Final Report

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Title: Investigating the biotic and abiotic factors affecting apple canker (*Neonectria ditissima*) symptom development

Environmental and microbial drivers of European apple canker

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1. Industry Summary

European apple canker, caused by the fungus *Neonectria ditissima*, is one of the most damaging diseases facing global apple production, yet the biological and environmental factors that drive the disease are poorly understood. This research investigated how the root microbial community, winter waterlogging, and orchard establishment decisions interact to influence canker outcomes.

The apple root-associated microbiome was strongly shaped by orchard site, but establishment decisions left a lasting imprint: trees planted in April following cold storage had both an altered root microbiome and higher canker incidence compared to trees planted in December without cold storage, and these microbiome differences were still detectable three years after planting. The composition of the root microbiome was associated with canker outcomes. Trees with higher abundances of beneficial fungi including *Epicoccum nigrum* and *Trichoderma* had fewer cankers, while trees with a higher abundance of *Fusarium* in the roots had more cankers. These findings suggest that establishing root populations of known biocontrol fungi at planting could help sustain protective microbial communities throughout the tree and across the orchard lifespan.

With wetter winters projected for the UK and Northern Europe, this research provided the first study of how waterlogging outside the summer growing season affects apple tree survival, growth, and canker susceptibility. Autumn waterlogging was identified as the highest-risk period, causing up to 50% rootstock mortality after just four weeks. Winter waterlogging was less consistently lethal, although prolonged waterlogging caused severe mortality in one experiment. Importantly, surviving trees showed no lasting growth penalty, and waterlogging during dormancy did not directly increase canker susceptibility under the controlled conditions tested. However, winter waterlogging left a persistent shift in the soil microbiome which was still detectable 6–9 months after drainage, indicating a hidden ecological impact that may affect long-term orchard health.

Genomic analysis of *Sphingomonas* bacteria isolated from apple trees revealed their potential as biological control agents. Despite showing low antagonism against *N. ditissima* in laboratory tests, whole-genome analysis identified genes associated with the production of antifungal compounds as well as novel gene clusters that may encode unknown antimicrobial metabolites. These bacteria appear well adapted to surviving and competing in the apple phyllosphere, making them promising candidates for targeted biological control of apple canker pending further field evaluation.

Together, these findings advance understanding of the interactions between environment, host physiology, and the microbial communities that influence European apple canker. Apple growers will benefit from clearer guidance on the risks associated with planting time and waterlogging, while biological control developers gain new leads. This research lays the groundwork for microbiome-informed, climate-resilient canker management strategies that benefit the entire UK apple industry.

2. Introduction

2.1. The Disease and Its Importance

European apple canker, caused by the fungal pathogen *Neonectria ditissima*, is among the most damaging diseases affecting apple production worldwide, threatening orchard establishment, reducing fruit yields, and increasing management costs (Saville and Olivieri, 2019). The pathogen spreads via wind- and splash-dispersed spores that infect through wounds – leaf and petal scars, pruning cuts, and growth cracks – before entering a latent period that can last from weeks to years (Weber and Børve, 2021). Infections from the nursery may remain undetected for up to three years after planting, and sudden, severe outbreaks in young orchards can result in the removal of up to 25% of trees after the first growing season (Weber and Børve, 2021). Autumn represents the highest-risk infection window, when an abundance of fresh wounds, peak spore availability, and cool, wet conditions combine to favour infection.

Existing management depends heavily on chemical fungicides and cultural practices – primarily the pruning out of infected wood. However, regulation of fungicide chemistry, dose, and application timing is increasingly stringent. Copper fungicides face increasing restrictions due to soil accumulation and aquatic toxicity concerns, carbendazim is no longer permitted in Europe, and resistance to methyl benzimidazole carbamate fungicides has been reported (Walter *et al.*, 2014; Saville and Olivieri, 2019). Despite considerable research effort, no reliable microbial biological control agent (BCA) for apple canker has yet been developed for commercial use. Early candidates such as *Bacillus subtilis* demonstrated antagonism to *N. ditissima* in vitro but failed to provide adequate control under orchard conditions (Swinburne and Brown, 1976; Walter *et al.*, 2017). More recently, fungal candidates including *Clonostachys rosea* and *Epicoccum nigrum* have shown promise, but challenges around persistent establishment in the apple phyllosphere have limited their practical effectiveness (Elena *et al.*, 2022; Papp-Rupar *et al.*, 2023b). The urgent need for sustainable, reduced-chemical management strategies provides the central motivation for this research.

2.2. The Microbiome and Canker Susceptibility

Research over the past decade has established that the apple phyllosphere microbiome – the community of bacteria and fungi colonising above-ground plant tissues – plays a role in canker susceptibility. Multiple studies have demonstrated that the endophyte communities of apple leaf scars and stems vary between scion cultivars and between sites, and that the composition of these communities correlates with canker susceptibility (Olivieri *et al.*, 2021; Papp-Rupar *et al.*, 2022, 2023a). Site – encompassing local soil, climate, and surrounding vegetation – consistently emerges as the dominant driver of apple endophyte community structure, dwarfing the

contributions of host genotype (Liu *et al.*, 2020; Olivieri *et al.*, 2021; Papp-Rupar *et al.*, 2023a). Some endophytes found in greater abundance in canker-resistant cultivars, including members of the *Sphingomonas* genus, have been associated with canker-tolerance traits such as increased canopy size in *N. ditissima*-inoculated trees (Papp-Rupar *et al.*, 2022, 2023a).

While this work on above-ground microbiomes has been informative, the role of root-associated microbial communities in European apple canker has received comparatively little attention. Root and rhizosphere microbiomes vary between apple rootstock genotypes (Van Horn *et al.*, 2021; Liu *et al.*, 2022) and are influenced by scion genotype through differences in root exudate chemistry (Chai *et al.*, 2022). Analogues of the canker system exist in other tree diseases: rootstock genotypes resistant to apple replant disease harbour distinct root microbiomes compared to susceptible genotypes (Van Horn *et al.*, 2021), and root microbiome composition has been linked to susceptibility to Valsa canker in rootstocks (Wang *et al.*, 2022). Arbuscular mycorrhizal fungi (AMF) inoculation has been shown to reduce susceptibility to *N. ditissima* by an average of 18% (Berdeni *et al.*, 2018), providing direct evidence that below-ground microbial interventions can influence canker outcomes. Despite this, a comprehensive investigation of the drivers of root microbiome variation in commercial apple orchards and their relationship with canker susceptibility had not yet been undertaken.

2.3. Waterlogging as an Emerging Abiotic Stressor

Climate projections indicate that the UK and Northern Europe will experience warmer, wetter winters and more frequent extreme rainfall events (Santos *et al.*, 2016; Bednar-Friedl *et al.*, 2022; Pope *et al.*, 2022), making waterlogging an increasingly important threat to temperate apple production. Waterlogging deprives roots of oxygen, forcing a shift to anaerobic fermentation, impairing hydraulic conductance, reducing stomatal opening, and ultimately limiting photosynthesis and carbon allocation throughout the tree (Dat *et al.*, 2006; Parent *et al.*, 2008; Zhou *et al.*, 2020). Apple rootstocks vary substantially in their waterlogging tolerance: genotypes such as G11, G814, and G935 recover quickly, while M9 and G202 suffer prolonged physiological disruption (Marchioretto *et al.*, 2018; Choi *et al.*, 2020). Repeated seasonal waterlogging has been shown to cause compounding declines in root dry weight, fruit yield, and stomatal conductance in grafted apple trees over three years (Olien, 1987).

Beyond direct physiological impacts, abiotic stresses including waterlogging can predispose plants to disease. Broader research in woody hosts has found that the majority of studies link water stress to increased expression of canker diseases. A meta-review by Desprez-Loustau et al. (2006) found that 83% of 64 papers reported drought increasing canker symptom severity. Waterlogging has specifically been found to increase susceptibility of trees to canker-causing opportunistic

pathogens such as *Botryosphaeria dothidea* and *Diplodia mutila* (Schoeneweiss, 1975, 1981). Abiotic stresses also reshape the plant microbiome through both direct environmental effects on microbes and indirect effects via changes in host root exudation and signalling (Hartman and Tringe, 2019; Trivedi *et al.*, 2020), adding a fourth dimension to the established host-pathogen-environment disease triangle (Pandey and Senthil-Kumar, 2019). Despite the increasing risk of winter waterlogging and its clear potential to affect apple physiology, the microbiome, and pathogen behaviour, no research had examined the effects of waterlogging on *N. ditissima* infection or apple canker symptom development, particularly during or around the dormant season.

2.4. Research Objectives

The collective gaps in understanding identified above – the role of the below-ground microbiome in canker susceptibility, the effects of dormant-season waterlogging on apple physiology and the microbiome, and the uncharacterised biocontrol potential of apple-associated *Sphingomonas* – provided the rationale for this research. The overarching aim was to investigate how biotic and abiotic factors affect the development of *N. ditissima* apple canker symptom expression, and to identify microbial taxa associated with canker susceptibility, waterlogging stress, and potential biological control.

This was pursued through four interconnected objectives. The first (Thesis Chapter 2) examined whether orchard establishment factors – site, planting time and cold storage, and scion genotype – affect root-associated fungal and bacterial diversity and community structure and explored whether variation in the root microbiome could be linked to canker symptom expression. The second objective (Thesis Chapter 3) assessed the impact of waterlogging before, during, and after winter dormancy on tree survival, growth, and canker susceptibility across two rootstock genotypes and grafted trees, providing the first experimental evidence of the effects of dormant-season waterlogging on *N. ditissima* infection outcomes. The third objective (Thesis Chapter 4) investigated the long-term impact of winter waterlogging on root and soil microbiome composition, testing whether repeated waterlogging events leave persistent ecological legacies that could affect orchard health. The fourth and final objective (Thesis Chapter 5) characterised the genomic potential of apple-derived *Sphingomonas* isolates for biological control activity, identifying gene homologues associated with plant colonisation, antifungal compound synthesis, and potentially novel biosynthetic gene clusters representing uncharacterised antimicrobial potential. Together, these objectives were designed to generate new understanding of the interconnected biotic and abiotic factors influencing apple canker, and to lay the groundwork for microbiome-informed, climate-resilient management strategies for this economically important disease.

3. Materials and methods

Refer to the thesis for a detailed description of the materials and methods of the below experiments. Brief descriptions of the methods are included in this report.

3.1. The interplay between scion genotype, root microbiome, and *Neonectria ditissima* apple canker

Root samples were collected from a field trial established at three commercial apple orchard sites in Kent, UK. Seven scion genotypes ('Royal Gala', 'Braeburn', 'Scifresh', 'Nicoter', 'Civni', 'Grenadier', and 'Golden Delicious') grafted onto M9 rootstocks were planted at each site in either December 2018 (without cold storage) or April 2019 (following approximately four months of cold storage at 2°C). Trees were uniformly inoculated with *N. ditissima* in the nursery before planting, and canker lesion counts were recorded in May 2021 shortly before root sampling. Fine root samples were collected from four of five blocks per site, pooled per subplot, washed, lyophilised, and ground to a powder for DNA extraction using the E.Z.N.A Soil DNA Kit. Fungal (ITS1) and bacterial (16S V5–V7) communities were characterised by amplicon sequencing on the Illumina NovaSeq platform and community sizes were estimated by qPCR. Sequence data were processed using DADA2-based pipelines to generate amplicon sequence variants (ASVs) with taxonomy assigned using SINTAX. Statistical analysis included ANOVA, permutation ANOVA, PERMANOVA, and negative binomial generalised linear models to assess the effects of site, planting date and cold storage, scion genotype, and canker lesion counts on microbiome composition and diversity.

3.2. Effects of waterlogging timing and duration on growth and *Neonectria ditissima* symptom expression in apple (*Malus* × *domestica*)

Four pot-based experiments were conducted at Niab East Malling to investigate the effects of waterlogging timing and duration on apple rootstock survival, growth, and canker susceptibility. MM106 rootstocks were used in Experiments 1 and 2, which were replicated in consecutive years (planted March 2022 and March 2023) to test the effects of winter waterlogging duration (control, 1-, 2-, 4-, and 8-weeks). Experiment 3 (planted March 2024) compared the effects of autumn, winter, and spring waterlogging on MM106 and M9 rootstocks. Experiment 4 (planted March 2024) tested the effects of waterlogging timing and duration on grafted 'Braeburn'/M9 trees. Plants were grown in pots of low-fertility sandy loam amended with slow-release fertiliser. Waterlogging was applied by submerging pots in buckets of water approximately 3 cm above the soil surface for the assigned duration. All plants were inoculated with *N. ditissima* conidial suspension at leaf fall on artificially wounded leaf scars and rasp wounds. Stem diameter and height were measured before

and after each growing season and canker lesions on inoculated wounds were assessed after each growing season. Mortality, growth, and canker incidence data were analysed using generalised linear models and mixed-effects models in R.

3.3. Effects of winter waterlogging on apple root and soil summer microbiome

Microbiome samples were collected from the pot-based waterlogging experiments described in Chapter 3 (Experiments 1 and 2). One-year-old MM106 rootstocks were planted in low-fertility sandy loam in March 2022 and March 2023, arranged in five blocks of five plots with three rootstocks per pot. After the first growing season, rootstocks were inoculated with *N. ditissima* and subjected to winter waterlogging treatments (control, 1-, 2-, 4-, and 8-weeks) in December/January. Root and soil core samples were collected 6–9 months after drainage from control and 4-week treatment plots. In the 2022 experiment, a second waterlogging treatment was applied the following winter and a second set of samples collected. Frozen samples were freeze-dried, separated into root and soil fractions, and ground before DNA extraction using the E.Z.N.A Soil DNA Kit. Fungal and bacterial communities were characterised by amplicon sequencing (Illumina NovaSeq) and community sizes estimated by qPCR. ASVs were called using DADA2 and taxonomy assigned using SINTAX. Statistical analyses included ANOVA, permutation ANOVA, PERMANOVA, principal component analysis, and DESeq2 differential abundance testing to assess the effects of waterlogging treatment, experimental replicate, and repeated waterlogging on microbiome composition and diversity.

3.4. The potential of *Sphingomonas* bacteria from apple to improve apple canker (*Neonectria ditissima*) management

One-year-old apple twigs were collected from five commercial scion cultivars (‘Braeburn’, ‘Discovery’, ‘Royal Gala’, ‘Saturn’, and ‘Queen Cox’) at the Niab East Malling site across four sampling events in 2023. Leaf scar and leaf disc tissues were homogenised and plated onto antibiotic-amended LB agar to isolate bacteria. Morphologically distinct yellow and orange colonies were selected, confirmed as *Sphingomonas* using a PCR assay targeting the *spt* gene, and genotyped by 16S rRNA Sanger sequencing. Seventy isolates were recovered, yielding seven unique 16S genotypes. A representative isolate from each genotype was selected for whole-genome sequencing using Illumina short-read 250 bp paired-end sequencing (60× coverage) by MicrobesNG. De novo genome assembly was performed using SPAdes and assembly completeness assessed with BUSCO. A preliminary in vitro co-culture antagonism assay was performed on the first 35 isolates to test inhibition of *N. ditissima* mycelial growth. Genome annotation was performed using Bakta, and genes associated with plant interaction and biocontrol traits were identified using PGPg_finder. Biosynthetic gene clusters (BGCs) were predicted using

antiSMASH. Apple-derived isolates were compared to nine reference *Sphingomonas* strains, two established biocontrol agents (*Bacillus velezensis* and *Pseudomonas protegens*), and *Escherichia coli* as a non-biocontrol baseline.

4. Results

4.1. The interplay between scion genotype, root microbiome, and *Neonectria ditissima* apple canker

A total of 2,401 fungal and 7,261 bacterial amplicon sequence variants (ASVs) were identified. The most abundant fungal ASVs included *Dactylonectria macrodidyma* and an unclassified Ascomycota, while *Streptomyces* was the most abundant bacterial taxon.

Across all analyses, orchard site was the dominant driver of both fungal and bacterial community size, alpha diversity, and beta diversity, explaining 9–40% of variance in community size, 24–51% of variance in alpha diversity, and approximately 31% of variance in beta diversity depending on the community and metric examined (Figure 1).

Cold storage and delayed planting (April planting after four months at 2°C) had a smaller but consistent and statistically significant effect on the root microbiome. Fungal community size was larger in trees planted in winter without cold storage ($F = 5.43$, $p = 0.025$), and the planting treatment left a detectable imprint on both fungal and bacterial community composition (beta diversity, Figure 2) that remained measurable three years after planting. This effect was site-dependent, with the most pronounced separation between planting treatments observed at sites 1 and 2. Scion genotype had only limited effects on the root microbiome, primarily on bacterial community composition in a site-dependent manner.

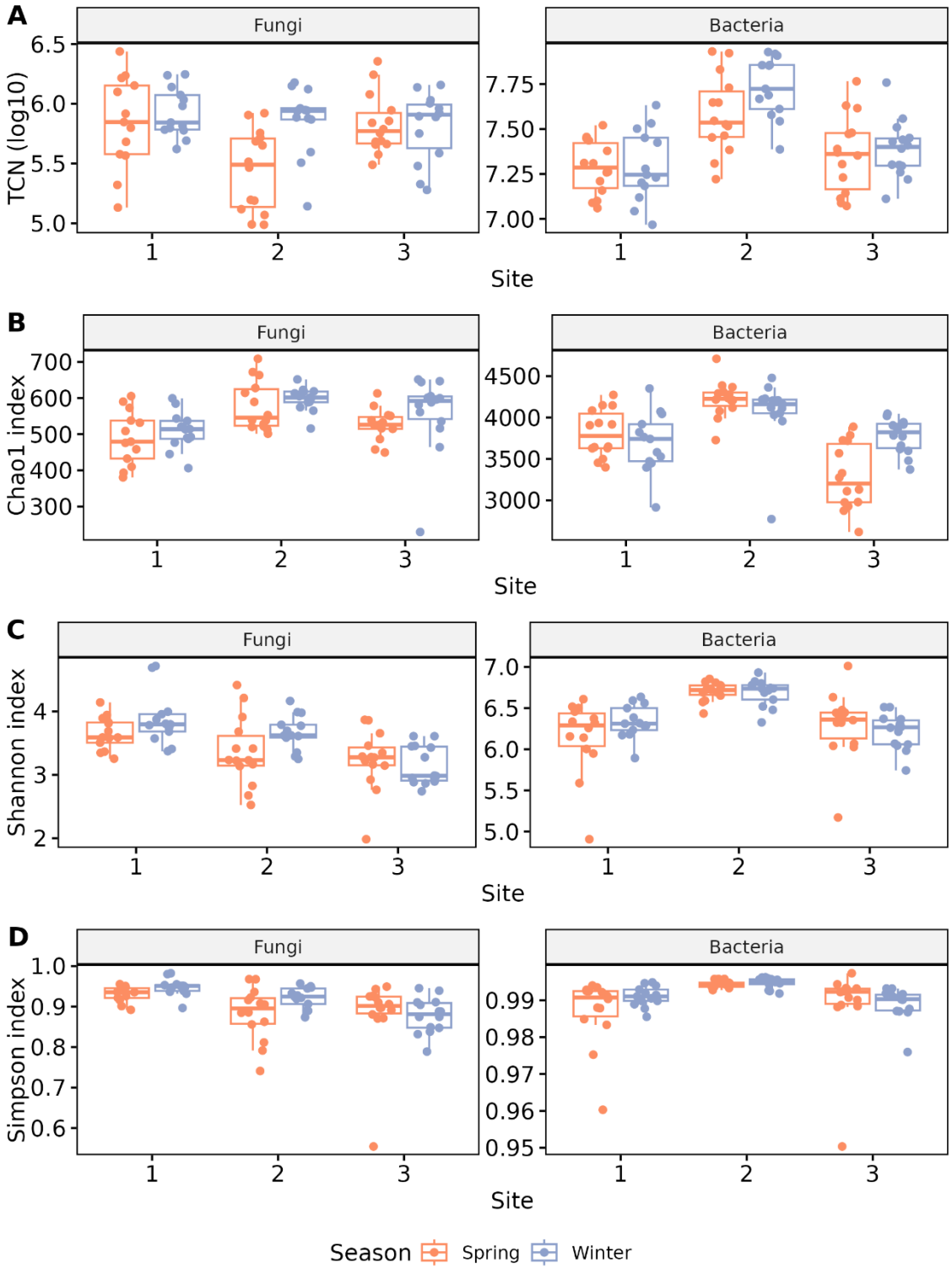

Figure 1. Size and α-diversity of root-associated communities. Box plots illustrating fungal and bacterial (A) estimated community size based on qPCR-derived theoretical copy number, (B) Chao1 richness, (C) Shannon diversity, and (D) Simpson evenness across sites and cold storage/planting season treatments.

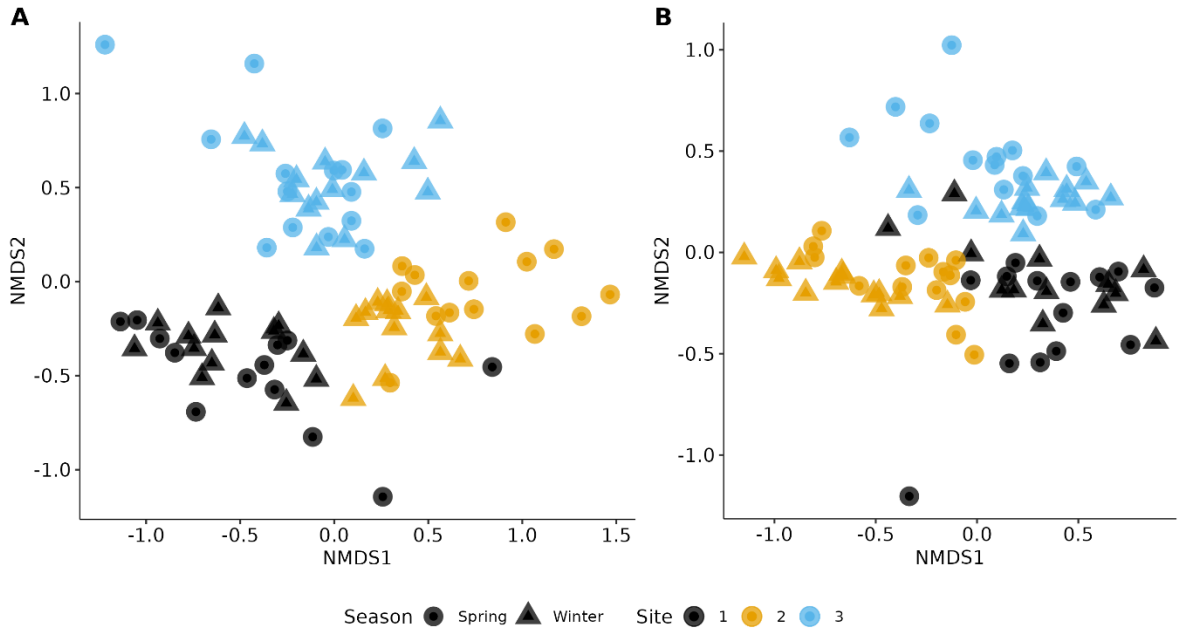

Figure 2. NMDS plots showing β-diversity of root-associated communities. Plots of the first two non-metric multidimensional scaling (NMDS) dimensions, illustrating Bray-Curtis (BC) dissimilarity of fungal (A), and bacterial (B) root-associated communities. Colours are assigned to sites (Site) and shapes to cold storage/planting season (Season).

After accounting for the effects of site, scion genotype, and planting treatment, multiple fungal and bacterial ASVs were significantly associated with canker lesion counts. Among fungi, higher abundance of *Rhizophagus irregularis*, *Epicoccum nigrum*, and Agaricales (order) in the root zone was significantly associated with fewer canker lesions. Conversely, higher abundance of *Fusarium* was associated with greater canker counts at site 1. Among bacteria, higher abundance of *Streptomyces*, *Amycolatopsis*, and *Bradyrhizobium* was associated with fewer canker lesions across all sites or within individual sites. Higher abundance of *Pseudomonas* was associated with greater canker counts.

4.2. Effects of waterlogging timing and duration on growth and *Neonectria ditissima* symptom expression in apple (*Malus × domestica*)

For MM106 rootstocks subjected to winter waterlogging of increasing duration (experiments 1 and 2), eight weeks of winter waterlogging caused 78.7% mortality in experiment 1 but only 4.2% in the replicated experiment 2 (Figure 3A). Repeated winter waterlogging in a second consecutive year did not significantly increase mortality in any treatment group (Figure 3B,C).

Four weeks of waterlogging in autumn caused 50.0% mortality in M9 rootstocks and 31.3% in MM106, both significantly higher than controls ($p < 0.001$). In contrast, winter and spring waterlogging for four weeks did not significantly increase mortality in either genotype (Figure 3D). Shorter durations of one to four weeks did not significantly increase mortality in either experiment. Mortality in grafted ‘Braeburn’/M9 trees was negligible (2 out of 56 trees, Figure 3E).

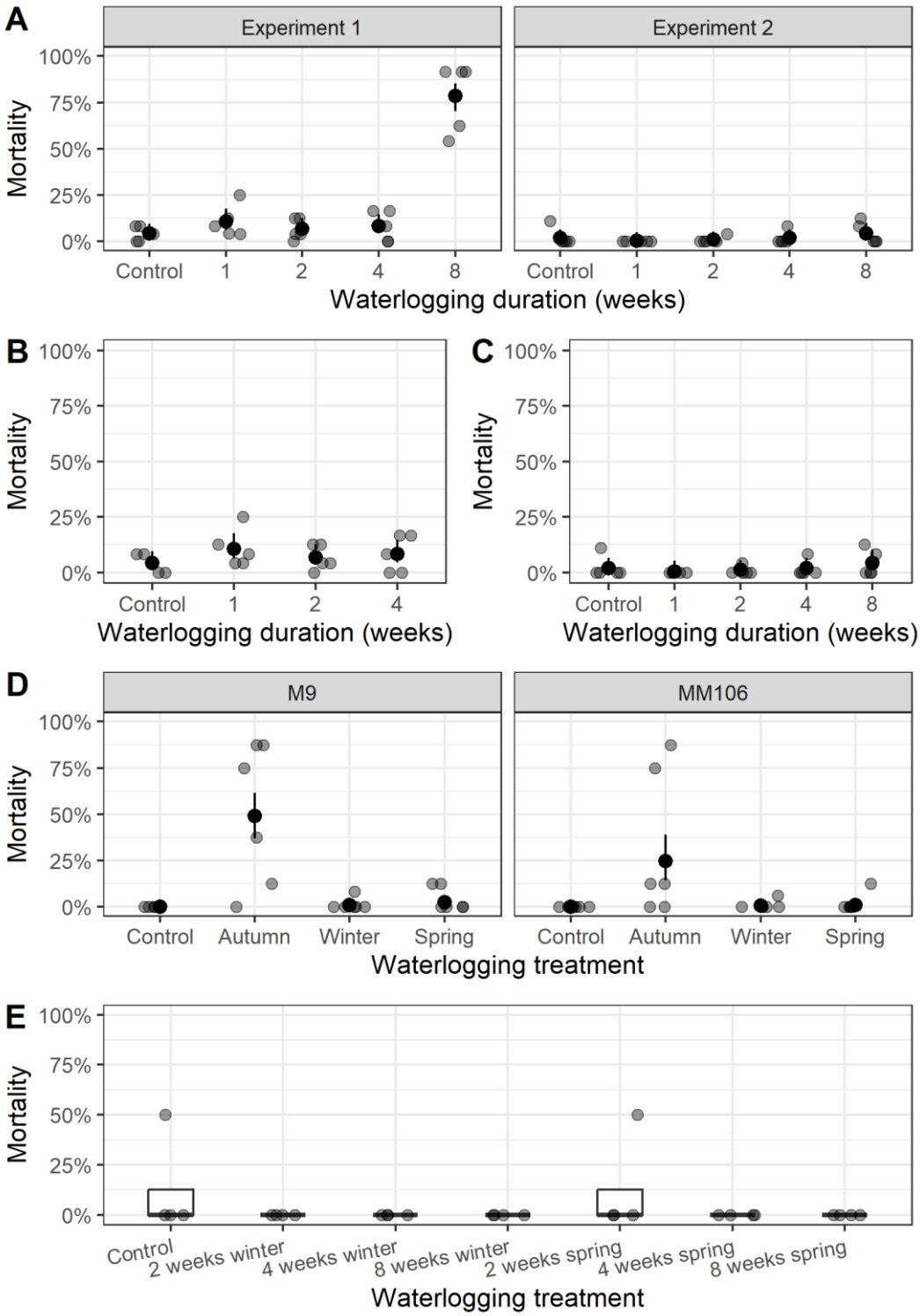

Figure 3. Mortality (% dead trees per plot) in September after the growing season following waterlogging treatments. Results shown for: (A) MM106 rootstocks after single winter waterlogging of 1–8 weeks (experiments 1 and 2); (B) MM106 after repeated waterlogging (experiment 1); (C) MM106 after repeated waterlogging (experiment 2); (D) M9 and MM106 rootstocks after 4-week waterlogging in different seasons; (E) 'Braeburn'/M9 grafted trees after winter or spring waterlogging. Black points show estimated mortality with 95% confidence intervals; grey points show plot-level data.

Across all four experiments, surviving rootstocks and grafted trees showed no significant reduction in stem diameter growth or height growth in the growing season following waterlogging, regardless of timing, duration, or whether it was a first or second repeated treatment (See thesis for figures).

Canker incidence on artificially inoculated leaf scar and rasp wounds was not significantly affected by waterlogging in any experiment (Figure 4 & 5). The one exception was a significant increase in total canker count (including background infections from unknown sources) after repeated 2-week winter waterlogging in experiment 1, but this was not observed for the 4-week treatment in the same experiment and was not replicated in inoculated wound incidence data. M9 rootstocks had 28.9 times higher canker incidence than MM106 regardless of treatment, and rasp wounds had 16.7–22.2 times higher incidence than leaf scars.

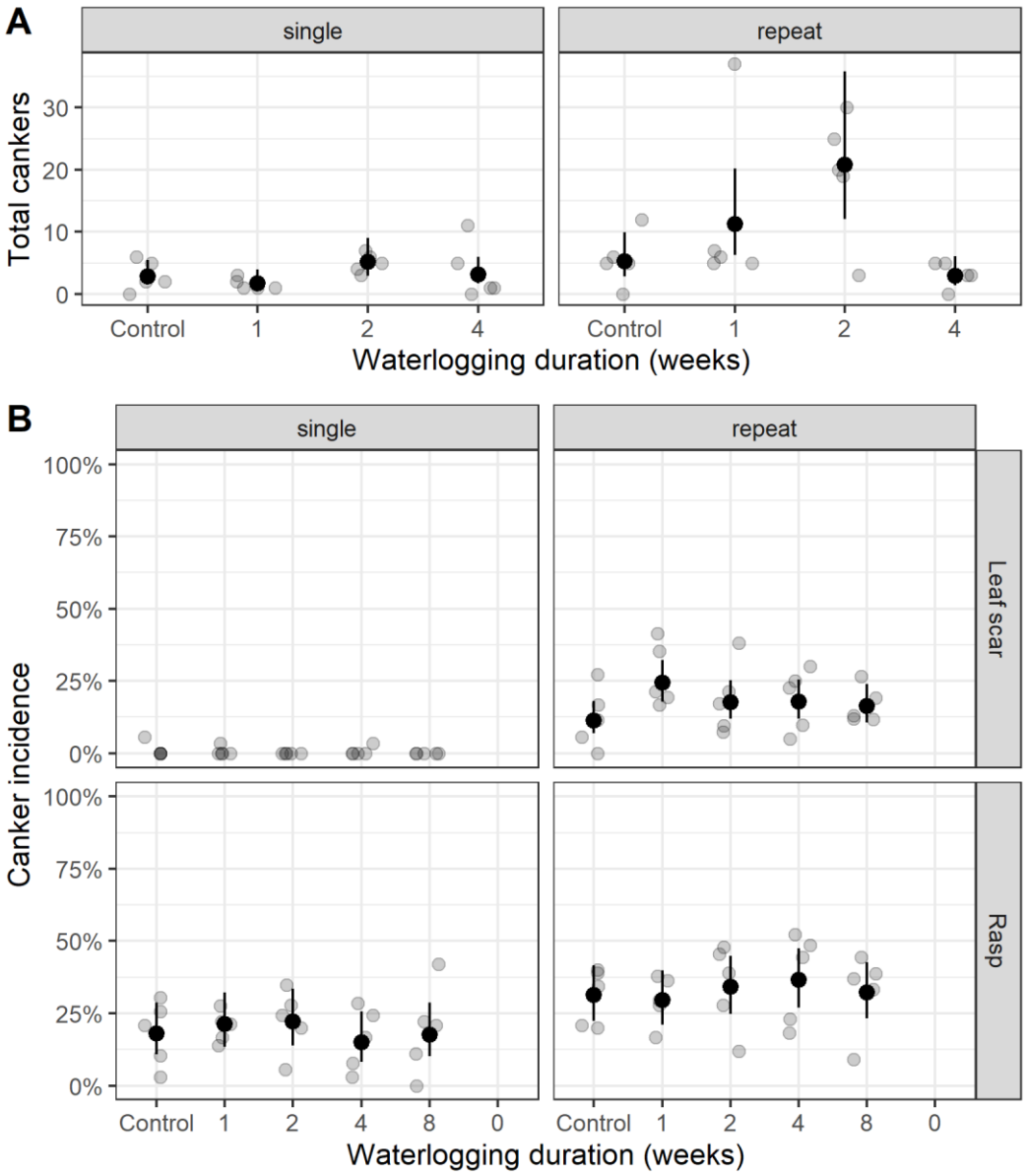

Figure 4. Total cankers in experiment 1 (A) and canker incidence in experiment 2 (B) after single and repeated winter waterlogging treatments. Black points show estimated total cankers (A) or canker incidence (B) with 95% confidence intervals; grey points show plot-level data.

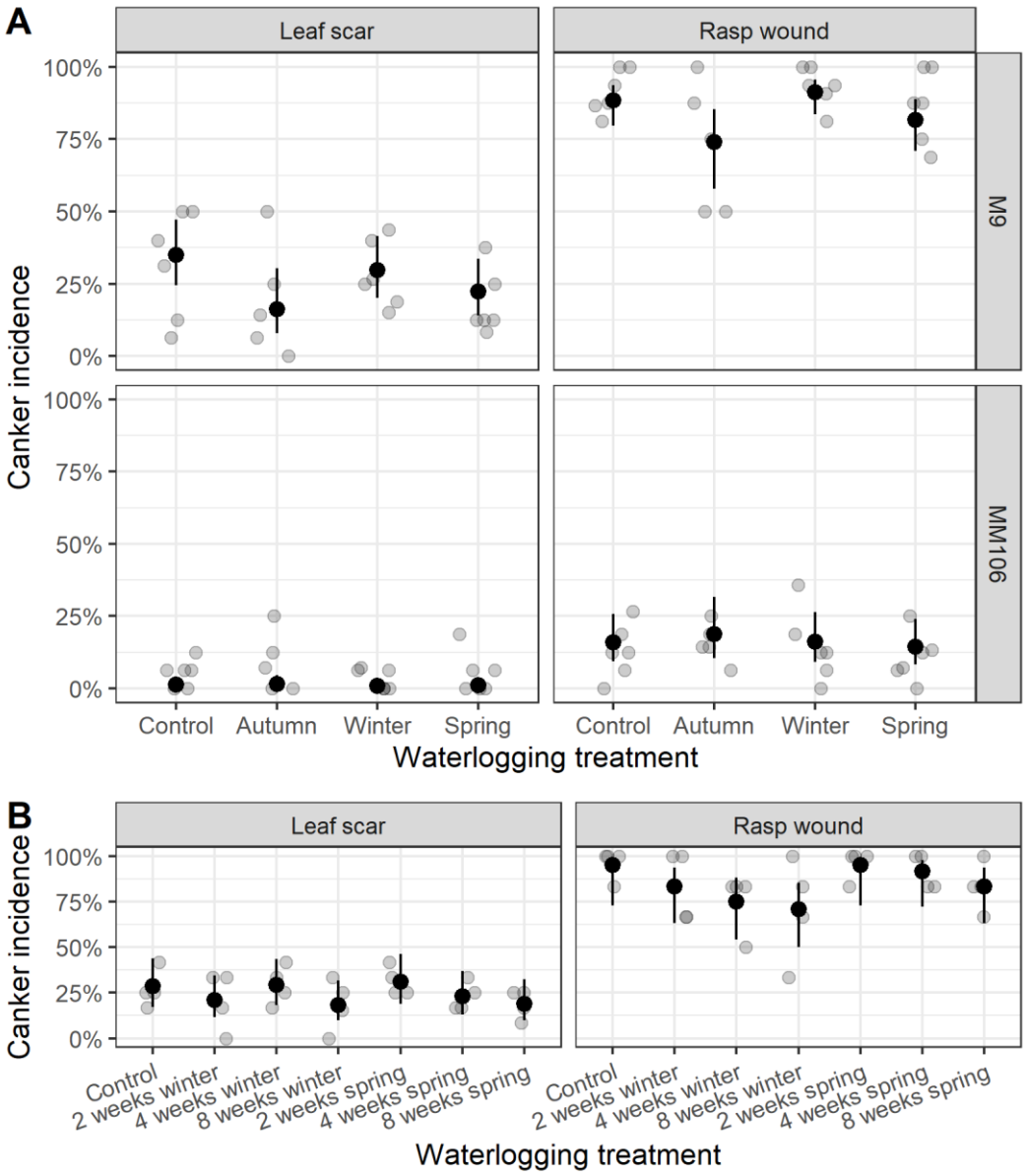

Figure 5. Canker incidence on inoculated leaf scars and rasp wounds for M9 and MM106 rootstocks in experiment 3 (A) and 'Braeburn'/M9 grafted trees in experiment 4 (B). Black points show estimated canker incidence with 95% confidence intervals; grey points show plot-level data.

4.3. Effects of winter waterlogging on apple root and soil summer microbiome

A total of 1,224 fungal and 4,359 bacterial amplicon sequence variants (ASVs) were identified. The most abundant fungal ASVs were Plectosphaerellaceae, while the most abundant bacterial ASVs were *Streptomyces* and Actinobacteria.

For single waterlogging across experiments, experimental year and spatial variation (block within experiment) dominated. Across single and repeated waterlogging in the 2022 experiment, temporal succession and spatial variation (block) dominated.

Winter waterlogging consistently reduced fungal soil diversity (Chao1, Shannon, Simpson; all $p < 0.01$), while bacterial root and soil richness increased in response to waterlogging (Chao1; $p < 0.01$), both effects persisting across independent experimental replicates sampled 6–9 months post-treatment (Figure 6). Across single and repeated waterlogging treatments in the 2022 experiment, waterlogging decreased bacterial soil evenness (Simpson, $p = 0.001$, Figure 7).

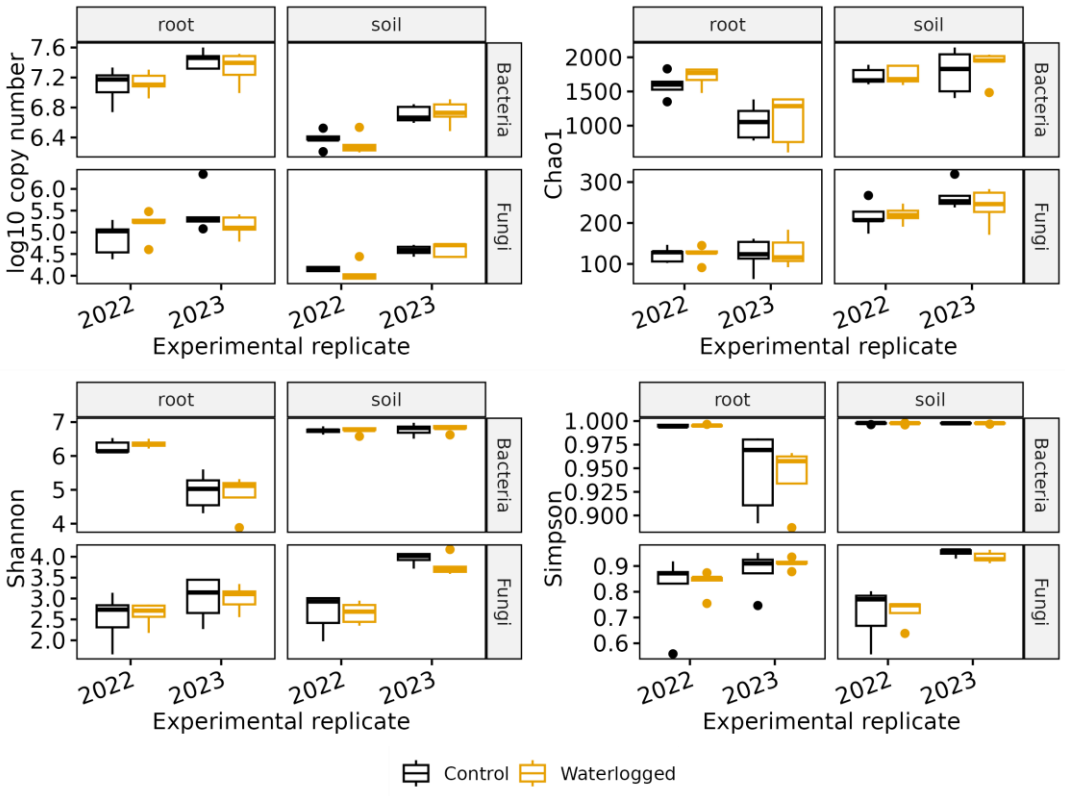

Figure 6. Size and α-diversity of communities across experimental replicates. Community size (log10 theoretical copy number), and alpha-diversity measures of richness (Chao1), diversity (Shannon), and evenness (Simpson) of fungal and bacterial root and soil communities for the 2022 and 2023 experimental replicates after single waterlogging compared to non-waterlogged control.

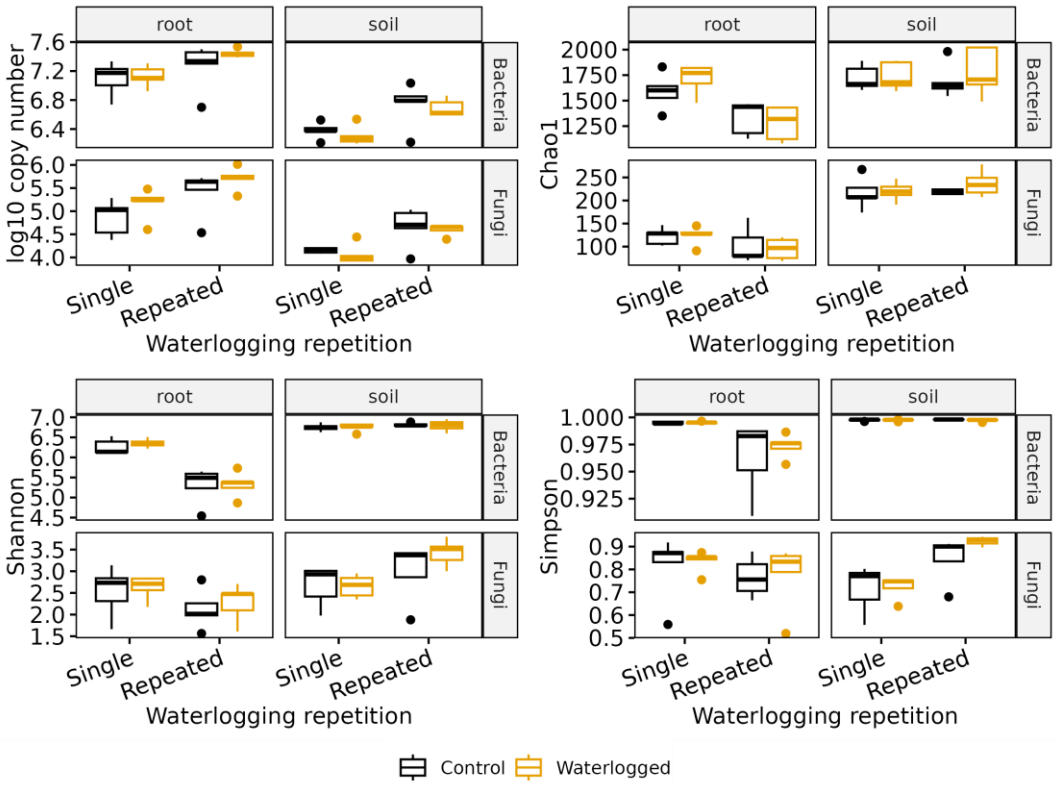

Figure 7. Size and α-diversity of communities for single and repeated waterlogging. Community size (log10 theoretical copy number), and alpha-diversity measures of richness (Chao1), diversity (Shannon), and evenness (Simpson) of fungal and bacterial root and soil communities in the 2022 experiment after first waterlogging in 2022/2023 (single) and second waterlogging in 2023/2024 (repeated) compared to non-waterlogged controls at each timepoint.

Beta diversity analysis confirmed small but statistically significant shifts in community structure in response to waterlogging. Fungal root community structure responded significantly to single waterlogging (PERMANOVA, $p = 0.043$, 6.2% of variation), while bacterial soil communities showed a consistent sub-population response captured by the third principal component (PC3; $p = 0.044$) (Figure 8). All communities shifted significantly in the 2022 experiment in response to both single and repeated waterlogging (PERMANOVA, $p < 0.05$, Figure 9), with bacterial root and soil community shifts again reflected in PC3.

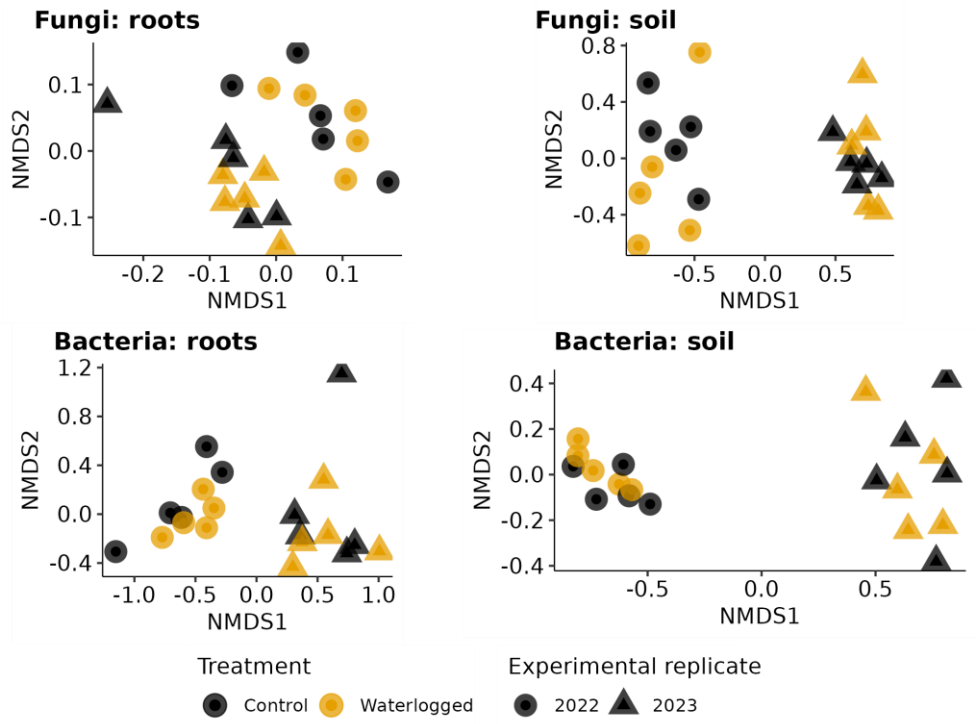

Figure 8. NMDS plots showing β-diversity across experimental replicates based on Bray-Curtis dissimilarity of fungal and bacterial root and soil communities across samples from the 2022 and 2023 experimental replicates after single waterlogging compared to non-waterlogged control.

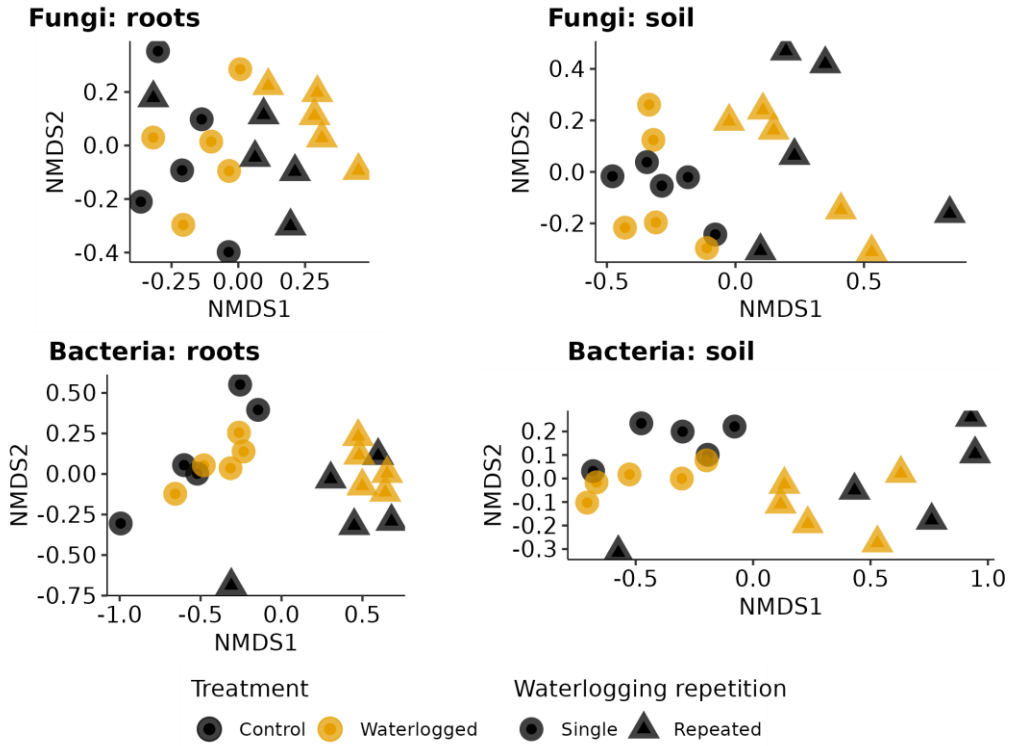

Figure 9. NMDS plots showing β-diversity across single and repeated waterlogging based on Bray-Curtis dissimilarity of fungal and bacterial root and soil communities in the 2022 experiment after first waterlogging in 2022/2023 (single) and second waterlogging in 2023/2024 (repeated) compared to non-waterlogged controls at each timepoint.

Differential abundance analysis identified the microbial taxa driving these shifts. In soil, four bacterial genera consistently increased in abundance in response to waterlogging across both experimental replicates: the obligate anaerobes *Geomonas*, *Geobacter*, *Desulfuromonas*, and *Clostridium*. In roots, the anaerobes *Geomonas* and *Cellulomonas* increased, while the strictly aerobic *Arenibacter* and *Lysinimonas* decreased (Figure 10). Analysis of the 2022 experiment across single and repeated waterlogging events revealed additional transient responders: the fungal biocontrol candidate *Chaetomium* and the beneficial bacterial genera *Massilia* and *Ramlibacter* increased after single waterlogging, but these responses were not sustained or amplified by repeated waterlogging. *Geomonas* was the only genus to respond to both single and repeated waterlogging in both root and soil compartments (Figure 11).

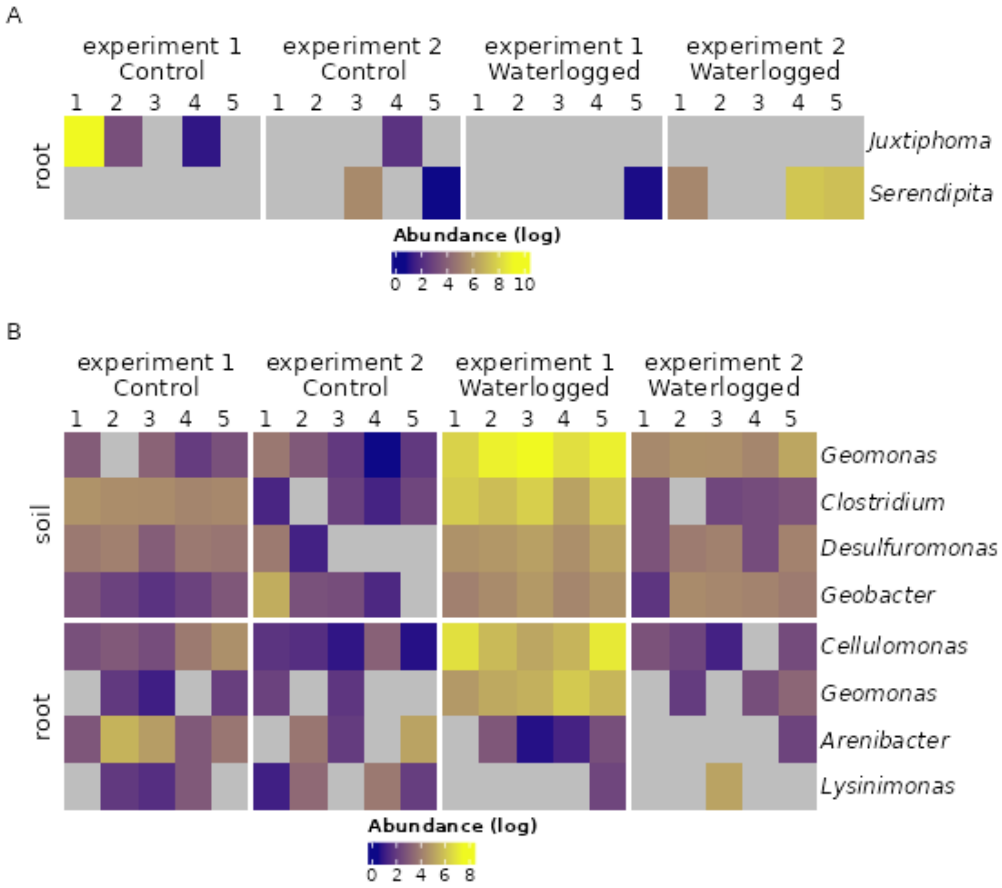

Figure 10. Heatmap of taxa that responded to waterlogging across experimental replicates. Fungal (A) and bacterial (B) genera with significant differential abundance between control and waterlogged samples across the 2022 and 2023 experimental replicates. Heatmap values show the natural log of abundance + 1; grey cells indicate the genus was not detected in the sample.

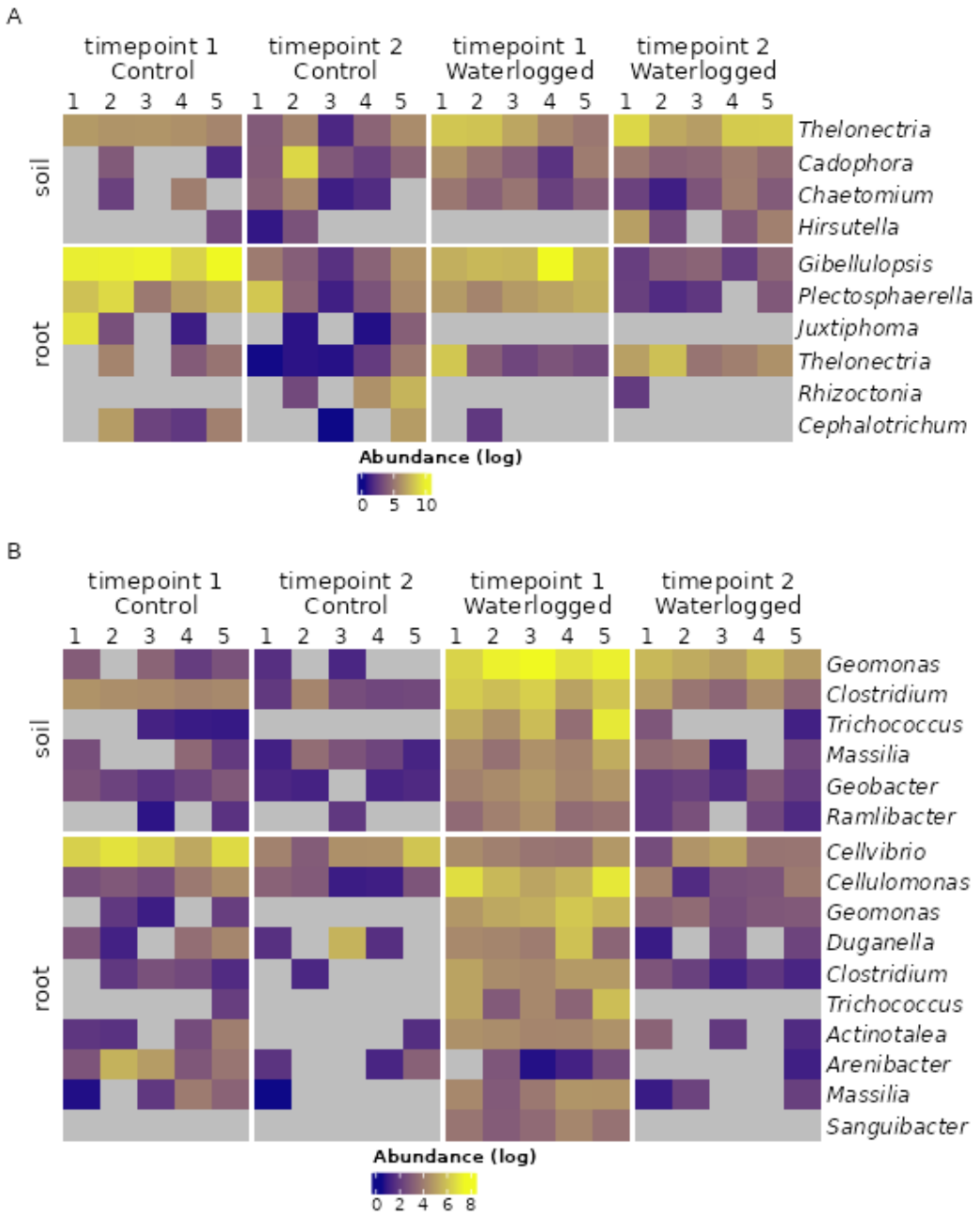

Figure 11. Heatmap of taxa that responded to single or repeated waterlogging. Fungal and bacterial genera with significant differential abundance after single (timepoint 1) or repeated (timepoint 2) waterlogging in the 2022 experiment. Heatmap values show the natural log of abundance + 1; grey cells indicate the genus was not detected.

4.4. The potential of *Sphingomonas* bacteria from apple to improve apple canker (*Neonectria ditissima*) management

In vitro antagonism assays on the first 35 of the 70 recovered *Sphingomonas* isolates showed that, while eight isolates produced a statistically significant reduction in *Neonectria ditissima* mycelial growth, the magnitude of inhibition was very small (maximum 7.4%), compared to 54.4% and 34.9% inhibition achieved by *Bacillus subtilis* positive controls. Several isolates showed slight negative inhibition, and in many cases *N. ditissima* mycelium grew directly over the *Sphingomonas* colonies.

Whole-genome sequencing of seven representative isolates (one per unique 16S genotype) revealed that six of the seven may represent previously undescribed *Sphingomonas* species, failing to meet the 70% digital DNA-DNA hybridisation (dDDH) threshold for assignment to any known species in the TYGS database (Figure 12). The exception was isolate B21, which was assigned to *Sphingomonas aurantiaca*. Two isolates (F69 and G66) had distinct 16S sequences but identical whole genomes, identifying them as clones. Isolates A32, D04, E36, and F69/G66 did not cluster with any available type strains.

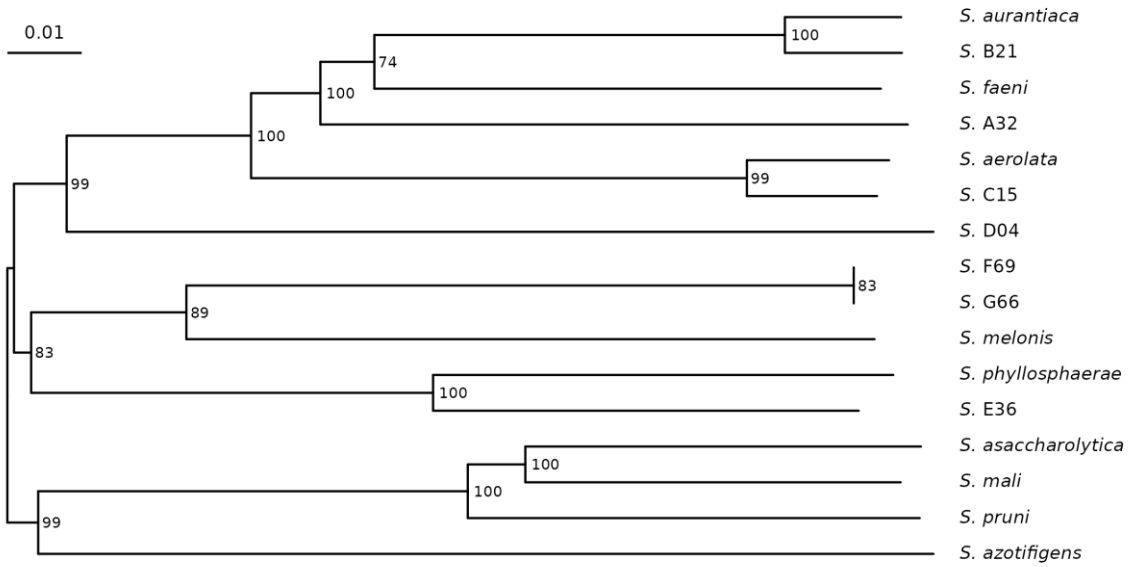

Figure 12. Balanced minimum evolution tree for Sphingomonas strains used in this study, based on intergenomic distances calculated by the Type (Strain) Genome Server (TYGS). Branch lengths are drawn to scale; the scale bar (0.01) represents a 1% change in genomic distance. Bootstrap values based on 100 pseudo-replicates are indicated at nodes.

Analysis of plant-associated genetic traits using the PLaBAse database revealed that all *Sphingomonas* isolates dedicated a substantial proportion of their genomes (53–59% of coding sequences) to plant-related functions. The functional profile of the *Sphingomonas* isolates most closely resembled that of *Pseudomonas protegens* rather than *Bacillus velezensis*, with enrichment

in plant colonisation, iron acquisition, phosphate solubilisation, and competitive exclusion traits. Several apple-derived isolates (E36, F69, G66) were enriched in chemotaxis genes. The *S. pruni/S. mali/S. asaccharolytica clade* – all isolated from Rosaceae roots – was particularly enriched in genes for detoxification of peroxidised compounds, antibiotic resistance, and N-acyl homoserine lactone (AHL) biosynthesis.

Among biocontrol-specific genetic traits, all *Sphingomonas* isolates contained gene homologues associated with biosynthesis of the antifungal compound pyrrolnitrin (specifically tryptophan 7-halogenase, prnA/rebH/ktzQ), with apple-derived isolates S. C15 and S. D04 each possessing 15–16 copies compared to three in *P. protegens* (Figure 13). Genes encoding phenazine-1-carboxylic acid (PCA) and chitinolytic enzymes were present in all isolates, most abundantly in the *S. pruni/S. mali/S. asaccharolytica* clade. Genes for stimulation of plant immune responses, including LPS elicitors and AvrXa21 effectors, were present in a subset of isolates.

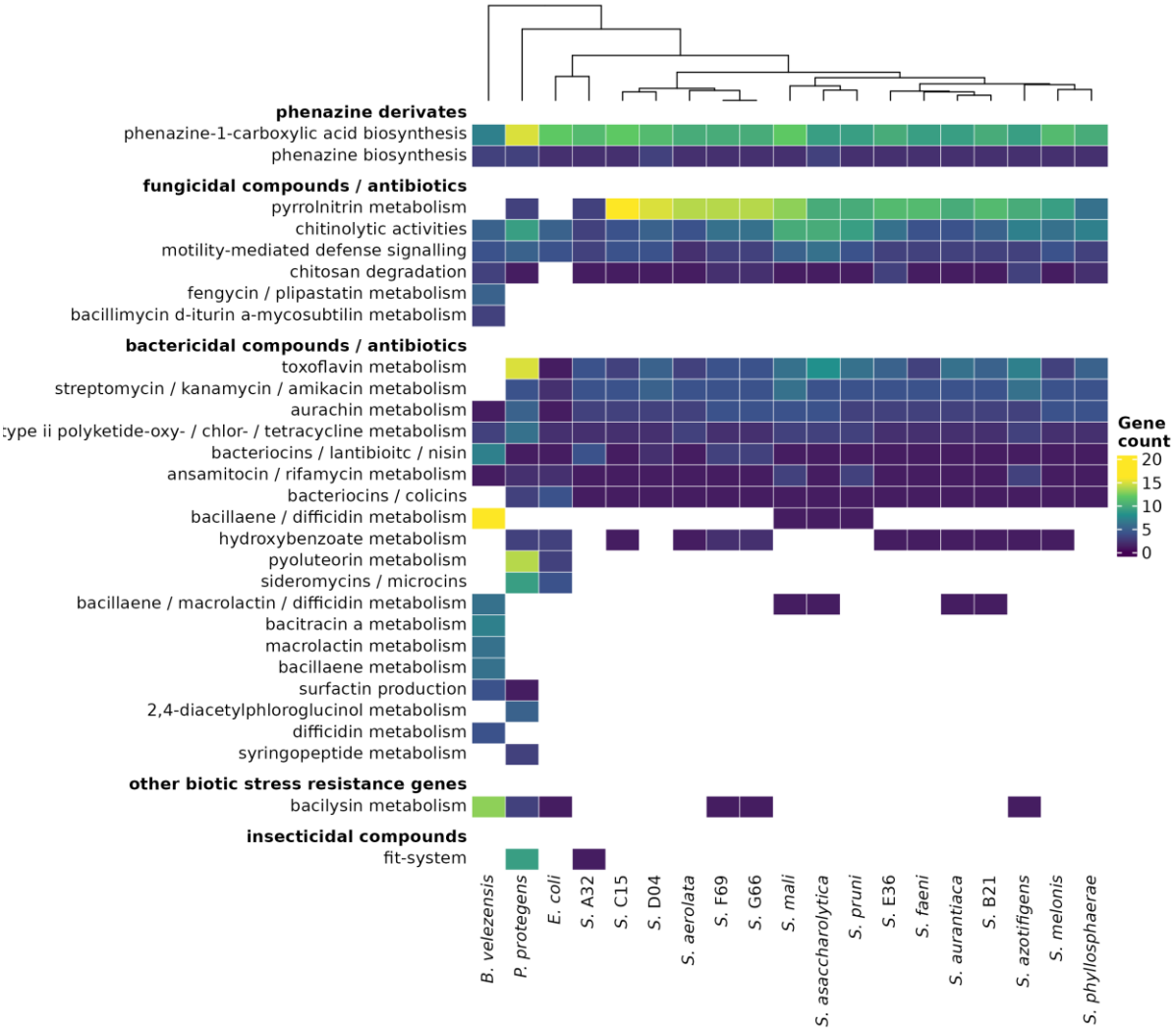

Figure 13. Count of genes assigned to 'biological control' categories in the PLaBAse database for each genome. Dendrograms reflect hierarchical clustering based on Euclidean distance.

Biosynthetic gene cluster (BGC) prediction using antiSMASH revealed that, in contrast to the well-characterised arsenals of *B. velezensis* (surfactin, fengycin, bacillaene, difficidin) and *P. protegens* (pyrrolnitrin, pyoluteorin, DAPG), the majority of BGCs in all *Sphingomonas* isolates lacked matches in the MIBiG database and therefore represent potentially novel secondary metabolites with uncharacterised biological activity (Figure 14). The *S. pruni/S. mali/S. asaccharolytica* clade had the highest predicted BGC counts (10–15 per genome), including pathways for RiPPs, NRPs, polyketides, and terpenes. A BGC with 100% similarity to rhizomide A/B/C – a non-ribosomal peptide associated with biocontrol activity in *Pseudomonas* and *Burkholderia* – was identified in *S. mali*. Carotenoid BGCs (likely for zeaxanthin, conferring oxidative stress tolerance) were identified across several isolates.

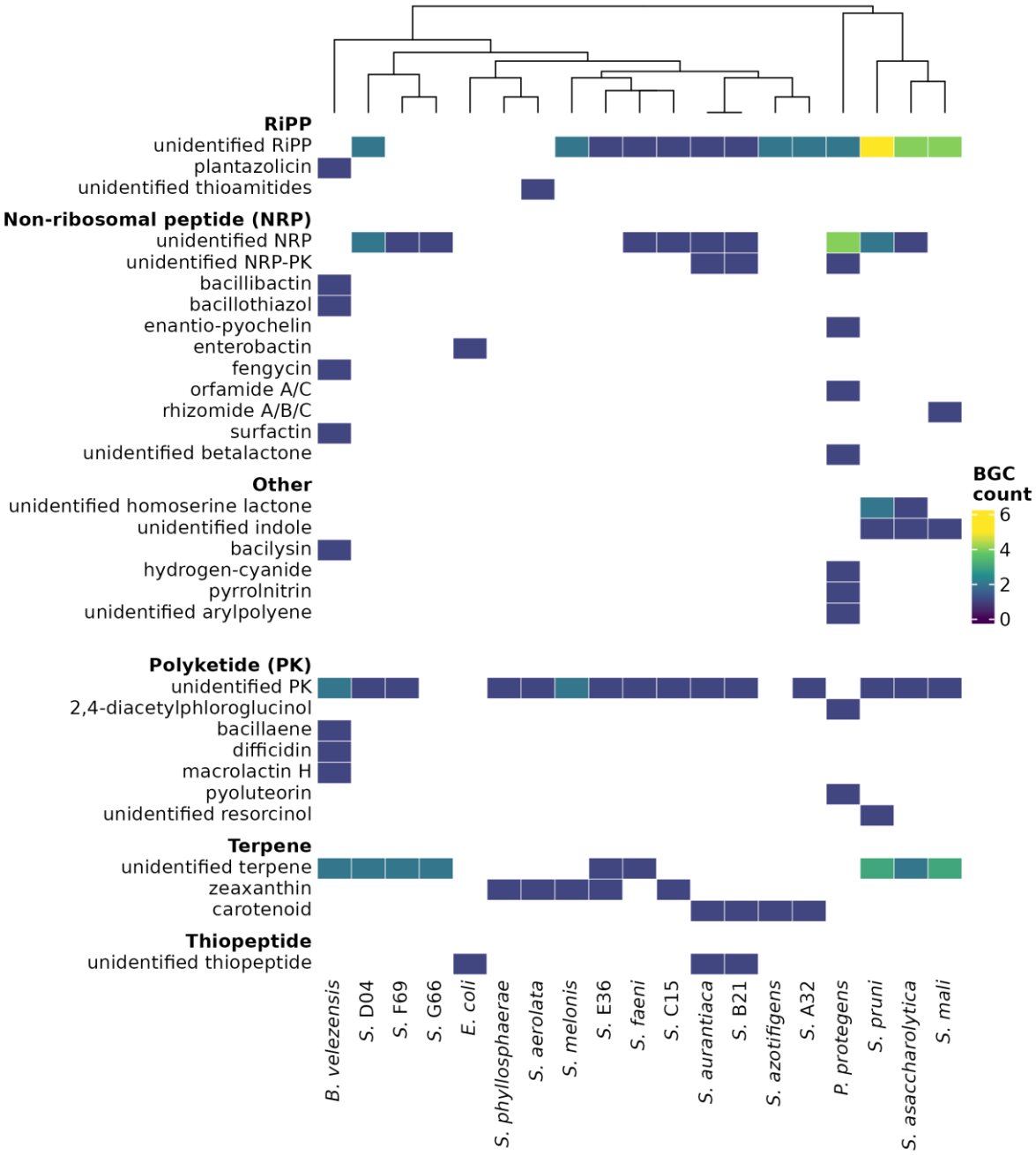

Figure 14. Count of biosynthetic gene cluster (BGC) categories identified by antiSMASH for each genome. BGCs with <50% identity to their closest MIBiG match are labelled as undefined and broadly categorised. Dendrograms reflect hierarchical clustering based on Euclidean distance.

5. Discussion

5.1. The interplay between scion genotype, root microbiome, and *Neonectria ditissima* apple canker

Site was the overwhelming driver of root microbiome variation, consistent with the established dominance of local soil and environmental conditions over host genotype across apple microbiome studies (Liu *et al.*, 2020; Olivieri *et al.*, 2021; Papp-Rupar *et al.*, 2023a). The finding that cold storage combined with delayed planting left a detectable imprint on root microbiome composition three years after planting is novel and aligns with the concept of a "founder effect" in microbiome assembly – the idea that initial community composition at establishment constrains the trajectory of long-term succession. This has practical relevance for growers: planting timing and pre-planting cold storage are routine logistical decisions, but this work suggests they may have lasting consequences for the microbial communities that influence tree health. Industry guidance on optimal planting protocols should consider these microbiome effects alongside the established agronomic and disease-management considerations. Scion genotype had only limited effects on root microbiome composition, likely because the uniform M9 rootstock exerted dominant control over the closely associated root endophyte communities sampled here; future studies examining the rhizosphere rather than root endosphere may reveal stronger scion effects. The associations identified between specific taxa and canker outcomes – particularly the beneficial associations with *Rhizophagus irregularis*, *Epicoccum nigrum*, *Trichoderma*, *Amycolatopsis*, and *Bradyrhizobium* – are promising but correlative. Establishing causality through targeted inoculation trials is the critical next step. These findings support the development of microbiome-enriching planting protocols – for example, arbuscular mycorrhizal fungi inoculation at planting, which has already been shown to reduce canker susceptibility by an average of 18% (Berdeni *et al.*, 2018) – or broader biological soil amendment strategies timed to orchard establishment. Such interventions represent a practical avenue for growers and advisors to explore, pending confirmation of causality from targeted experimental work.

5.2. Effects of waterlogging timing and duration on growth and *Neonectria ditissima* symptom expression in apple (*Malus* × *domestica*)

This study provides the first experimental evidence of the effects of dormant-season waterlogging on apple survival, growth, and canker susceptibility, addressing an important gap given the projected increase in winter rainfall events in the UK and Northern Europe. The clear finding that autumn waterlogging was the most lethal period – causing up to 50% mortality in M9 rootstocks after just four weeks – is consistent with the understanding that metabolically active transitions, such as the entry into dormancy, are more vulnerable to oxygen deprivation than the established

dormant state (Olien, 1987; Nicoll and Coutts, 1998). The inconsistency in mortality outcomes across replicated winter waterlogging experiments (high mortality in the 2022 experiment, negligible in the 2023 experiment) suggests that ambient temperature during waterlogging could be an important modifying factor, with colder winters with more frost days likely compounding hypoxic stress. This interaction warrants further investigation under controlled conditions. Critically for orchard management, surviving trees showed no lasting growth penalty regardless of waterlogging timing or duration, suggesting that moderate waterlogging events during dormancy do not persistently compromise tree vigour. Waterlogging did not significantly increase canker incidence on artificially inoculated wounds under the experimental conditions tested, indicating that dormant-season waterlogging alone is not a major direct driver of *N. ditissima* infection. These experiments used a single soil type under controlled conditions, so field validation is needed; orchard soils with poorer drainage may sustain hypoxia for longer after water recedes, which could alter outcomes. Future work should also test whether waterlogging that coincides with active infection periods or the growing season increases canker susceptibility. For growers and advisors, the key practical message is that autumn waterlogging represents the highest-risk period: investment in drainage infrastructure and selection of waterlogging-tolerant rootstocks should prioritise protection during the autumn transition to dormancy.

5.3. Effects of winter waterlogging on apple root and soil summer microbiome

This chapter demonstrates that the ecological legacy of a single winter waterlogging event persists well beyond drainage, with significant shifts in soil bacterial and fungal communities detectable 6–9 months later. The consistent enrichment of obligate anaerobes – particularly *Geomonas*, *Geobacter*, and *Desulfuromonas* – across both experimental replicates identifies these genera as reliable indicators of waterlogging history in apple orchard soils, consistent with their identification as waterlogging biomarkers in wheat and rapeseed (Gschwend *et al.*, 2020; Francioli *et al.*, 2021). The persistence of anaerobic bacteria long after re-oxygenation is likely driven by stable anaerobic microsites within soil aggregates and biofilms. For orchard management, this suggests that even a single significant waterlogging event can alter the soil microbial environment in ways that carry over into the subsequent growing season, with potential knock-on effects for nutrient cycling. The transient enrichment of potentially beneficial aerobic taxa – *Chaetomium* (a known antagonist of *N. ditissima* in vitro), *Ramlibacter*, and *Massilia* – after single waterlogging is intriguing and may reflect a host-mediated recruitment of beneficial microbes during recovery. This enrichment was not sustained after repeated waterlogging, suggesting that orchards subject to repeated winter flooding may progressively lose this compensatory response. The finding that potential fungal pathogens such as *Gibellulopsis* and *Plectosphaerella* declined after waterlogging is reassuring. Future research should examine whether these persistent microbiome shifts ultimately affect

disease outcomes when waterlogging coincides with active *N. ditissima* infection periods, and whether microbiome trajectories after waterlogging can be steered by soil amendment to favour beneficial taxa.

5.4. The potential of *Sphingomonas* bacteria from apple to improve apple canker (*Neonectria ditissima*) management

Despite the very low direct antifungal activity observed in vitro – with maximum inhibition of *N. ditissima* mycelial growth reaching only 7.4% compared to 54.4% for *Bacillus subtilis* positive controls – genomic analysis reveals that apple-derived *Sphingomonas* isolates possess a substantial suite of genetic traits associated with biocontrol activity and phyllosphere persistence. This contrast between low in vitro antagonism and high genomic potential echoes the broader challenge in biocontrol research, where in vitro assays frequently fail to predict field performance (Walter *et al.*, 2017; Papp-Rupar *et al.*, 2023b). The enrichment of pyrrolnitrin biosynthesis genes – present at 15–16 copies in some apple isolates compared to three in *Pseudomonas protegens* – is particularly noteworthy given pyrrolnitrin's critical role in *Pseudomonas* biocontrol against fungal pathogens (Zameer *et al.*, 2025). The fact that the majority of biosynthetic gene clusters (BGCs) in *Sphingomonas* lack matches in the MIBiG reference database suggests these bacteria may produce novel secondary metabolites whose biocontrol activity is entirely uncharacterised, representing potential targets for future discovery. The finding that six of the seven sequenced isolates likely represent previously undescribed species further underscores the novelty of this material. The genomic profile of apple *Sphingomonas* – with high investment in plant colonisation, iron acquisition, chemotaxis, and competitive exclusion – resembles that of *P. protegens* more than *B. subtilis*, suggesting these bacteria are equipped to establish persistent phyllosphere populations. Persistent colonisation is a recognised bottleneck for canker biocontrol (Papp-Rupar *et al.*, 2023b), and the apparent native adaptation of *Sphingomonas* to the apple environment is an advantage that warrants exploitation. Priority next steps for R&D include identifying the environmental conditions under which antifungal secondary metabolites (particularly pyrrolnitrin) are expressed *in planta*; characterising the products of novel BGCs through metabolomics approaches; and progressing to leaf scar inoculation trials under orchard conditions to test whether naturally adapted *Sphingomonas* populations can suppress *N. ditissima* infection in the field.

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