
49. Anwar A, Anwar S, Muhammad A, Nawaz F, Hyder S, Noman K, Malik I. 2019. Climate change and infectious diseases: evidence from highly vulnerable countries. Iranian journal of public health 48:2187.
50. Nnadi NE, Carter DA. 2021. Climate change and the emergence of fungal pathogens. PLoS pathogens 17:e1009503.
51. Ashrafi R, Bruneaux M, Sundberg LR, Pulkkinen K, Valkonen J, Ketola T. 2018. Broad thermal tolerance is negatively correlated with virulence in an opportunistic bacterial pathogen. Evolutionary Applications 11:1700-1714.
52. Huot B, Castroverde CDM, Velásquez AC, Hubbard E, Pulman JA, Yao J, Childs KL, Tsuda K, Montgomery BL, He SY. 2017. Dual impact of elevated temperature on plant defence and bacterial virulence in Arabidopsis. Nature communications 8:1808.
53. Baron C, Domke N, Beinhofer M, Hapfelmeier S. 2001. Elevated temperature differentially affects virulence, VirB protein accumulation, and T-pilus formation in different *Agrobacterium tumefaciens* and *Agrobacterium vitis* strains. Journal of Bacteriology 183:6852-6861.
54. Brauner A, Fridman O, Gefen O, Balaban NQ. 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nature Reviews Microbiology 14:320-330.
55. Munita JM, Arias CA. 2016. Mechanisms of antibiotic resistance. Virulence mechanisms of bacterial pathogens:481-511.
56. O'Neill J. 2016. Tackling drug-resistant infections globally: final report and recommendations.
57. Yang L-N, He M-H, Ouyang H-B, Zhu W, Pan Z-C, Sui Q-J, Shang L-P, Zhan J. 2019. Cross-resistance of the pathogenic fungus *Alternaria alternata* to fungicides with different modes of action. BMC microbiology 19:1-10.

58. Snelders E, Camps SM, Karawajczyk A, Schaftenaar G, Kema GH, Van der Lee HA, Klaassen CH, Melchers WJ, Verweij PE. 2012. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. PloS one 7:e31801.
59. Bottery MJ, van Rhijn NJ, Chown H, Rhodes JL, Celia-Sanchez BN, Brewer MT, Momany M, Fisher MC, Knight CG, Bromley MJ. 2023. Elevated mutation rates in the multi-azole resistant *Aspergillus fumigatus* clade drives rapid evolution of antifungal resistance. bioRxiv:2023.12. 05.570068.
60. Zhang H, Song J, Zheng Z, Li T, Shi N, Han Y, Zhang L, Yu Y, Fang H. 2023. Fungicide exposure accelerated horizontal transfer of antibiotic resistance genes *via* plasmid-mediated conjugation. Water Research 233:119789.
61. Liao H, Li X, Yang Q, Bai Y, Cui P, Wen C, Liu C, Chen Z, Tang J, Che J. 2021. Herbicide selection promotes antibiotic resistance in soil microbiomes. Molecular Biology and Evolution 38:2337-2350.
62. Kurenbach B, Marjoshi D, Amábile-Cuevas CF, Ferguson GC, Godsoe W, Gibson P, Heinemann JA. 2015. Sublethal exposure to commercial formulations of the herbicides dicamba, 2, 4-dichlorophenoxyacetic acid, and glyphosate cause changes in antibiotic susceptibility in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. MBio 6:10.1128/mbio. 00009-15.
63. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clinical infectious diseases 48:1-12.
64. MacFadden D, McGough S, Fisman D, Santillana M, Brownstein J. 2018. Antibiotic resistance increases with local temperature. Nat Clim Chang 8: 510–514.
65. Rodríguez-Verdugo A, Gaut BS, Tenaillon O. 2013. Evolution of *Escherichia coli* rifampicin resistance in an antibiotic-free environment during thermal stress. BMC evolutionary biology 13:1-11.
66. Bullivant A. 2022. Evolution Under Thermal Stress Affects *Escherichia coli*'s Resistance to Antibiotics. University of California, Los Angeles.
67. Cruz-Loya M, Kang TM, Lozano NA, Watanabe R, Tekin E, Damoiseaux R, Savage VM, Yeh PJ. 2019. Stressor interaction networks suggest antibiotic resistance co-opted from stress responses to temperature. The ISME journal 13:12-23.
68. Xun W, Liu Y, Li W, Ren Y, Xiong W, Xu Z, Zhang N, Miao Y, Shen Q, Zhang R. 2021. Specialized metabolic functions of keystone taxa sustain soil microbiome stability. Microbiome 9:1-15.

69. Wiesmeier M, Urbanski L, Hobley E, Lang B, von Lützow M, Marin-Spiotta E, van Wesemael B, Rabot E, Ließ M, Garcia-Franco N. 2019. Soil organic carbon storage as a key function of soils-A review of drivers and indicators at various scales. Geoderma 333:149-162.
70. Hayat R, Ali S, Amara U, Khalid R, Ahmed I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of microbiology 60:579-598.
71. Wu G-L, Zhang M-Q, Liu Y, López-Vicente M. 2020. Litter cover promotes biocrust decomposition and surface soil functions in sandy ecosystem. Geoderma 374:114429.
72. Kardol P, Cregger MA, Campany CE, Classen AT. 2010. Soil ecosystem functioning under climate change: plant species and community effects. Ecology 91:767-781.
73. Nikitin D, Semenov M, Chernov T, Ksenofontova N, Zhelezova A, Ivanova E, Khitrov N, Stepanov A. 2022. Microbiological indicators of soil ecological functions: a review. Eurasian Soil Science 55:221-234.
74. Philippot L, Spor A, Hénault C, Bru D, Bizouard F, Jones CM, Sarr A, Maron P-A. 2013. Loss in microbial diversity affects nitrogen cycling in soil. The ISME journal 7:1609-1619.
75. Bhattacharyya PN, Jha DK. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World Journal of Microbiology and Biotechnology 28:1327-1350.
76. Filimon MN, Voia SO, Popescu R, Dumitrescu G, Ciochina LP, Mituletu M, Vlad DC. 2015. The effect of some insecticides on soil microorganisms based on enzymatic and bacteriological analyses. Romanian Biotechnological Letters 20:10439-10447.
77. Wang X, Lu Z, Miller H, Liu J, Hou Z, Liang S, Zhao X, Zhang H, Borch T. 2020. Fungicide azoxystrobin induced changes on the soil microbiome. Applied Soil Ecology 145:103343.
78. Shahid M, Khan MS. 2018. Cellular destruction, phytohormones and growth modulating enzymes production by *Bacillus subtilis* strain BC8 impacted by fungicides. Pesticide biochemistry and physiology 149:8-19.
79. Ahemad M, Khan MS. 2012. Effect of fungicides on plant growth promoting activities of phosphate solubilizing *Pseudomonas putida* isolated from mustard (*Brassica compestris*) rhizosphere. Chemosphere 86:945-950.

80. Walia A, Mehta P, Guleria S, Chauhan A, Shirkot C. 2014. Impact of fungicide mancozeb at different application rates on soil microbial populations, soil biological processes, and enzyme activities in soil. The Scientific World Journal 2014.
81. Roman DL, Voiculescu DI, Filip M, Ostafe V, Isvoran A. 2021. Effects of triazole fungicides on soil microbiota and on the activities of enzymes found in soil: A review. Agriculture 11:893.
82. Steinweg JM, Dukes JS, Paul EA, Wallenstein MD. 2013. Microbial responses to multi-factor climate change: effects on soil enzymes. Frontiers in microbiology 4:47630.
83. Roper MM. 1985. Straw decomposition and nitrogenase activity (C2H2 reduction): effects of soil moisture and temperature. Soil Biology and Biochemistry 17:65-71.
84. Ali N, Swarnkar MK, Veer R, Kaushal P, Pati AM. 2023. Temperature-induced modulation of stress-tolerant PGP genes bioprospected from *Bacillus* sp. IHBT-705 associated with saffron (*Crocus sativus*) rhizosphere: A natural-treasure trove of microbial biostimulants. Frontiers in Plant Science 14:1141538.
85. Fanin N, Mooshammer M, Sauvadet M, Meng C, Alvarez G, Bernard L, Bertrand I, Blagodatskaya E, Bon L, Fontaine S. 2022. Soil enzymes in response to climate warming: Mechanisms and feedbacks. Functional Ecology 36:1378-1395.
86. Rillig MC, Ryo M, Lehmann A. 2021. Classifying human influences on terrestrial ecosystems. Global Change Biology 27:2273-2278.
87. Orr JA, Rillig MC, Jackson MC. 2022. Similarity of anthropogenic stressors is multifaceted and scale dependent. Natural Sciences 2:e20210076.
88. Rillig MC, Van der Heijden MG, Berdugo M, Liu Y-R, Riedo J, Sanz-Lazaro C, Moreno-Jiménez E, Romero F, Tedersoo L, Delgado-Baquerizo M. 2023. Increasing the number of stressors reduces soil ecosystem services worldwide. Nature Climate Change 13:478-483.
89. Kelbrick M. 2023. Exploring Microbial Eco-Evolutionary Responses to Environmental Stress. Doctorate. University of Liverpool.
90. Yang X, Cheng J, Franks AE, Huang X, Yang Q, Cheng Z, Liu Y, Ma B, Xu J, He Y. 2023. Loss of microbial diversity weakens specific soil functions, but increases soil ecosystem stability. Soil Biology and Biochemistry 177:108916.
91. Kurm V, van der Putten WH, Pineda A, Hol WG. 2018. Soil microbial species loss affects plant biomass and survival of an introduced bacterial strain, but not inducible plant defences. Annals of Botany 121:311-319.

92. Maron P-A, Sarr A, Kaisermann A, Lévêque J, Mathieu O, Guigue J, Karimi B, Bernard L, Dequiedt S, Terrat S. 2018. High microbial diversity promotes soil ecosystem functioning. Applied and Environmental Microbiology 84:e02738-17.
93. Aukour FJ, Bani Hani N. 2022. Plant biomass and water use efficiency of winter barley (Hordeum vulgare L.) under biosolid applications. Water Science and Technology 85:2350-2357.
94. Kennedy J, Stroyan H. 1959. Biology of aphids. Annual Review of Entomology 4:139-160.
95. Shih P-Y, Sugio A, Simon J-C. 2023. Molecular mechanisms underlying host plant specificity in aphids. Annual Review of Entomology 68:431-450.
96. Ng JC, Perry KL. 2004. Transmission of plant viruses by aphid vectors. Molecular plant pathology 5:505-511.
97. Pineda A, Kaplan I, Bezemer TM. 2017. Steering soil microbiomes to suppress aboveground insect pests. Trends in Plant Science 22:770-778.
98. Wolfgang A, Tack AJ, Berg G, Abdelfattah A. 2023. Reciprocal influence of soil, phyllosphere, and aphid microbiomes. Environmental Microbiome 18:63.
99. Sanchez-Mahecha O, Klink S, Rothballer M, Sturm S, Weisser WW, Zytynska S, Heinen R. 2023. Microbe-induced plant resistance against insect pests depends on timing of inoculation but is consistent across climatic conditions. Functional Ecology.
100. Blubaugh CK, Carpenter-Boggs L, Reganold JP, Schaeffer RN, Snyder WE. 2018. Bacteria and competing herbivores weaken top–down and bottom–up aphid suppression. Frontiers in plant science 9:388029.
101. Feng J-L, Zhang J, Yang J, Zou L-P, Fang T-T, Xu H-L, Cai Q-N. 2021. Exogenous salicylic acid improves resistance of aphid-susceptible wheat to the grain aphid, *Sitobion avenae* (F.)(Hemiptera: Aphididae). Bulletin of Entomological Research 111:544-552.
102. Pangesti N, Reichelt M, van de Mortel JE, Kapsomenou E, Gershenzon J, van Loon JJ, Dicke M, Pineda A. 2016. Jasmonic acid and ethylene signaling pathways regulate glucosinolate levels in plants during rhizobacteria-induced systemic resistance against a leaf-chewing herbivore. Journal of Chemical Ecology 42:1212-1225.
103. Tabatabai M, Bremner J. 1970. Factors affecting soil arylsulfatase activity. Soil Science Society of America Journal 34:427-429.

104. BÁRCENAS-MORENO G, GÓMEZ-BRANDÓN M, Rousk J, Bååth E. 2009. Adaptation of soil microbial communities to temperature: comparison of fungi and bacteria in a laboratory experiment. Global Change Biology 15:2950-2957.
105. Matuschek E, Brown DF, Kahlmeter G. 2014. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clinical microbiology and infection 20:O255-O266.
106. Hall JP, Wood AJ, Harrison E, Brockhurst MA. 2016. Source–sink plasmid transfer dynamics maintain gene mobility in soil bacterial communities. Proceedings of the National Academy of Sciences 113:8260-8265.
107. Boyd H. 1971. Manganese toxicity to peanuts in autoclaved soil. Plant and soil 35:133-144.
108. Team RC. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www R-project org/.
109. Dinno A. 2017. dunn. test: Dunn's test of multiple comparisons using rank sums. R package version 1:1.
110. Wickham H, Wickham H. 2016. Data analysis. Springer.
111. Auguie B, Antonov A. 2017. gridExtra: miscellaneous functions for "grid" graphics. R package version 2.
112. Bellón-Gómez D, Vela-Corcía D, Pérez-García A, Torés JA. 2015. Sensitivity of *Podosphaera xanthii* populations to anti-powdery-mildew fungicides in Spain. Pest management science 71:1407-1413.
113. López MJ, Jurado MM, López-González JA, Estrella-González MJ, Martínez-Gallardo MR, Toribio A, Suárez-Estrella F. 2021. Characterization of thermophilic lignocellulolytic microorganisms in composting. Frontiers in microbiology 12:697480.
114. Miyatake F, Iwabuchi K. 2005. Effect of high compost temperature on enzymatic activity and species diversity of culturable bacteria in cattle manure compost. Bioresource technology 96:1821-1825.
115. Dacal M, Delgado-Baquerizo M, Barquero J, Berhe AA, Gallardo A, Maestre FT, García-Palacios P. 2022. Temperature increases soil respiration across ecosystem types and soil development, but soil properties determine the magnitude of this effect. Ecosystems 25:184-198.

116. Eng AY, Narayanan A, Alster CJ, DeAngelis KM. 2023. Thermal adaptation of soil microbial growth traits in response to chronic warming. Applied and Environmental Microbiology 89:e00825-23.
117. Gordo I, Perfeito L, Sousa A. 2012. Fitness effects of mutations in bacteria. Journal of molecular microbiology and biotechnology 21:20-35.
118. Rodríguez-Verdugo A, Carrillo-Cisneros D, González-González A, Gaut BS, Bennett AF. 2014. Different tradeoffs result from alternate genetic adaptations to a common environment. Proceedings of the National Academy of Sciences 111:12121-12126.
119. Xing Y, Wu S, Men Y. 2020. Exposure to environmental levels of pesticides stimulates and diversifies evolution in *Escherichia coli* toward higher antibiotic resistance. Environmental science & technology 54:8770-8778.
120. Zhang H, Chen S, Zhang Q, Long Z, Yu Y, Fang H. 2020. Fungicides enhanced the abundance of antibiotic resistance genes in greenhouse soil. Environmental pollution 259:113877.
121. Song J, Zhang H, Wu Z, Qiu M, Zhan X, Zheng C, Shi N, Zhang Q, Zhang L, Yu Y. 2023. A novel bidirectional regulation mechanism of mancozeb on the dissemination of antibiotic resistance. Journal of Hazardous Materials 455:131559.
122. Sim JX, Doolette CL, Vasileiadis S, Drigo B, Wyrsch ER, Djordjevic SP, Donner E, Karpouzas DG, Lombi E. 2022. Pesticide effects on nitrogen cycle related microbial functions and community composition. Science of the Total Environment 807:150734.
123. Ahemad M, Khan MS. 2012. Effects of pesticides on plant growth promoting traits of Mesorhizobium strain MRC4. Journal of the Saudi Society of Agricultural Sciences 11:63-71.
124. Hol WG, De Boer W, Termorshuizen AJ, Meyer KM, Schneider JH, Van Dam NM, Van Veen JA, Van Der Putten WH. 2010. Reduction of rare soil microbes modifies plant–herbivore interactions. Ecology letters 13:292-301.
125. Fiodor A, Ajijah N, Dziewit L, Pranaw K. 2023. Biopriming of seed with plant growth-promoting bacteria for improved germination and seedling growth. Frontiers in Microbiology 14:1142966.
126. Zytynska SE, Eicher M, Rothballer M. 2020. Microbial-mediated plant growth promotion and pest suppression varies under climate change. Frontiers in plant science 11:573578.