

CRANFIELD UNIVERSITY

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HAS PLANT DOMESTICATION DECOUPLED BENEFICIAL
PLANT-MICROBE INTERACTIONS IN WHEAT?

SCHOOL OF WATER, ENERGY AND ENVIRONMENT
PhD in Environment and Agrifood

PhD
Academic Year: 2021 - 2022

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the degree of PhD

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ABSTRACT

Domestication of plant species has substantially contributed to human civilization, but also caused a strong decrease in the genetic diversity of modern crop cultivars by selecting for high-yielding dwarf crops which are reliant on unsustainable levels of inorganic fertilizer. This transition from natural to agricultural systems has played an important role in the development of agriculture over the last 10,000 years but may have affected the ability of plants to establish beneficial associations with rhizosphere microbes.

Plant growth-promoting rhizobacteria (PGPR) are essential for plant health and fitness and play an important role in the sustainable intensification of agriculture. This thesis assesses the impacts of domestication on beneficial plant-microbial interactions in wheat with a view to developing robust microbial inocula for enhanced crop growth.

Culture dependent and independent approaches were used to explore the influence of widely used nitrogen-phosphate-potassium (NPK) fertilizer on the abundance of wheat rhizobacterial genera with plant growth-promoting traits. Putative plant growth-promoting rhizobacteria (PGPR) were reduced in fertilized wheat plants. Approximately 1,500 bacterial isolates were amassed from the rhizosphere and rhizoplane of Cadenza wheat plants grown in low nutrient soil either supplemented with or without fertilizer in pot greenhouse experiments, representing an inherently more controlled design than many literature field studies. PGPR were taxonomically identified by Sanger sequencing of the 16S rRNA gene and functionally characterized using single colony functional bioassays (nitrogen, phosphate, potassium, iron, and zinc solubilization) and subsequently identified in high throughput 16S rRNA gene amplicon sequence derived culture-independent community datasets, which revealed a significantly lower abundance of nutrient-solubilizing rhizobacteria in fertilized plants. The same methods were applied to rhizoplane samples from 19 ancestral and domesticated wheat genotypes grown in the same conditions which resulted in isolation of approximately 15,000 bacterial isolates. Notably, there was a significantly lower abundance of PGPR isolated from unplanted control pots (bulk

soil) compared to plants and no difference between fertilization conditions. Moreover, differences in the abundance of PGPR under contrasting fertilization conditions were more pronounced in domesticated wheat, which we hypothesize is due to a loss of plant-microbe signalling pathways as the wheat genome underwent expansion. Key genera differentially more abundant in non-fertilized wheats included members of the phylum Cyanobacteria (*Nostoc*) and Proteobacteria (*Bradyrhizobium*, *Pseudomonas*) compared to fertilized wheats which were richer in Actinobacteria (*Arthrobacter*, *Catenulispora*, *Leifsonia*, and *Streptomyces*).

Introduction of the *Reduced height* (*Rht*) genes during the Green Revolution has been hypothesized to reduce PGPR selection due to their influence on the plant hormone gibberellin (GA). 16S rRNA gene amplicon sequencing revealed a markedly different rhizosphere microbiome in severe Cadenza wheat *Rht* dwarf mutants which became more pronounced with mineral fertilizer addition. There was a higher differential abundance in Acidobacteria, Chloroflexi and Gemmatimonadetes, phyla more commonly associated with bulk soil, in *Rht* mutant cultivars compared with a higher differential abundance of Bacteroidetes, Firmicutes and Proteobacteria, phyla more commonly associated with plant growth promotion, in wildtype Cadenza wheat.

The research presented in this thesis contributes to our understanding of the impact domestication has had on plant-microbe interactions in the presence and absence of agriculturally important but potentially environmentally deleterious chemicals, as well as presenting a method for functional characterization of microbiomes. This knowledge will benefit the development of more targeted ecologically benign biofertilization strategies.

Keywords: Wheat, rhizobacteria, fertilizer, plant growth-promoting rhizobacteria, PGPR, domestication, ploidy, *Reduced height*, *Rht*

DECLARATION

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“To my beloved parents, David and Catherine Reid”

*“There can be no life without soil and
no soil without life; they have evolved
together.”*

-Charles E. Kellogg
Biography, 1965

*“How can I stand on the ground every
day and not feel its power? How can
I live my life stepping on this stuff and
not wonder at it?”*

-W. B. Logan
Dirt: the Ecstatic Skin of the Earth

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACT	Alignment, Classification and Tree
ANOVA	Analysis of variance
ASV	Amplicon sequence variant
AVK	Aleksandrov agar
BS	Bulk soil
CAP	Canonical analysis of principle coordinates
CAS	Chrome Azural S
CD	Culture-dependent
CFU	Colony forming unit
CI	Culture-independent
DF	Degrees of freedom
FAO	Food and Agriculture Organization
GA	Gibberellin
HDTMA	Hexadecyltrimethylammonium
IAA	Indole-3-acetic acid
JA	Jasmonic acid
NGS	Next-generation sequencing
NPK	Nitrogen-phosphorous-potassium
OTU	Operational taxonomic units
ox	Oxidase
PC1	First principal coordinate
PC2	Second principal coordinate
PCoA	Principal coordinate analysis
PD	Phylogenetic diversity
PERMANOVA	Permutational analysis of variance
PGPR	Plant growth-promoting rhizobacteria
PVK	Pikovskaya agar
QIIME	Quantitative insights in microbial ecology
QTL	Quantitative trait loci
<i>Rht</i>	<i>Reduced height</i>
SA	Salicylic acid
SDW	Sterile distilled water
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TWYE	Tap water yeast extract
TYG	Tryptone yeast extract
wt	wild-type
YEM	Yeast extract mannitol

1 INTRODUCTION

1.1 Background and motivation

1.1.1 The making of modern wheat

Since plant domestication by humans began ca.19,000 years ago, edible plants such as cereals have been bred extensively (1, 2). Wheat domestication originated in the Near East and the crop has undergone a series of crosses and modifications leading to the hexaploid bread species, *Triticum aestivum* L (3). During the Green Revolution, Reduced height (*Rht*) dwarfing genes were introduced in modern wheat cultivars (4), resulting in high-yielding wheat plants, which, when combined with agrochemical management and optimal conditions, increased yields, without productivity losses caused by lodging (5). Overtime the aboveground phenotypical appearance of wheat changed dramatically and has been well documented (Figure 1-1).

Wheat is a key crop for global food security, providing 20% of dietary requirements for calories and protein (6). However, current high-yielding dwarf crop varieties rely on unsustainable levels of inorganic nitrogen and phosphorous fertilizers, pesticides and other chemical inputs which are environmentally harmful and increasingly costly (7, 8). Wheat is the third most cultivated cereal in the world; the FAO predicts that by 2050 the global population will reach 9.73 billion meaning food production must be increased by 50% assuming current dietary patterns (9, 10). By 2027, demand for wheat will increase to 833 million tons, which is 10% above the annual current production (11).



Figure 1-1: Phenotypical evolution of wild, domesticated, and modern wheat ears.

1.1.2 The soil microbiome

Soil is considered one of the most diverse habitats on Earth containing billions of bacterial and millions of fungal species (comprising thousands of taxa), as well as larger organisms such as nematodes, ants, or moles (12). Plants and bacteria make up ~80% and ~10% of global biomass, respectively (13). Belowground biomass accounts for ~40% of total global biomass, composed mostly of plant roots and microbes residing in soil and deep subsurfaces (13). This suggests that the relationship between the soil microbiome with plant roots is a significant factor in driving plant biomass productivity which shows the promise that manipulating the soil microbiome holds in driving agricultural productivity (14-16).

Soil microorganisms carry out important processes, including cycling of carbon and other nutrients and support of plant growth, health, and resource use efficiency, especially the subset that coexist and are selected to form the root microbiome (16-19). A gradient of intimacy between plant roots and microbes extends away from the root: plant influence over the microbial community increases nearer the root surface. Root surface microbes are said to inhabit the rhizoplane, and those in soil closely associated with the root, the rhizosphere (20, 21).

The soil environment surrounding the plant root is a zone of maximum microbial activity resulting in a confined nutrient pool in which essential macro- and micronutrients are extracted. The microbial population present in the rhizosphere is relatively different from that of its surroundings due to the presence of root exudates that function as a source of nutrients for microbial growth (22). Weller and Thomashow (23) prove that the narrow rhizosphere zone is rich in nutrients for microbes compared to the bulk soil; this is shown by the quantity of bacteria that are present surrounding the roots of the plants, generally 10 to 100 times higher than in the bulk soil. The range of microbes colonizing the rhizosphere includes bacteria, fungi, actinomycetes, archaea, phages, protozoa, and algae. However, bacteria are the most abundant microbes present in the rhizosphere and colonization of the root occurs despite a sophisticated plant immune system,

suggesting a finely tuned discrimination of mutualists and commensals from pathogens. (24).

1.1.3 Plant growth-promoting rhizobacteria

Root associated microorganisms can benefit crop plants in several ways including improved plant nutritional status and protection against biotic and abiotic stresses (Figure 1-2) (25-28), so are termed plant growth-promoting rhizobacteria (PGPR) (29). Inoculating plants with PGPR can stimulate crop growth, forming the basis for the biofertilizer industry providing green alternatives to synthetic fertilizers and agrochemicals (30). Understanding the effect of agricultural practices, such as fertilization regime, on PGPR populations is essential to microbiome optimization for the sustainable intensification of agriculture (31, 32).

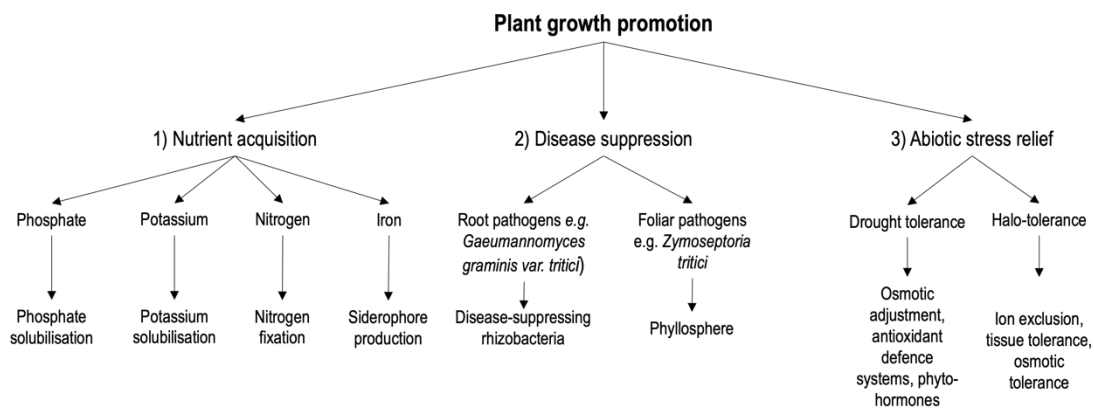


Figure 1-2: Factors contributing to growth promotion in plants

1.1.4 Plant-microbe coevolution

Approximately 3.22 billion years ago, the first life forms colonized landmass on Earth as unicellular microorganisms, starting the evolution of bacteria as we know today. Land colonization by plants, some 450 million years ago, provided the basis for terrestrial animal life on Earth but was only made possible with the help of beneficial soil mutualistic microbes (33). All plants establish mutualistic relationships with microorganisms which are subsequently transmitted between host generations, the association of which is key to ensure holobiont fitness within its environment (12). So, if microbial symbionts play such an important role in the

lives of their plant hosts, why should they not also play a role in the evolution of these higher organisms? The hologenome theory of evolution suggests that the holobiont (the host and its symbiotic microbiota) with its hologenome, should be considered a unit of selection in evolution (34-36). The theory focuses on the holobiont as a single dynamic entity in which a vast amount of the genetic information and variability is contributed by the microorganisms, but wheat breeding programmes have largely focused on improvements of above ground parts of plants, with little attention to below ground parts or plant-microbe interactions (37, 38). Additionally, variation in the hologenome can be brought about by changes in either the host or the microbiota genomes (35). So given the vast expansion of the wheat genome from diploid to allopolyploid and the impact of long-term chemical fertilization on the soil microbiota, it is conceivable that the ability of crop plants to select beneficial rhizobacteria has been undermined by plant domestication (39, 40). It has also become clear that future breeding programs should consider plant-microbe interactions as plants are complex holobiont organisms influenced both positively and negatively by their microbial communities.

1.1.5 Has chemical fertilization decoupled plant-microbe coevolution?

Chemical-only fertilization is ubiquitous in contemporary conventional agriculture even though sustainability of this agricultural practice is increasingly being questioned because of the current observed soil degradation (41-46). Long-term chemical-only fertilization results in deep selection pressure on soil microbial communities (40). The soil microbiota has clearly changed as a consequence of the fertilization regime. Moreover, fertilizer alters microbial community structure in the root environment (47-54). The diagnoses of the functional consequences of these evolutionary changes in relation to agricultural practices are key to imagining agriculture in the time ahead and especially regarding future efforts for the conservation, restoration, and management of the soil microbiota reservoir which is key to the fertility of the ecosystem.

1.1.6 Combined use of culture -independent and -dependent methods to study the microbiome

A common culture-independent method for evaluation of bacterial diversity and abundance in biological samples uses sequencing of environmental DNA. Next-generation sequencing (NGS), which was first coined in 2005 (55, 56), provided a higher-throughput alternative to Sanger sequencing, which enabled a massive reduction in the per-base cost of sequencing. This rendered the technology more accessible to the scientific research community, leading to a widespread use of DNA sequencing for multiple applications, including characterisation of microbial consortia and microbiomes, clinical diagnostics procedures, and metagenomic analysis of environmental samples (56).

Culture-dependent analysis can be highly dependent on the medium used for isolation and the incubation conditions. A paradigm in microbial ecology is that classical dilution plating culture-based techniques only recover 1-10% of the true bacterial diversity within an environment and that molecular surveys of bacterial communities yield dramatically different results than traditional culture approaches. Yet, it's been shown that despite a large difference between isolated bacterial species and overall number of operational taxonomic units (OTUs) obtained from pyrosequencing (31 vs. 634 respectively), proportionally many of the dominant taxa identified by the pyrosequencing approach were represented by isolates (57). Therefore, combined use of culture-dependent and culture-independent approaches such as NGS technologies may provide a better characterization of microbiome composition (58, 59) than that obtained when using each approach independently. An essential, parallel step would be to determine the proportion of the microbiome that can be easily isolated and cultured to be subsequently tested and potentially exploited as biocontrol/biofertilization agents. The advantage of culture-based approaches is that isolates can be functionally screened, and the results compared with culture independent approaches. Also, cultures can be mutagenized to prove function which is not possible with culture independent approaches.

1.1.7 Let the core microbiota be functional

Taxa collectively constitute an evolutionary conserved core root microbiome. The core microbiota can be defined as a subset of the microbiota associated with a given host irrespective of the macroenvironment (e.g. soil type) and that encompasses microbial hosts carrying genes with essential functions for holobiont fitness.

To date, microbial community studies have focused on taxonomic composition, but the functional potential of the microbiome may be more important to ensure key functions for holobiont fitness (60, 61). With advances in NGS technologies, the wheat microbiome has mostly been defined based on culture-independent methods, however, little has been done to link taxonomic structure of the wheat microbiome to its functional ability.

1.2 Aims and objectives

The aim of this PhD is to investigate whether beneficial plant-microbe interactions in wheat have been hindered by domestication and intensive mineral fertilizer application, by assessing both genotypic and treatment effects through culture-independent and -dependent methods. In consequence, there are three linked objectives in this PhD project.

Objective 1: Assess the root-soil microbiome with and without chemical fertilizer addition to test whether the addition of fertilizer reduces the abundance of PGPR because the plant does not exert a selective pressure on nutrient acquiring rhizobacteria when there are ample nutrients available..

Hypothesis 1: The abundance of rhizobacteria with plant growth-promoting traits is reduced for fertilized relative to unfertilized wheat.

Objective 2: Assess root-soil microbiome selection associated with ancestral wheat species through to modern cultivated varieties grown in agricultural soil with contrasting fertilizer application.

Hypothesis 2: The ability of crop plants to recruit beneficial rhizosphere microbes was undermined by the introduction of chemical fertilization and plant

domestication and breeding, and ancestral plants can select and sustain more beneficial microbial interactions than cultivated plants.

Objective 3: Assess the effect that mutations in the *Rht* genes, amongst other hormone signalling mutants in abscisic acid, auxin, brassinosteroid, ethylene, GA, and strigolactone, have had on rhizobacterial taxonomic composition and diversity and the ability of wheat to select for PGPR.

Hypothesis 3: Genetic changes which occurred because of plant breeding during the Green Revolution have reduced selection for beneficial root microbes in wheat.

1.3 Novelty and linkage of project outputs

To reach the objectives established for this research project, several contributions have been made to the scientific body of knowledge. These have been reported in this thesis in the style of 3 research articles; the linkage among them is presented in Figure 1-3.

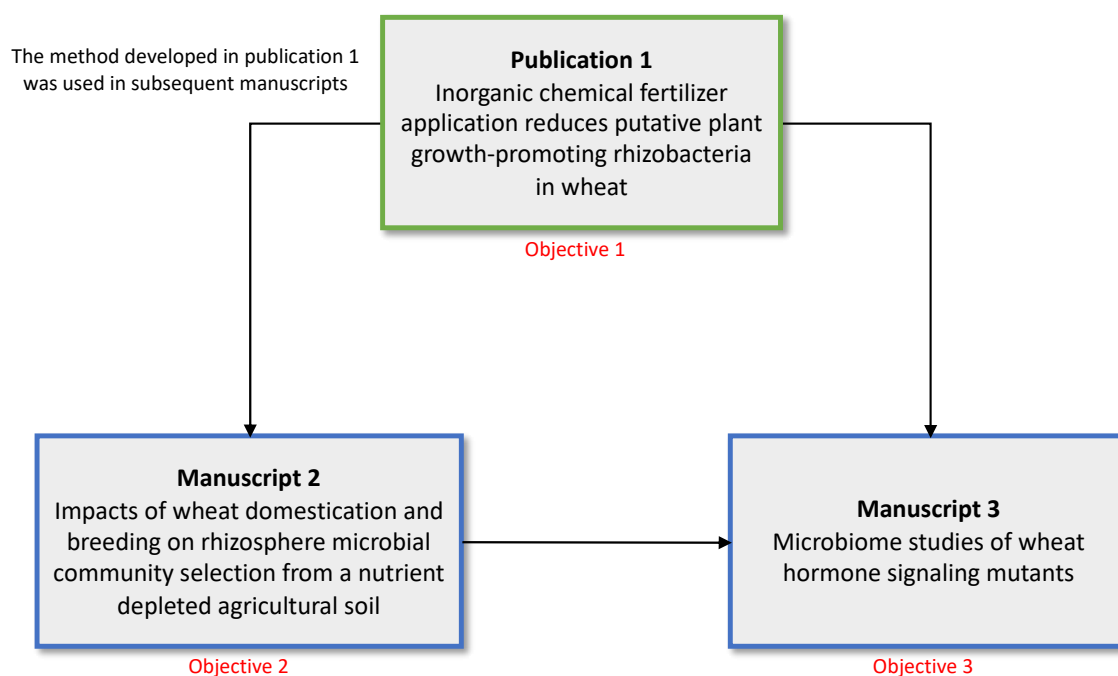


Figure 1-3: Interconnections of the project outputs and manuscripts presented in this thesis

1.4 Outline of PhD Thesis

The structure of this doctoral thesis with a brief description of the content of each chapter is detailed below:

- **Chapter 1** the background and motivation of the research project are detailed in this chapter, as well as the aims and objectives and an overview of the novelty and how the publications are linked with the aims and objectives.
- **Chapter 2** presents the methodology and results of culture -independent and -dependent analysis of rhizobacterial communities from Cadenza wheat grown under contrasting fertilization conditions. Additionally, it presents a method to identify culturable PGPR in culture-independent 16S rRNA gene datasets.
- **Chapter 3** describes the methodology and results of culture -independent and -dependent analysis of rhizobacteria from seventeen varieties of wheat representative of genetic changes that occurred during domestication, along with the evolutionary genome donor species *Aegilops*, grown under contrasting fertilization conditions. Chapter 3 is a published journal paper in *Frontiers in Microbiology*.
- **Chapter 4** details the methodology and results of culture-independent analysis of rhizobacteria from a variety of mutant hormone signalling wheat varieties, including severe mutants in the Reduced height (*Rht*) genes, grown under contrasting fertilization conditions. Chapter 4 is a draft journal paper to be submitted to the ISME journal.
- **Chapter 5** presents a general discussion of all the work done through the course of this research linking all the chapters together. Chapter 5 is a draft journal paper to be submitted to Scientific reports.
- **Chapter 6** deals with the concluding remarks, contributions to existing knowledge and recommendations for future research based on the outcomes detailed in this thesis.

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2 INORGANIC FERTILIZER APPLICATION TO WHEAT REDUCES THE ABUNDANCE OF PUTATIVE PLANT GROWTH-PROMOTING RHIZOBACTERIA

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Statement of contributions of joint authorship

TR, TM, IC, and VK designed the experiments. TR, VK, and MA performed the experiments and collected the data. TR analyzed the data with input from AT-B, VK, and MA for bioinformatic analyses. TR wrote the manuscript. All co-authors edited and commented on the manuscript.

Abstract

The profound negative effect of inorganic chemical fertilizer application on rhizobacterial diversity has been well documented using 16S rRNA gene amplicon sequencing and predictive metagenomics. We aimed to measure the function and relative abundance of readily culturable putative plant growth-promoting rhizobacterial (PGPR) isolates from wheat root soil samples under contrasting inorganic fertilization regimes. We hypothesized that putative PGPR abundance will be reduced in fertilized relative to unfertilized samples. *Triticum aestivum* cv. Cadenza seeds were sown in a nutrient depleted agricultural soil in pots treated with and without Osmocote® fertilizer containing nitrogen-phosphorous-potassium (NPK). Rhizosphere and rhizoplane samples were

collected at flowering stage (10 weeks) and analysed by culture-independent (CI) amplicon sequence variant (ASV) analysis of rhizobacterial DNA as well as culture-dependent (CD) techniques. Rhizosphere and rhizoplane derived microbiota culture collections were tested for plant growth-promoting traits using functional bioassays. In general, fertilizer addition decreased the proportion of nutrient-solubilizing bacteria (nitrate, phosphate, potassium, iron, and zinc) isolated from rhizocompartments in wheat whereas salt tolerant bacteria were not affected. A “PGPR” database was created from isolate 16S rRNA gene sequences against which total amplified 16S rRNA soil DNA was searched, identifying 1.52% of total community ASVs as culturable PGPR isolates. Bioassays identified a higher proportion of PGPR in non-fertilized samples [rhizosphere (49%) and rhizoplane (91%)] compared to fertilized samples [rhizosphere (21%) and rhizoplane (19%)] which constituted approximately 1.95 and 1.25% in non-fertilized and fertilized total community DNA, respectively. The analyses of 16S rRNA genes and deduced functional profiles provide an in-depth understanding of the responses of bacterial communities to fertilizer; our study suggests that rhizobacteria that potentially benefit plants by mobilizing insoluble nutrients in soil are reduced by chemical fertilizer addition. This knowledge will benefit the development of more targeted biofertilization strategies.

Keywords: rhizosphere, rhizoplane, fertilizer, microbiome, bacteria, plant growth-promoting rhizobacteria

2.1 Introduction

Since anthropogenic plant domestication began ca. 19,000 years ago, edible plants e.g. cereals have been extensively bred (1, 2). However, current high-yielding dwarf crop varieties rely on unsustainable levels of inorganic nitrogen and phosphorous fertilizers, pesticides and other chemical inputs which are environmentally harmful (3, 4). Wheat is the third most cultivated cereal in the world; the FAO predicts that by 2050 the global population will reach 9.73 billion meaning food production must be accordingly increased by 50 % (5). By 2027, demand for wheat will increase to 833 million tons, which is 10 % above the annual current production (6).

Soil microbial communities influence plant growth, health, and resource use efficiency, especially the subset that coexist and are selected to form the root microbiome (7-10). A gradient of intimacy between plant roots and microbes extends away from the root: plant influence over the microbial community increases nearer the root surface. Root surface microbes are said to inhabit the rhizoplane, and those in soil closely associated with the root, the rhizosphere (11, 12). Rhizosphere and rhizoplane microorganisms can benefit crop plants in several ways including improved plant nutritional status and protection against biotic and abiotic stresses (13-16), so are termed plant growth-promoting rhizobacteria (PGPR) (17). Inoculating plants with PGPR can stimulate crop growth, forming the basis for the biofertilizer industry providing green alternatives to synthetic fertilizers and agrochemicals (18). Understanding the effect of agricultural practices, such as fertilization regime, on PGPR populations is essential to optimize microbiome function in the sustainable intensification of agriculture (19).

To date, microbial community studies have focused on taxonomic composition, but the functional potential of the microbiome may be more important to ensure key functions for holobiont fitness (20, 21). With advances in next-generation sequencing technologies, the wheat microbiome has mostly been defined based on Cl methods; host genotype (22), fertilization regime (23, 24), land management and seed load (25), irrigation (26), growth stage (24) and dwarfing

(27) all affect the rhizosphere community structure. However, little has been done to link taxonomic structure of the wheat microbiome to its functional ability.

Using CI and CD methods, we studied the effect of chemical fertilizer on putative PGPR abundance in the commercial wheat variety, Cadenza, from a low input agricultural soil depleted in most nutrients, in which beneficial microorganisms are important to sustain crop production. We hypothesized that the abundance of rhizobacteria with plant growth-promoting traits would be reduced for fertilized relative to unfertilized wheat due to differences in plant nutrient status. Addition of NPK would mean that plants no longer need to interact with beneficial rhizobacteria to provide nutrients to sustain growth. Our aim was to characterize culturable bacteria with plant growth-promoting traits; determine their abundance within culturable communities; and characterize CI and CD 16S rRNA gene DNA to assess the impact of the widely used NPK fertilizer on putative PGPR populations. This was achieved by creating isolate libraries from each soil sample and subjecting them to a range of bioassays which test key traits in nutrient acquisition to establish the abundance of isolates with beneficial traits. 16S rRNA gene sequences from isolates were used to create a 'PGPR' database against which total CI amplified 16S rRNA soil DNA was searched in order to determine the relative abundance of culturable PGPR within total community DNA.

2.2 Materials and methods

2.2.1 Soil collection, experimental setup, and harvesting

We evaluated the rhizosphere and rhizoplane soil from wheat grown with and without NPK fertilizer. Soil was collected from Stackyard bare-fallow soil mine (LATLONG 52.000293N, -0.614308E), a well-draining sandy loam soil from the Rothamsted Research experimental farm at Woburn, Bedfordshire (UK). The soil is a Cottenham series (28) classified as a Cambic Arenosol (FAO), chosen to reduce the legacy effect of prior cropping systems. Soil was sieved (2 mm mesh), mixed thoroughly, and stored at 4 °C in polythene bags prior to use. *Triticum aestivum* cv. Cadenza seeds were surface sterilized (70% ethanol, 10 min; 1.5 % active chlorine, 1 h; 5 x rinse, sterile distilled water (SDW); overnight imbibition, sterile water, 4 °C) before germination on filter paper. Seedlings were planted (1x seedling/pot; 9x9x10 cm pots, ~500 g soil) with and without NPK granules [15% N, 9% P₂O₅, 11% K₂O, 2% MgO with micro-nutrients (B, Cu, Fe, Mn, Mo, Zn); Osmocote, UK] (~2.5 g per pot). Four replicate pots were prepared for each treatment. Plants were grown in a glasshouse (20 °C, 16 h/day light regime) and watered daily with tap water.

Pots were harvested at the start of flowering (Zadoks growth stage 61; ten weeks post germination) (29), resulting in 8 rhizosphere samples and 8 root samples. Height (from soil surface to head of longest stem) was measured, then soil was gently tipped from the pot onto a fresh polythene bag. Loose soil was discarded and non-rhizospheric soil carefully removed. Roots were vigorously shaken in a bag to release tightly attached soil (i.e. rhizosphere) and mixed to homogenize. The root system was excised, cut vertically in half and placed in sterile 10 ml vials for subsequent rhizoplane work. One half was frozen (-20 °C) for soil DNA extraction and the other stored (4 °C) for culture work. Around 5 g of rhizosphere soil and 1 g of root was collected per plant. The remainder of the plant was dried (80 °C, 24 h) and dry foliar plant biomass measured.

2.2.2 Isolation of bacteria

To obtain a library of rhizospheric bacteria, 1 g of each rhizosphere soil sample was diluted 10-fold (SDW) and shaken vigorously for 10 min using a shaker. To increase the diversity and number of culturable isolates returned in this study, rhizosphere samples were plated onto both 10TSA and an additional 7 agar types (Supplementary Table A-1) (20). Soil suspensions were serially diluted and 100 µl of final dilution spread on agar, (6 replicates per agar type). Different dilutions were plated depending on agar type (Supplementary Table A-2) and incubated (25 °C) for 4 days. Individual colonies were picked and inoculated in 500 µl tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) (1/10th concentration) in deep well 96-well plates (Supplementary Figure A-1) and incubated (25 °C, 2 days). For rhizoplane-colonizing bacteria, root samples were weighed and 900 µl SDW was added for every 0.1 g root. Samples were shaken vigorously for 10 min using a shaker, serially diluted, plated onto 10TSA and incubated (25 °C, 4-6 days). Individual colonies were picked and inoculated in 500 µl TSB (1/10th conc.) (96-well plates, 25 °C, 2 days) prior to functional analysis.

In addition, once colonies had been hand-picked from agar plates the remaining microbial biomass was resuspended in 5 ml SDW using a sterile spreader and transferred to a 50 ml centrifuge tube. Suspensions from technical replicates of the same agar (Supplementary Figure A-2) were combined and each sample vortexed (10 min, high speed) to ensure homogeneity. Sub-samples of each were stored (2 ml Eppendorf tube, -20 °C) for genomic DNA extraction.

2.2.3 Bioassays for plant growth-promoting traits

2.2.3.1 Bioassay inoculation

Plant growth-promoting functions were tested using previously established bioassays. A sterile 48-prong inoculating manifold was used to spot individual inoculated isolates from the 96-well plate liquid cultures onto agar (2x technical replicates per 96-well plate). Assays were incubated (25 °C, 5-7 days); positive isolates were counted per sample for each functional assay.

2.2.3.2 Hydrolyzation of casein

Casein agar is used to detect peptide bond hydrolyzing microorganisms (30). Isolate cultures were spot inoculated onto agar supplemented with skimmed milk powder as the casein source. Casein agar (Hardy Diagnostics, Santa Maria, CA, USA): 5% skimmed milk powder, 0.5% pancreatic digest of casein, 0.25% yeast extract, 0.1% D-glucose, 1.25% agar. Hydrolyzing isolates produced a clear halo in the surrounding medium.

2.2.3.3 Solubilization of insoluble phosphate, potassium, and zinc

Isolate cultures were spot inoculated onto agar plates containing: tricalcium phosphate as an insoluble phosphate source (31); potash feldspar (Bath potters, UK) as an insoluble potassium source (32); and zinc oxide as an insoluble zinc source (HiMedia M2023) (33). Pikovskayas agar (31): 0.05% yeast extract, 1% D-glucose, 0.5% $\text{Ca}_3(\text{PO}_4)_2$, 0.05% $(\text{NH}_4)_2\text{SO}_4$, 0.02% KCl, 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00001% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.00001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5% agar. Aleksandrov agar (34): 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% CaCO_3 , 0.2% potash feldspar, 0.5% D-glucose, 0.0005% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.2% $\text{Ca}_3(\text{PO}_4)_2$, 2% agar, pH 7.0-7.2. Zinc solubilizing agar (33): 1% D-glucose, 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.02% KCl, 0.01% K_2HPO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% ZnO, 1.5% agar. The plates were incubated at 25 °C for 5-7 days and observed for the formation of halo zone around the colonies.

2.2.3.4 Production of siderophores

Iron solubilization was tested using agar containing chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) which form a blue color complex with ferric iron; a color change to orange is observed when a strong iron chelator such as a siderophore removes iron from the dye complex (35, 36). The medium was prepared as outlined in (36). Iron solubilization was denoted as either 'positive' or 'negative' based on the presence, or absence of an orange halo surrounding the colony.

2.2.3.5 Salt tolerance

In vitro screening of the isolates tolerance to salt stress was tested by culturing strains on 10TSA supplemented with 5% (w/v) sodium chloride (NaCl). 10TSA with no additional NaCl was included as a control.

2.2.3.6 Statistical analyses for culture-dependent work

Statistical differences in the frequency of positive vs. negative isolates (n=376) between non-fertilized and fertilized wheat were performed in R 3.6.1 (<http://www.r-project.org>) using the 'chisq.test' function. Box plots were created in GraphPad Prism version 8 for Mac (San Diego, CA: GraphPad Software, Inc). This software was also used to calculate two-way analysis of variance (ANOVA) and pair-wise t-tests with Šidák correction for bacterial abundance and absolute abundance of nutrient-solubilizing isolates. Data were first normalized by logarithmic transformation. Normality was confirmed by quantile-quantile normality plots and Shapiro-Wilk test; homogeneity of variances was confirmed by residuals versus fits plots and Spearman's test for heteroscedasticity.

2.2.4 DNA analysis

2.2.4.1 Mixed culture DNA extraction and quantitation

Each mixed culture sample was subjected to Sigma GenElute Bacterial Genomic DNA extraction kit using the lysozyme utilizing Gram-positive bacterial preparation method to ensure lysis of both Gram-positive and Gram-negative cells, according to the manufacturer's instructions. DNA purity and concentrations were established by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, United States), and a Qubit 2.0 Fluorimeter using the dsDNA HS assay kit (Thermo Scientific), respectively.

2.2.4.2 Soil DNA Extraction and Quantitation

For each sample, total soil DNA was extracted from approximately 0.25 g homogenized soil (Qiagen DNeasy PowerSoil DNA isolation kit (Venlo, Netherlands)), according to the manufacturer's instructions using the MP Biomedicals FastPrep-24 machine twice (30 s, 5.5 m s⁻¹). DNA concentrations and purity were determined as above.

2.2.4.3 Amplicon library preparation and sequencing

To assess the impact of fertilizer on microbial community composition, bacterial 16S rRNA gene amplicons were subjected to Illumina® sequencing using the MiSeq platform. Amplicons (~460 bp) spanning the V3-V4 hypervariable region of the 16S rRNA gene were produced using primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (37). Rhizosphere DNA was sent to Novogene (HK) (Wan Chai, Hong Kong) for 2 x 250 bp paired-end sequencing on a MiSeq instrument. Rhizoplane and bacterial samples were sequenced in-house (2 x 300 bp paired-end sequencing) on a MiSeq instrument; see SUPPLEMENTARY METHODS for full details. Amplicon preparation followed the protocol of Kozich et al. (38).

2.2.4.4 Processing of 16S rRNA gene amplicon sequence data

Demultiplexing of raw sequences was performed by CASAVA data analysis software (Illumina). Paired-end sequences were merged using the `vsearch merge_pairs` function (39) then filtered, de-replicated, and denoised to identify ASVs using the DADA2 1.2 (40) pipeline with Quantitative Insights into Microbial Ecology (QIIME2) (version 2018.11.0) default parameters (41). The resulting ASV table retained high quality nonchimeric reads and was used to build a phylogenetic tree using the `align-to-tree-mafft-fasttree` command in QIIME2. Taxonomy was assigned using the SILVA132 database (42, 43). All non-bacterial ASVs were removed for further analysis.

2.2.4.5 Colony PCR for identification of rhizobacterial isolates

To identify individual rhizobacterial isolates, amplicons (~1500 bp) spanning almost the full length of the 16S rRNA gene were produced using primers `fD1` (5'-AGAGTTTGATCCTGGCTCAG-3') and `rD1` (5'-AAGGAGGTGATCCAGCC-3'). From a total of 1,504 rhizobacterial isolates (94 isolates per rhizosphere sample; 94 isolates per rhizoplane sample), 541 isolates were sequenced to gain a representative population of species present in non-fertilized and fertilized wheat. Amplicons were produced by colony PCR and sent to Eurofins Genomics Germany for purification and Sanger sequencing; see SUPPLEMENTARY METHODS for full details. Amplicon sequences were processed in Geneious

Prime version 2020.1.1; taxonomically assigned using the SILVA Alignment, Classification and Tree (ACT) service (www.arb-silva.de/act); and deposited in NCBI GenBank (for individual accession numbers see Supplementary Data).

2.2.4.6 Isolate database creation

The resulting 541 16S rRNA gene sequences and corresponding taxonomy were used to create QIIME2 taxonomy and ASV files to identify whether isolates matched major ASVs within the 16S rRNA gene amplicon dataset. The classify-consensus-blast command in QIIME2 was used at 100% sequence identity to search the 16S rRNA gene amplicon sequences against the isolate database. Secondly, a 'PGPR' database was created which only included isolate sequences identified as 'putative PGPR' (as they were found to be positive for at least one functional trait) to identify the relative abundance of potentially plant beneficial isolates within CI and CD amplicon datasets. ASVs identified as 'Bacteria' were considered rhizobacterial isolates.

2.2.5 Data visualization and statistical analyses

2.2.5.1 Total 16S rRNA gene dataset

The resulting ASV table was analyzed in R 3.6.1 using Phyloseq (v1.30.0) (available at <https://joey711.github.io/phyloseq/>) (44). Amplicon sequencing data were normalized using DESeq2 to account for differences in sequencing bias (45), except for alpha diversity analysis, which was calculated by normalizing sequence number to minimum sample size (8,554) by random subsampling. The subsampling of sequences still yielded sufficient resolution of bacterial communities, as suggested by rarefaction curve analysis (Supplementary Figure A-3). Two-way type III ANOVA was performed using the R function 'aov' with fertilizer and rhizocompartment as factors, to determine the dominant factor contributing to variation in means for alpha diversity data. Normality was confirmed by quantile-quantile normality plots and the Shapiro-Wilk test; homogeneity of variances was confirmed by residuals versus fits plots and the Levene's test (R package: car (v3.0-6)). Beta diversity was determined by principle Coordinate Analysis (PCoA) which was employed on weighted UniFrac distance matrices using the 'ordinate' function in the Phyloseq package;

significantly different clusters were determined using 'adonis' with the 'betadisper' test to check for equal variance (R package: vegan (v2.5.6)) (46). Phylum level community composition was investigated by relative abundance of normalized data, after removal of bacterial ASVs only classified to Kingdom level and ASVs assigned as 'environmental samples' for visualization purposes. Unique and shared ASVs were determined in Excel. To identify ASVs preferentially associated with fertilization and vice versa, the differential relative abundances (fold changes) of ASVs between the different groups were determined. Low abundance ASVs with less than 3 counts in <20% of the samples were removed. This was performed individually for each data set with the DeSeq2 package, using the Wald significance test and the Benjamini-Hochberg *p* value correction. All graphs were rendered in Prism 8.

2.2.5.2 Isolate and PGPR 16S rRNA gene dataset

The resulting isolate ASV table and PGPR ASV table were analyzed using Phyloseq (as described above), normalized using DESeq2 and transformed to relative abundances. Heatmaps were created in Excel. ASVs identified as 'Bacteria' were considered rhizobacterial isolates or putative PGPR; the remaining ASVs were unassigned. To identify whether isolates matched ASVs preferentially associated with fertilization and vice versa, the differential relative abundances (fold changes) of ASVs between the different groups were determined, as described above. Statistical differences between mean putative PGPR abundance between treatments were assessed using two-way ANOVA and pair-wise t-tests with Šidák correction. Normality was confirmed by quantile-quantile normality plots and Shapiro-Wilk test; homogeneity of variances was confirmed by residuals versus fits plots and Spearman's test for heteroscedasticity. Box plots were created in Prism 8.

2.3 Results

2.3.1 Plant phenotypical data

Fertilizer addition increased aerial biomass of wheat from 1.27 ± 0.32 g to 4.11 ± 0.95 (Welch's two-sample t-test: $t=-5.645$, $df=3.67$, $p=0.006$) (Supplementary Figure A-4). There were no statistical differences in mean height of non-fertilized vs. fertilized wheat plants ($p>0.05$) (Supplementary Figure A-4).

2.3.2 Rhizobacterial community composition in culture -independent and -dependent communities

Amplicon-based analysis of the V3-V4 region of the 16S rRNA gene generated a total of 902,187 sequences from 16 CI samples (median 50,658 sequences per sample) and 279,558 sequences from 16 CD samples (median 17,235 sequences per sample). Filtering, denoising, and removal of chimeras resulted in 521,098 (CI) and 206,960 (CD) high-quality sequences, retaining 58% and 70% of initial reads, respectively. The number of sequences per sample ranged from 20,809 to 49,203 (CI) and 12,623 to 24,165 (CD). One CD sample was removed due to low sequence number (<100). ASV analysis using the SILVA132 database generated 4,879 ASVs from CI DNA and 426 ASVs from CD DNA (after removal of ASVs that were not classified as bacteria (archaeal, eukaryote, metagenome, unassigned)). The CD approach retrieved 4.9% of ASVs from the CI total community; 244 (4.9%) ASVs were found using both CI and CD methods indicating that 182 (3.6%) ASVs detected by the CD method, were absent from the CI DNA (Figure 2-1).

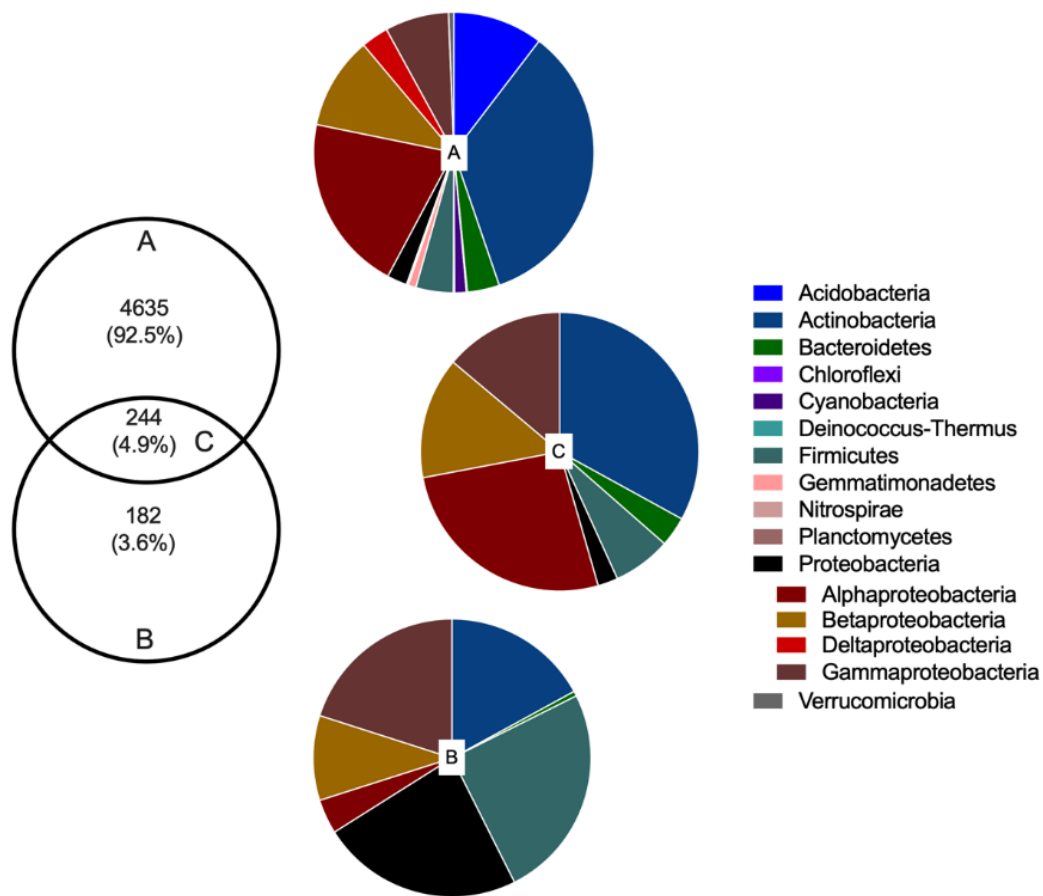


Figure 2-1: Rhizobacterial taxonomic composition in culture-independent and -dependent communities of wheat grown in soils with and without fertilizer addition.

Venn diagram showing the number and proportion of unique ASVs (at 97% similarity) in (A) culture-independent and (B) culture-dependent communities and (C) shared ASVs detected with both methods. Pie charts correspond to the percentage of bacterial phyla and classes of Proteobacteria assigned to each ASV.

2.3.3 Fertilizer treatment and niche effects on rhizobacterial community diversity

Rhizobacterial community beta-diversity, as measured by weighted UniFrac distances, was influenced by both rhizocompartment and fertilizer. PCoA of CI communities showed clustering of samples by rhizocompartment along the first principal coordinate axis (PC1) and clustering by fertilization along the second principal coordinate axis (PC2) (Figure 2-2, CI). Two-way permutational analysis of variance (PERMANOVA) indicated that both rhizocompartment and fertilizer effect were significant in CI rhizobacterial communities, as well as their interaction (Supplementary Table A-3). Rhizocompartment accounted for 49% of the variance ($p=0.001$) and fertilizer accounted for 24% of the variance ($p=0.001$). The two-way PERMANOVA of CD rhizobacterial community beta diversity indicated a significant effect of rhizocompartment (20% of variance, $p=0.002$), whereas the effect of fertilization was weaker, although still statistically significant (13% of variance, $p=0.035$), but the interaction was not significant (Supplementary Table A-4). PCoA of the CD rhizobacterial community revealed that samples also clustered by rhizocompartment along PC1 (Figure 2-2, CD), whereas there was some overlap between treatment groups along PC2, consistent with the small effect size in the PERMANOVA analysis.

Rhizobacterial CI community alpha diversity, as measured by the Shannon diversity index, was consistent across rhizocompartments but lower in samples from fertilized wheat (Figure 2-2, CI). Two-way type III ANOVA indicated that the effect of fertilizer was significant ($p=0.0024$), while the effects of rhizocompartment and interaction were not (Supplementary Table A-5). Rhizobacterial CD communities showed lower alpha diversity compared to CI communities (Figure 2-2, CD), with fertilizer and rhizocompartment causing less change in variation ($p>0.05$) (Supplementary Table A-6).

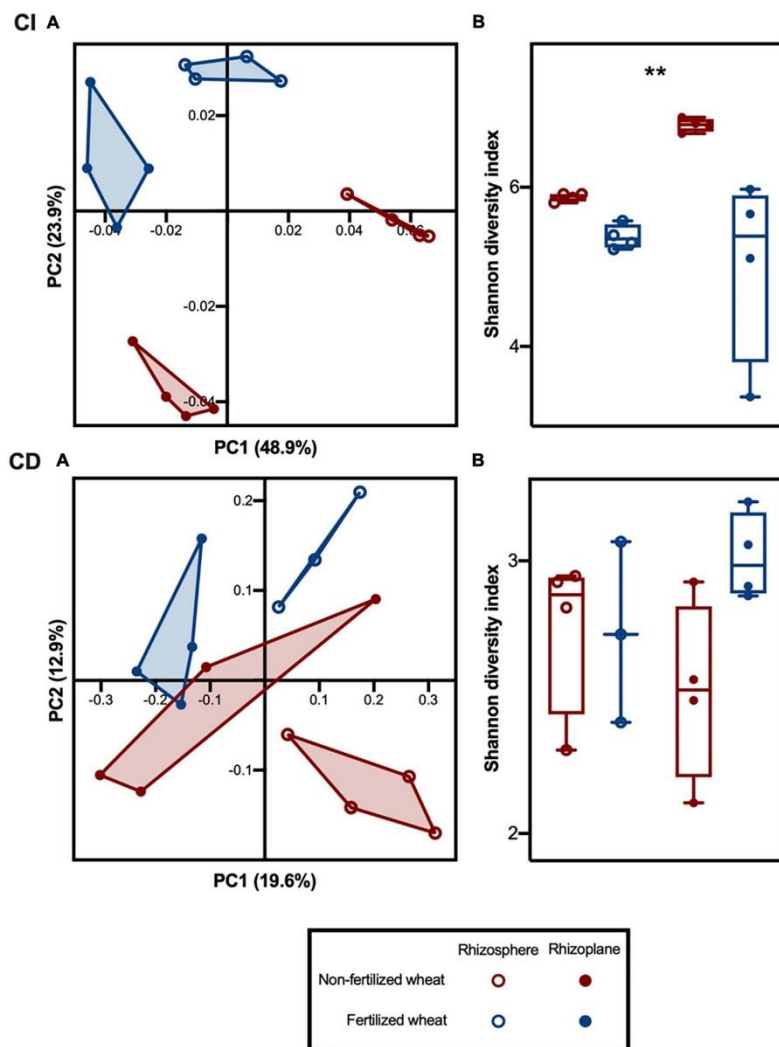


Figure 2-2: Culture-independent (CI) and culture-dependent (CD) bacterial community diversity in the rhizosphere and rhizoplane of wheat grown in soils with and without fertilizer addition.

(A) PCoA plots of bacterial composition based on weighted UniFrac distances for CI and CD communities at ASV level. The percentage shown in each axis corresponds to the proportion of variation explained. (B) Alpha diversity estimates at ASV level for rhizosphere and rhizoplane bacterial communities in non-fertilized and fertilized wheat; with median (line) and hinges at first and third quartiles (25th and 75th percentiles). Significant differences as determined by two-way type III ANOVA are shown by ‘***’ for $p < 0.01$ between treatment groups. Symbols represent rhizosphere (open circles) and rhizoplane (closed circles) samples from non-fertilized (red) and fertilized (blue) wheat.

2.3.4 Comparison of isolation media in assessing the influence of fertilizer on rhizosphere bacterial communities

In total, 894 bacterial ASVs were identified from 63 mixed culture samples (4 biological replicates from 8 agar-types for non-fertilized and fertilized wheat rhizosphere samples (1 outlier removed)). The use of 7 additional agar types, as well as 10TSA, retrieved 8.6% of CI ASVs compared to 4.2% from just 10TSA.

Effect of fertilizer was more apparent when a larger number of agar types were used to isolate rhizobacteria. Overall, mean alpha diversity was greater in samples from non-fertilized wheat (2.95 ± 0.31) compared to fertilized wheat (2.66 ± 0.31) (d.f=1, $F=21$, $p<0.0001$) however, there were no statistical differences between individual means for each agar-type (d.f=7, $F=3.6$, $p>0.05$) (A in Figure 2-3). Furthermore, fertilizer was the dominant factor contributing to variation in beta diversity (PERMANOVA, $F=40$, $r^2=0.35$, $p=0.001$), accounting for 61.7% variation along PC1 compared to 16.7% variation along PC2 as caused by agar-type (PERMANOVA, $F=2.6$, $r^2=0.16$, $p=0.004$) (B in Figure 2-3), with no significant interaction. The relative abundance of different taxonomic groups in mixed cultures shifted from non-fertilized wheat plants and fertilized wheat plants (C in Figure 2-3). Most notable, was the higher abundance of Actinobacteria in the rhizosphere of fertilized wheat plants across all media types compared to a higher abundance of Proteobacteria in the rhizosphere of non-fertilized wheat plants.

Agar type had minimal effect on bacterial diversity within treatment groups. There were no statistical differences in mean alpha diversity between different agar types for a given sample from either non-fertilized or fertilized wheat. Furthermore, PCoA showed no distinct groupings between agar type for non-fertilized wheat (A in Supplementary Figure A-5) (PERMANOVA, $F=1.1$, $r^2=0.24$, $p=0.38$) however, fertilized wheat samples from TWYE agar formed a clear grouping compared to the other agar types (B in Supplementary Figure A-5). With TWYE samples present, media type significantly affected bacterial community structure (PERMANOVA, $F=11.9$, $r^2=0.29$, $p=0.001$). However, when TWYE

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samples were removed from PCoA, agar type had no significant effect on bacterial community structure (PERMANOVA, $F=0.86$, $r^2=0.056$, $p=0.59$).

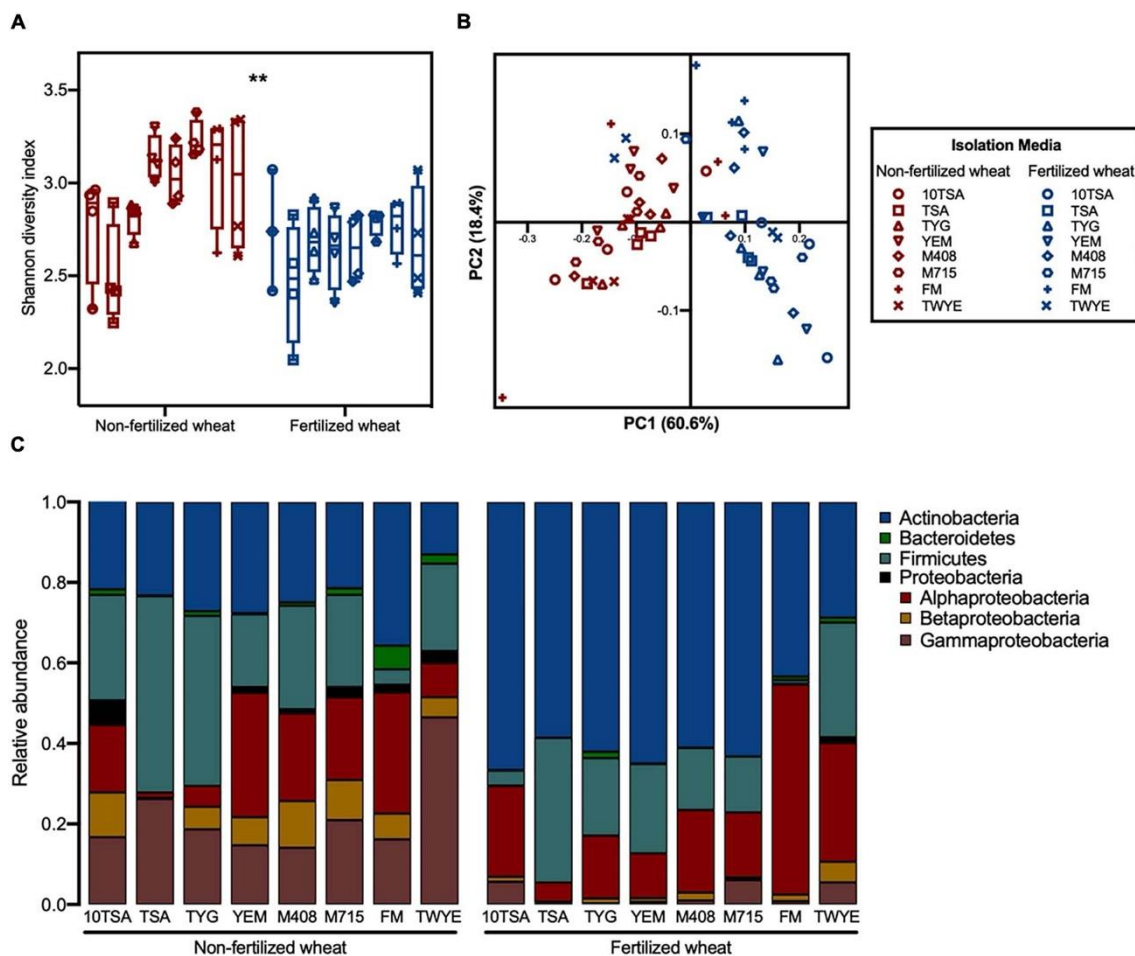


Figure 2-3: Influence of different culture-agar on bacterial community diversity and structure in the rhizosphere of wheat grown in soils with and without fertilizer.

Alpha diversity estimates at ASV level for rhizosphere bacterial communities grown on 8 agar types with median (line) and hinges at first and third quartiles (25th and 75th percentiles). Significant differences as determined by two-way type III ANOVA are shown by ‘**’ for $p < 0.01$ between treatment groups. (B) PCoA plot of bacterial community composition based on weighted UniFrac distances at ASV level, colored by fertilizer treatment. Symbols represent the isolation media that bacteria were cultured on from rhizosphere samples: TSA (10th conc.) (circles), TSA (squares), TYG (up-triangles), YEM (down-triangles), M408 (diamond), M715 (hexagon), FM (plus), TWYE (cross). (C) Average relative abundances of bacterial phyla within the bacterial 16S V3-V4 sequences. Identifications are based on the SILVA database for prokaryotes.

2.3.5 Effect of fertilizer on abundance of culturable rhizobacteria with plant growth-promoting traits

Based on previous evidence of fertilization altering the structure of wheat rhizosphere bacterial communities (23), we hypothesized that addition of fertilizer would reduce, specifically, the presence of putative plant growth-promoting rhizobacteria because the plant does not exert a selective pressure on nutrient acquiring rhizobacteria when there are ample nutrients available..

In general, rhizobacteria that tested positive for solubilization of plant macronutrients: organic N (casein), inorganic phosphate ($\text{Ca}_3(\text{PO}_4)_2$) and potassium (potash feldspar), and plant micronutrients: iron ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and zinc (ZnO), had a statistically higher relative abundance in isolate libraries cultured from non-fertilized wheat samples compared to fertilized wheat samples (A and B in Figure 2-4) (Supplementary Table A-7). However, there was no significant difference in the relative abundance of salt-tolerant rhizobacteria in non-fertilized wheat samples compared to fertilized wheat samples (B in Figure 2-4) (Supplementary Table A-7). Relative abundance of nutrient solubilizing isolates were greater in rhizosphere ($49.2 \pm 13\%$; two-tailed chi-square test: $X^2 = 116$, $p < 0.0001$, d.f = 1) and rhizoplane ($90.7 \pm 9\%$; $X^2 = 389$, $p < 0.0001$, d.f = 1) samples from non-fertilized wheat compared to fertilized wheat ($21.5 \pm 2\%$ and $19.1 \pm 14\%$ from rhizosphere and rhizoplane, respectively) (C in Figure 2-4).

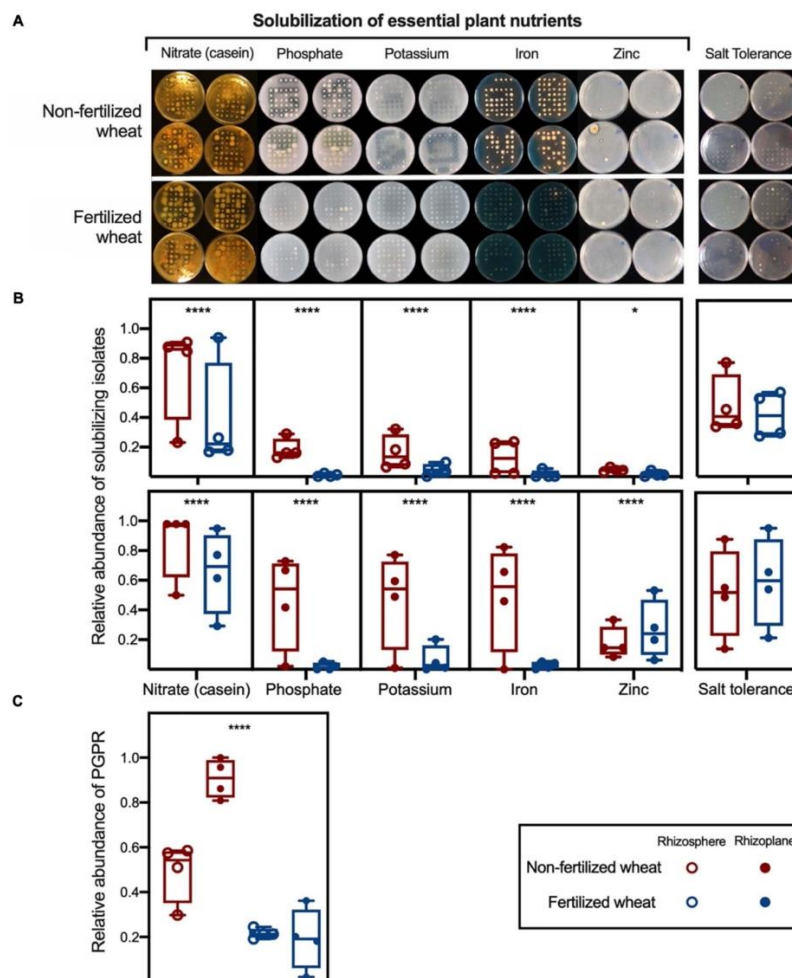


Figure 2-4: Relative abundance of culturable rhizobacteria with plant growth-promoting traits isolated from wheat grown in soils with and without fertilizer.

(A) Nitrate-, phosphate-, potassium-, iron-, and zinc-solubilization as determined by casein, PVK, AVK, CAS, and zinc carbonate agar, respectively. Casein, phosphate, potassium and zinc solubilization are indicated by clear halos surrounding the colony whereas orange halos indicate iron solubilization. Salt tolerance as determined by 10TSA supplemented with 5% NaCl. Box plots show relative abundance of (B) solubilizing isolates and salt tolerant isolates and (C) relative abundance of PGPR isolates, as determined by functional bioassays; with median (line) and hinges at first and third quartiles (25th and 75th percentiles). Each point represents an isolate library (n=94) derived from rhizosphere (open circles) and rhizoplane (closed circles) samples. Single colonies were isolated from different non-selective agar and 10TSA respectively and inoculated in TSB (1/10th conc.) in a 96-well plate; sterile TSB (1/10th conc.) in 2x wells were left uninoculated to act as a negative control. Significant differences as determined by chi-squared tests are shown by '*' and '****' for p<0.05 and p<0.0001.

Absolute abundance of bacteria isolated from the rhizoplane (mean log CFU counts.g soil⁻¹: 8.84 ± 0.34) was higher compared to the rhizosphere of wheat (mean log CFU counts.g soil⁻¹: 6.70 ± 0.05) (two-way ANOVA: $F=18.3$, d.f=1, $p<0.0001$) whereas fertilizer addition had no significant effect on rhizobacterial abundance ($p>0.05$) (A in Figure 2-5). In comparison, absolute abundance of nutrient-solubilizing bacteria isolated from rhizocompartments in wheat were statistically higher from rhizoplane samples ($F=113$, d.f=1, $p<0.0001$) and were also statistically higher in non-fertilized compared to fertilized wheat samples ($F=20.7$, d.f=1, $p=0.0007$) (B in Figure 2-5). Post-hoc multiple comparison tests with Šidák correction showed differences in means in rhizosphere (log CFU counts.g soil⁻¹: 8.18 ± 0.08 and 7.24 ± 0.67 in non-fertilized and fertilized wheat, respectively) ($p=0.0289$) and rhizoplane (mean log CFU counts.g soil⁻¹: 6.12 ± 0.09 and 5.37 ± 0.27 in non-fertilized and fertilized wheat, respectively) ($p=0.0075$) samples. However, there were no statistical differences between individual assays for absolute abundance of isolates from non-fertilized vs. fertilized wheat (Supplementary Figure A-6).

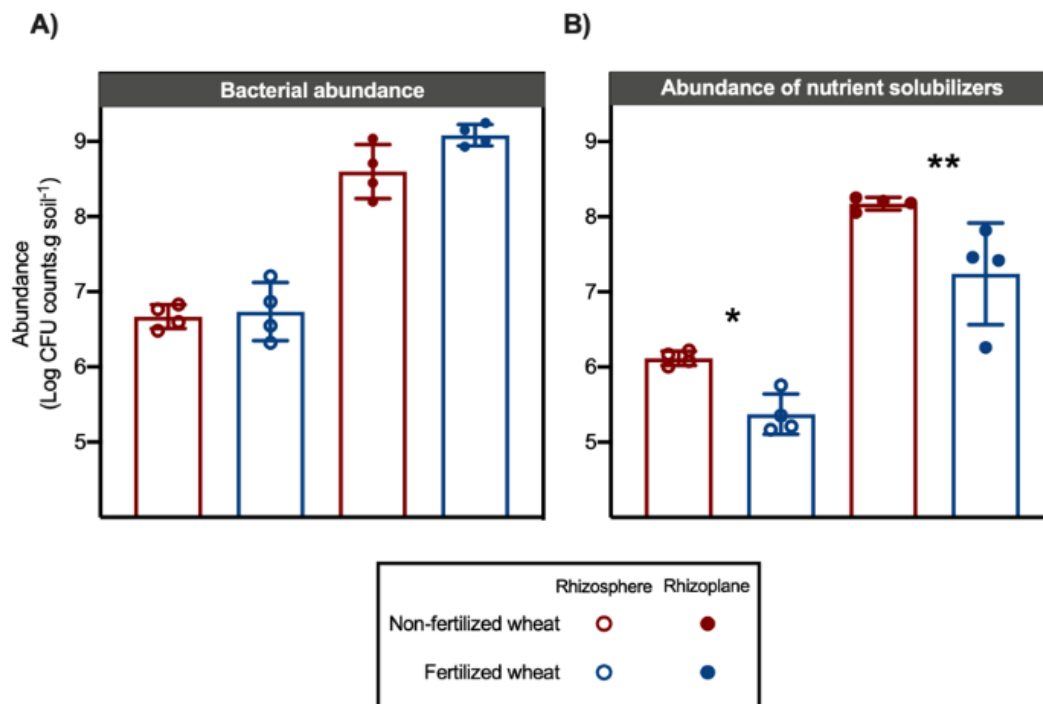


Figure 2-5: Effect of chemical fertilizer (NPK) on absolute abundance of culturable bacteria isolated from the rhizosphere and rhizoplane of wheat grown in low nutrient soil.

(A) Absolute abundance (log CFU counts.g soil⁻¹; CFU is colony-forming units) of culturable rhizobacteria isolated from soil samples on 10TSA. (B) Absolute abundance (log CFU counts.g soil⁻¹) of nutrient solubilizing rhizobacteria as determined by functional bioassays. Significant differences as determined by post-hoc multiple comparison tests with Šidák correction are shown by '*' and '**' for p<0.05 and p<0.01, respectively.

2.3.6 Identification of culturable isolates within culture-independent and -dependent amplicon datasets

An isolate database was curated from 541 isolates (275 from non-fertilized wheat; 266 from fertilized wheat) which consisted of a total of 27 genera (Figure 2-6). Rhizobacterial isolates that displayed growth-promoting abilities were identified as being mostly *Bacillus* species (48%) in the rhizosphere and *Pseudomonas* (50%) in the rhizoplane of non-fertilized wheat (Figure 2-6A). For fertilized wheat plants the majority of isolates were also identified as *Bacillus* (52%) in the rhizosphere and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (48%) in the rhizoplane (A in Figure 2-6).

In total, 96 (1.92%) isolate bacterial ASVs were also identified as being present in the total community amplicon 16S rRNA gene dataset from this work. The remaining 4,906 ASVs could not be assigned to any culturable isolates from this work; 88 (1.84%) bacterial ASVs were identified in the CI amplicon dataset and 58 (13.6%) bacterial ASVs were identified in the CD amplicon dataset (B in Figure 2-6). Comparatively, at ninety-nine percent sequence identity, 246 (4.5%) isolate ASVs were classified as bacteria from 5,456 ASVs. However, to increase accuracy of isolate identification, one hundred percent sequence identity was used. Overall, 23 genera were identified in the CI and CD amplicon datasets, when searched against the isolate database; no ASVs were classified as *Rhodococcus*, *Fictibacillus*, *Paraburkholderia* and *Agrobacterium*. The PGPR isolate database identified 76 ASVs (1.52%) as PGPR within the CI and CD communities (C in Figure 2-6).

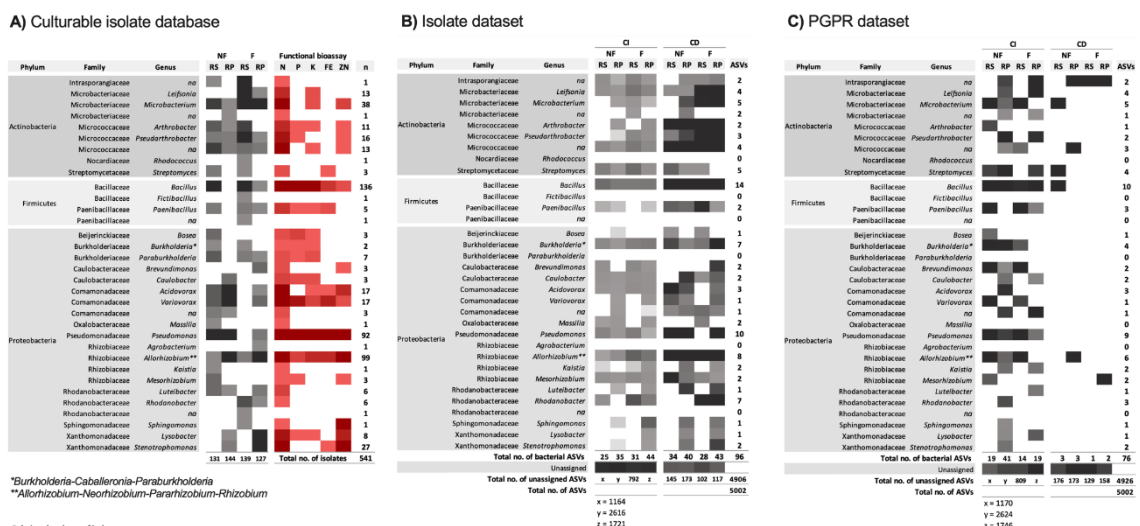


Figure 2-6: Heatmaps representing abundance and associated functional ability of bacterial isolates from rhizosphere (RS) and rhizoplane (RP) samples from non-fertilized (NF) and fertilized (F) wheat.

(A) The full region of the 16S rRNA gene was amplified and sequenced for each rhizobacterial isolate. Identifications are based on the SILVA database for prokaryotes. Gray indicates relative abundance of genera in each sample (number of isolates per sample shown below columns). Red indicates relative abundance of positively solubilizing isolates from total number of isolates sequenced (541). “n” shows number of genera identified from total number of isolates sequenced. (B) All culturable rhizobacterial isolate sequences and (C) plant growth-promoting rhizobacteria (PGPR) isolate sequences were used to create a database with which the culture-independent (CI) and culture-dependent (CD) amplicon datasets were searched against using blast at 100% sequence identity. *Burkholderia** stands for *Burkholderia-Caballeronia-Paraburkholderia*; *Allorhizobium*** stands for *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*.

Overall, relative abundance of PGPR isolates were relatively low (<5%) however, there was a statistically higher mean abundance of PGPR in non-fertilized CI samples (0.019 ± 0.006) compared to fertilized CI samples (0.012 ± 0.002) (two-way ANOVA: $F=22.7$, $df=1$, $p=0.0005$) (Figure 2-7). Compartment also contributed to difference in means and was statistically higher in the rhizosphere (0.020 ± 0.006) compared to the rhizoplane (0.013 ± 0.003) (two-way ANOVA: $F=14.7$, $df=1$, $p=0.0024$). There was no significant interaction with fertilization regime. Post-hoc multiple comparison tests with Šidák correction showed differences in means in rhizosphere (0.024 ± 0.002 and 0.016 ± 0.004 in non-fertilized and fertilized wheat, respectively) ($p=0.0009$) but not in rhizoplane (0.015 ± 0.003 and 0.011 ± 0.003 in non-fertilized and fertilized wheat, respectively) ($p>0.05$) samples.

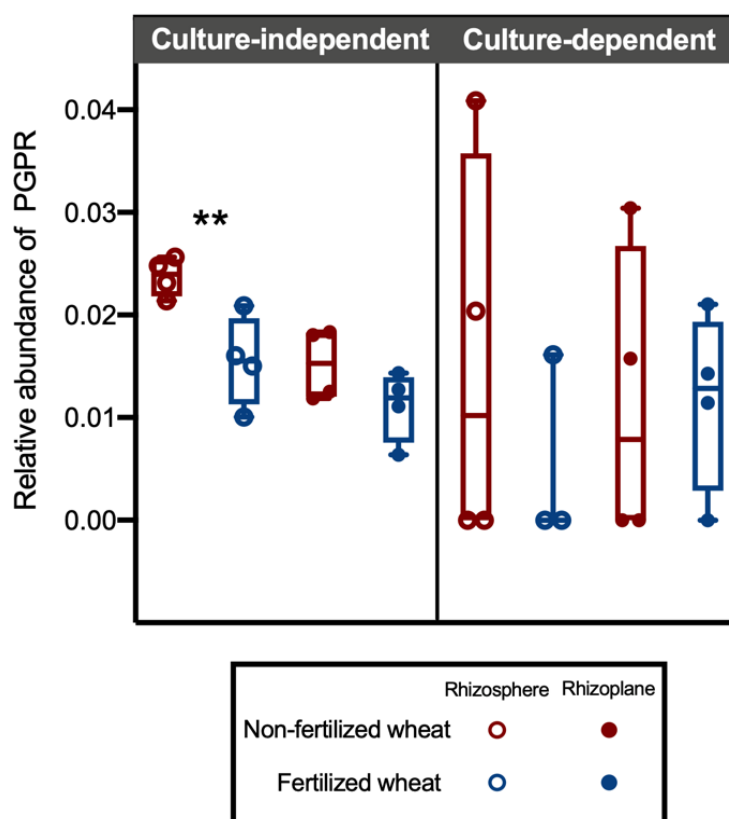


Figure 2-7: Box plots showing relative abundance of ASVs identified as putative plant growth-promoting rhizobacteria (PGPR) in culture-independent and -dependent isolate datasets.

A PGPR database was created from 16S rRNA gene sequences from culturable bacterial isolates with acquisitional traits for key plant nutrients. Each point represents the proportion of ASVs that were identified as Bacteria in CI and CD datasets, with median (line) and hinges at first and third quartiles (25th and 75th percentiles). Significant differences as determined by post hoc multiple comparison tests with Šidák correction are shown by “***” for $p < 0.01$.

2.3.7 Major ASVs within wheat rhizobacterial communities

Differential abundance analysis identified ASVs that were highly associated with non-fertilized wheat compared to fertilized wheat in rhizosphere and the rhizoplane samples. Overall: 54 ASVs (3.5% of all ASVs identified in CI rhizosphere samples); 115 ASVs (3.5% of all ASVs identified in CI rhizoplane samples); 17 ASVs (6.4% of all ASVs identified in CD rhizosphere samples); and 16 ASVs (6.0% of all ASVs identified in CD rhizoplane samples) were differentially abundant between non-fertilized and fertilized samples (Supplementary Table A-8 and Supplementary Table A-9). Six genera (15 ASVs) were enriched in non-fertilized samples ($p < 0.01$) and they included members of the Proteobacteria (46.97%), Firmicutes (9.09%) and Actinobacteria (7.58%) (Figure 2-8). Conversely, nineteen genera (36 ASVs) were enriched in fertilized samples and they included members of the Proteobacteria (49.26%), Actinobacteria (31.62%), Bacteroidetes (10.29%) and Firmicutes (4.41%) (Figure 2-8). In particular, 9 ASVs identified as *Pseudomonas* were enriched in non-fertilized samples compared to fertilized samples where 4 ASVs identified as *Catenulispora*, *Leifsonia* and *Rhodanobacter* were enriched (Figure 2-8).

Key ASVs that were differentially abundant in both the amplicon dataset and isolate dataset were identified ($p < 0.01$). They included: ASV_3448_*Pseudomonas*, ASV_1275_*Pseudomonas*, and ASV_4785_*Pseudomonas* in non-fertilized wheat samples and ASV_1051_*Rhizobium*, ASV_4593_*Rhizobium*, ASV_4049_*Pedobacter*, ASV_1557_*Leifsonia*, ASV_1818_*Rhodanobacter*, ASV_1979_*Rhodanobacter*, ASV_4187_*Paraburkholderia* and ASV_2089_*Lysobacter* in fertilized wheat samples (Figure 2-8). Four ASVs (ASV_4593, ASV_4049, ASV_1979, ASV_4187) were differentially more abundant in fertilized wheat samples in CI and CD amplicon datasets and CI and CD isolate datasets.

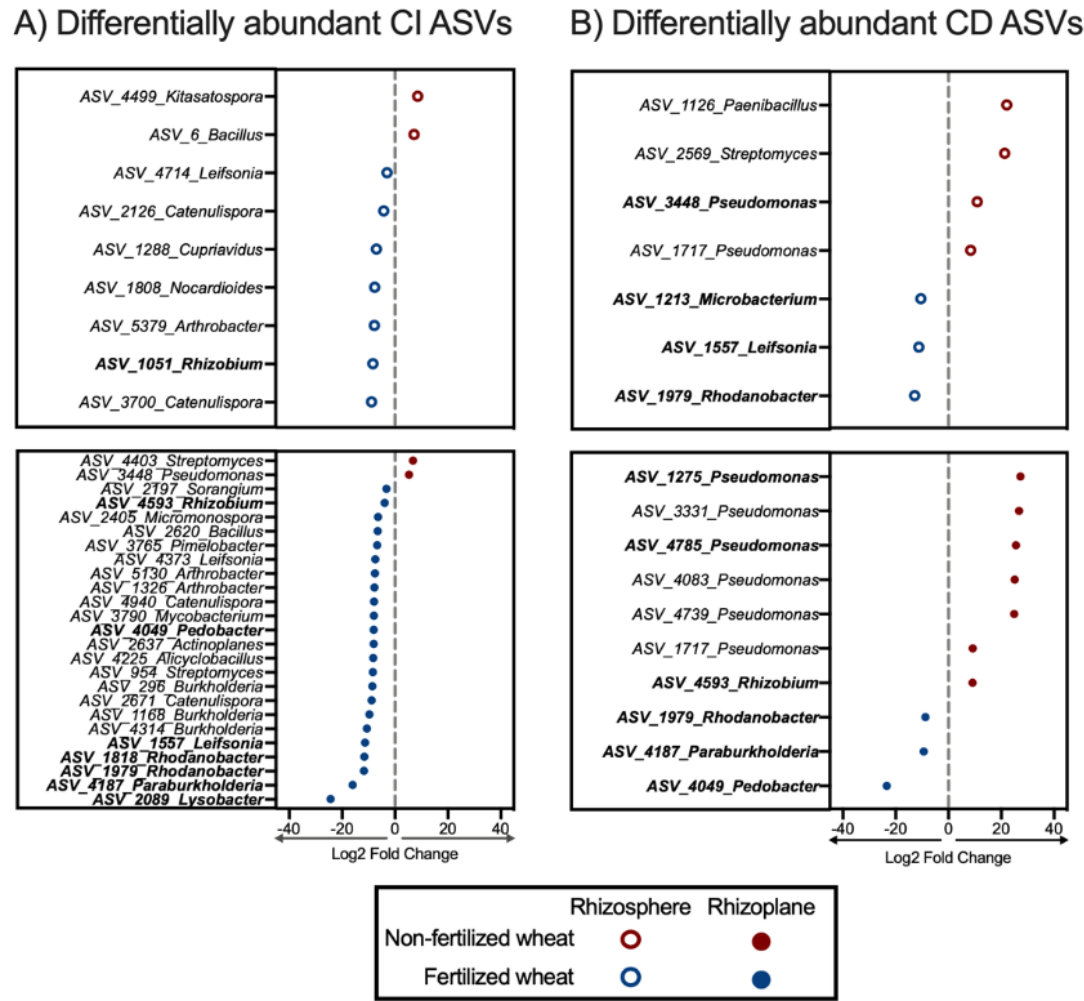


Figure 2-8: ASVs that significantly differ in abundance in non-fertilized wheat vs. fertilized wheat.

Differentially abundant ASVs detected by DESeq2 at a significance level of p -adjusted < 0.05 which were found to be enriched in (A) culture-independent (CI) and (B) culture-dependent (CD) rhizosphere (open circles) and rhizoplane (closed circles) communities in non-fertilized and fertilized wheat. ASVs depicted in bold were also detected in the isolate dataset. Only ASVs classified to genus level are pictured; for full list see Supplementary Material.

2.4 Discussion

We hypothesized that the addition of chemical fertilizer would reduce putative PGPR populations in wheat. We found that the abundance of rhizobacteria with acquisitional traits for key plant nutrients (endogenous nitrogen, phosphate, potassium, iron and zinc mobilization) were significantly reduced in wheat grown in soils treated with NPK fertilizer.

We combined both culture-independent and -dependent methods to study the impact of fertilizer on microbiome community composition and diversity. Our CI results confirmed previous studies showing that fertilizer alters community structure and reduces bacterial alpha diversity in the root environment (23, 24, 47-52). Our CD results support this as beta diversity was influenced and alpha diversity reduced by fertilizer. We decided to analyze CI rhizosphere and rhizoplane datasets together despite their having been sequenced using different approaches because, whilst inter-sequencing center variation can be significant (53), treatment effect consistently outweighs run variation (54). Thus, we acknowledge that compartment effect (in CI samples) could be partially due to bias in sequencing run; however, it is unlikely only due to this given the high percentage variability in beta-diversity, and previous evidence that different soil compartments have distinct microbial compositions (55). Additionally, our main aim was to study the effect fertilizer had on rhizobacterial diversity which is distinct in both compartments. Finally, we have previously found that fertilizer application reduces microbial species richness in the rhizosphere (23) and when re-examining the dataset from this study there is also a reduction in species richness in the bulk soil of plots receiving high levels of inorganic N fertilizer, but this effect is exacerbated in the rhizosphere. Therefore, we conclude that despite fertilizer also influencing bacterial community structure in bulk soil (56-61) it also has a profound effect on the rhizo-microbiome.

We tested how much the use of multiple agar types increased the percentage of total community DNA retrieved when compared to commonly used 10TSA only. The agar types were selected from Bai et al. (2015) which characterized extensive culture collections isolated from *Arabidopsis* leaf and root microbiomes

and demonstrated that the majority of leaf- and root-dwelling microbes were amenable to culture. Whilst Bai et al. used colony picking, as well as limiting dilution and cell sorting to purify bacterial isolates we were more interested in mixed culture taxonomy that resulted from the use of different isolation media, similar to the method used in Kavamura et al. (2019). In fact, the use of seven additional agar types increased the percentage of ASVs retrieved twofold compared to 10TSA only which, considering 10TSA retrieved half the ASVs from all agar types combined, supports its representability as an isolation medium. We reported a higher percentage of culturable ASVs (4.2%) retrieved in total community DNA, in contrast to Stefani et al. (2015) and Kavamura et al. (2019) who retrieved 2.4% and 2.2%, respectively, using similar isolation methods. Both studies analyzed 16S rRNA gene datasets by clustering sequences into operational taxonomic units; the increased percentage retrievability which we report could be due to improved taxonomic resolution from ASV analysis (62). Additionally, we found that agar type had little effect on the diversity and composition of rhizobacterial isolates cultured from soil. We conclude that this is likely due to the agar types used being less selective, not genera-specific and similar in composition which would explain the lack of diversity yielded. It would be interesting in future work to test microbial composition isolated from agar with similar conditions as found in soil, such as soil extract, root exudate and plant extract media.

Putative PGPR were characterized using a variety of functional bioassays to test a representative population of the culturable rhizo-microbiome. We found a clear difference in relative abundance of nutrient-solubilizing bacteria isolated from rhizosphere and rhizoplane samples between fertilized and non-fertilized wheat. There was a marked reduction in both relative and absolute abundance of nutrient solubilizing bacteria in fertilized samples. We hypothesized that this would be the case since fertilized plants and microorganisms can utilize easily available NPK source and do not need to solubilize NPK. Indeed, key enzymes involved in microbial solubilization of P, alkaline phosphatases, were shown to be strongly decreased by P fertilization in the rhizosphere (63) which suggests that fertilizer reduces hydrolytic enzyme-producing microorganisms or enzyme production in

organisms capable of such function. The role of fertilization on soil enzymatic activities has been investigated in detail over the last 40 years (64) where increasing evidence suggests that chemical fertilization can inhibit or slow down synthesis of hydrolytic enzymes. However, linking enzyme production to individual microbiome members or groups is particularly challenging (65). Our study suggests that genera such as *Bacillus*, *Pseudomonas*, *Rhizobium* and *Streptomyces* are producers of hydrolytic enzymes in soil. Of course, this study does not exclusively show that the culturable rhizobacteria with plant growth-promoting traits contributed to plant growth, and it is possible that the bacteria were releasing nutrients for their own consumption. However, spatial differentiation of microbes in the rhizosphere has been demonstrated to reduce plant-microbe competition as much as possible (66). Additionally, taxonomically and functionally similar bacteria to those we isolated in this study have been shown to increase plant growth when inoculated in soils (67-70).

We hypothesized that there is a host selection process for nutrient-solubilizing bacteria, driven by poor nutrient availability conditions and that fertilizer reduces this selection as wheat can utilize the readily available nutrients in soil and no longer need to interact with beneficial bacteria. Our results show a higher abundance of nutrient-solubilizing rhizobacteria in the rhizoplane, a compartment more intimately associated with the plant host than the rhizosphere, which could suggest a plant mediated selection process. Alternatively, the competition for nutrients might be heightened in the rhizoplane as compared to the rhizosphere, and nutrient-solubilizing organisms could be under a stronger selection when nutrients are depleted. We also found bacteria with known plant growth-promoting properties such as *Paenibacillus*, *Streptomyces* and *Pseudomonas* to be more abundant in root-associated soil from non-fertilized wheat (71, 72). However, it is unclear why fertilizer addition would inhibit root colonization by these bacteria. It is possible that rhizobacteria are less able to metabolize primary nutrients in the form presented in agricultural fertilizers than other members of the soil microbiome. If this is the case, it would follow that they are also less competitive in this environment and this would be reflected in their lower abundance. *Pseudomonas*, for example, colonize plants through chemotaxis into

the rhizosphere along a gradient of root exudates, followed by surface association and migration on the rhizoplane to ultimately form a bacterial biofilm, which would explain their increased abundance in the rhizoplane. In addition, many *Pseudomonas* spp. produce enzymes and other signaling molecules such as lipopolysaccharides to successfully colonize the plant rhizosphere and manipulate plants by activating symbiotic pathways (73). It has been shown that nutrient starvation triggers plants to activate symbiotic pathways with bacteria (74), so addition of fertilizer could mean plants no longer need to activate such pathways and thus no longer interact with beneficial bacteria, such as *Pseudomonas*. Chen et al. 2019 showed that the order Pseudomonadales in rhizosphere samples responded negatively to organic acid released by wheat in response to N fertilization (24) which correlate with our results that show a reduced abundance of *Pseudomonas* in soil samples from fertilized wheat and suggest that organic acid release by wheat as a response to fertilizer addition indirectly inhibits selection and colonization of *Pseudomonas* of the root system. The higher abundance of pseudomonads was more apparent in culturable rhizobacterial communities. Other non-culturable species may play just as important a role but are not as amenable to study.

Reducing environmental and financial costs of conventional agriculture requires novel management and breeding strategies to shift current high input to more sustainable biological methods. Soil microbiome manipulation promises to contribute to more environmentally benign agriculture by promoting plant growth and suppressing pathogenic microorganisms (10) and show promise in reducing chemical fertilizer application without influencing crop growth (67, 69). Our work provides a binary method to determine abundance of bacteria with beneficial traits in soil samples. Furthermore, we endeavored to link individual isolates cultured from soil samples to total soil community DNA. Culturable bacteria with plant growth-promoting traits are invaluable to the commercialization of biostimulants for sustainable agriculture (18). Therefore, establishing their abundance within total soil DNA will be useful to determine the overall impact that bioinoculants have on the microbiome as well as to determine optimal inoculation dose. However, first we must understand plant host evolutionary and

domestication history to identify plant traits linked to microbial recruitment by wild relatives (75). Significant knowledge gaps limit our understanding of the soil microbiome. The field needs to move beyond simple descriptions of community diversity to identify patterns in this complexity and recognize when that complexity is important (21). This will enable us to make soil microbiome research of practical utility to human undertakings, towards a cooperative relationship with terrestrial ecosystems instead of our current one-sided and often self-destructive relationship.

2.5 Conclusions

This study contributes to our understanding of the impact of fertilizer on wheat rhizobacteria and supports previous studies showing the deleterious effect of chemical fertilizer on plant rhizobacteria, particularly through highlighting the greater abundance of putative PGPR in unfertilized plants. It is assumed that wild relatives have co-evolved with the microbial community of native soils, selecting microbes beneficial to growth and health. Here, we show the probability that wheat plants can select growth-promoting bacteria to their roots to establish mutually beneficial associations and that chemical fertilizer reduces this selection. We propose a culture-dependent method to characterize the functional ability of the microbiome, and link this to culture-independent total soil DNA. We hope that this work contributes to the shift from simple microbiome studies on taxonomic characterization to conceptual framework analysis identifying and explaining patterns in the soil microbiome in agriculture and natural systems (76).

2.6 References

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3 IMPACTS OF WHEAT DOMESTICATION AND BREEDING ON RHIZOBACTERIAL MICROBIAL COMMUNITY SELECTION FROM A NUTRIENT DEPLETED AGRICULTURAL SOIL

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Statement of contributions of joint authorship

TR, TM, IC, and VK designed the experiments. TR performed the experiments and collected the data. TR analyzed the data with input from AT-B and VK for bioinformatic analyses. TR wrote the manuscript. All co-authors edited and commented on the manuscript.

Abstract

The transformation of natural habitats to agricultural systems increased food production but was accompanied by reduced wheat genetic diversity by selecting for high-yielding dwarf crops which are reliant on unsustainable levels of inorganic fertilizer. We previously showed that in the commercial variety of wheat, Cadenza, root-associated bacteria that beneficially contributed to plant nutritional status were reduced in fertilized plants. Therefore, we aimed to investigate the relationship between genetic changes during domestication of wheat, and the effect of fertilizer addition, on putative plant growth-promoting rhizobacteria (PGPR). Diploid, tetraploid and hexaploid varieties of wheat, along with the evolutionary genome donor species *Aegilops*, were grown in nutrient-depleted agricultural soil in pots without, and treated with, fertilizer. Rhizosphere and rhizoplane samples were collected at flowering stage (10 weeks) and analyzed by culture-independent amplicon sequence variant (ASV) analysis of

rhizobacterial DNA as well as culture-dependent techniques. Fertilization was the dominant factor shaping the microbiome. Other factors such as niche (rhizosphere/rhizoplane), plant species, and plant genotype, contributed significantly, but were associated with less variation in microbial communities. Chemical fertilization also reduced rhizosphere bacterial species richness and diversity. Key genera differentially more abundant in non-fertilized wheats included members of the phylum Cyanobacteria (*Nostoc*) and Proteobacteria (*Bradyrhizobium*, *Pseudomonas*) compared to fertilized wheats which were richer in Actinobacteria (*Arthrobacter*, *Catenulispora*, *Leifsonia*, and *Streptomyces*). After fertilization and niche, the expansion of the wheat genome from diploid to allopolyploid caused the greatest variation in microbial community structure (between 7-18%). Rhizoplane-derived microbiota culture collections, which resulted in isolation of approximately 15,000 bacterial isolates, were tested for plant growth-promoting traits using functional bioassays. Notably, unplanted control pots (bulk soil) showed a significantly lower abundance of PGPR compared to planted pots, and no difference between fertilization conditions. Moreover, differences in the abundance of PGPR under contrasting fertilization conditions were more pronounced in domesticated wheat, which we hypothesize is due to a loss of plant-microbe signalling pathways as the wheat genome expanded. We suggest that the influence of plants on the composition of their associated microbiota is an integral factor, hitherto overlooked, but intrinsic to selection during wheat domestication.

Keywords: rhizosphere, rhizoplane, fertilizer, microbiome, plant growth-promoting rhizobacteria, ploidy, domestication

3.1 Introduction

Bread wheat (*Triticum aestivum*), one of the world's leading sources of food (1), is an allopolyploid ($6x = AABBDD = 42$) composed of the genomes of three different species. The A genome is derived from *Triticum urartu* (2), the B from *Aegilops speltoides* or a closely related species (3-7) and the D genome from *Aegilops tauschii* (8). The final hybridization event between tetraploid wheat (AABB) and *Ae. tauschii* (DD) is thought to have occurred only once or twice,

~8000–10 000 years ago (summarized in Figure 3-1). Wheat is unique in this way amongst cereal crops in that its genome underwent huge expansion and as a result, wheat has gone through a significant genetic bottleneck through loss of nucleotide diversity since domestication (9-11). Additionally, during the Green Revolution mutant alleles of the reduced height (*Rht*) dwarfing genes were introduced into modern wheat cultivars, resulting in high-yielding wheat plants, which, when combined with agrochemical management and optimal conditions, increased yields, without productivity losses caused by lodging (12). Thus, the significant yield gains achieved by wheat breeders to date have been via the exploitation of genetic variation that has arisen via gene mutation over the last 8000–10 000 years and rare outcrossing events with tetraploid wheat.

Plants coexist with a large diversity of microorganisms. Understanding the impact of artificial plant selection during domestication and genetic plant divergence on the wheat-associated microbiota is important for the development of future wheat cultivars. The underlying genetics of wheat domestication has been described in detail, including the strong reduction in genetic diversity of domesticated species, polyploidization, and strong directional selection on loci encoding desired traits (9, 13). More recently, comparative genome analyses have allowed identification of domestication signatures along the *T. aestivum* genome (14). *T. aestivum* has been dispersed worldwide with wheat cultivation and constitutes a major crop on all continents (15). Wild relatives of wheat occur naturally in grassland vegetations in the Near East, including tetraploid wild emmer, *Triticum dicoccoides*, diploid einkorn, *Triticum monococcum*, and red wild einkorn, *Triticum urartu* (16, 17). The well-documented domestication history and close relatedness of wild and domesticated wheat provide a unique framework for comparative analyses of plant-associated microbial communities. Moreover, they allow us to address the evolution of associated microbial communities under cultivation.

It is becoming increasingly apparent that the microbiota associated with wheat root underwent significant changes during domestication (18-22). For example, Tkacz et al., 2020 observed three distinct groups of prokaryotic and eukaryotic

microbiota from the rhizosphere of bread wheat, synthetic hybrid wheat, and wild grasses and pasta wheat (21). Spor et al., 2020 revealed a depletion of nitrifiers and of arbuscular mycorrhizal fungi in elite tetraploid wheat cultivars compared to primitive domesticated forms (20). Kavamura et al., 2020 demonstrated how wheat rhizosphere bacterial communities from tall cultivars were distinct from those associated with semi-dwarf cultivars, associated with the development of dwarf cultivars during the Green Revolution (18). Valente et al., 2020 investigated the interactions of a model PGPR strain with 199 ancient and modern wheat genotypes revealing that PGPR colonization was higher overall in ancient relative to modern genotypes (22). It was also shown that vertically transmitted bacteria, but not fungi, of domesticated bread wheat species *T. aestivum* are less diverse and more inconsistent among individual plants compared to those of the wild emmer wheat species *T. dicoccoides* (19). Taken together, these results suggest how modern breeding, which is typically carried out under optimal agronomic conditions, has likely limited the potential for beneficial plant-microbe interactions (23-25). The majority of previous studies use either culture-independent or culture-dependent methods to study the effect of domestication on the wheat root microbiome and do not account for the effect that the introduction of chemical fertilizer during the Green revolution had on the co-evolution of plant-microbe interactions. In this study, we use culture- independent and -dependent methods to investigate the possible impacts of plant breeding on rhizosphere microbial selection of beneficial rhizobacteria in an agricultural soil under contrasting fertilization conditions. We analyze the rhizobacterial communities associated with 17 *Triticum* as well as 2 wild grass *Aegilops* accessions representing three plant genetic groups (diploid, tetraploid and hexaploid wheat varieties) along an evolutionary transect of wheat domestication and breeding. We utilise a culture-independent approach to determine microbiome community structure and complement this with a culture dependent approach to identify putative PGPR to improve our understanding of the extent to which plant-microbial interactions may have been impacted during wheat evolution and breeding. Our aim was to determine whether there were any identifiable shifts in microbial community composition and network structure associated with the transitions of

domestication and breeding represented by these plant groups. Understanding these changes has the potential to open new avenues to identify and promote beneficial interactions, towards ecologically intensified agroecosystems and more sustainable, lower input agriculture. It will also guide further studies to ultimately incorporate plant-microbial interactions into plant breeding programs.

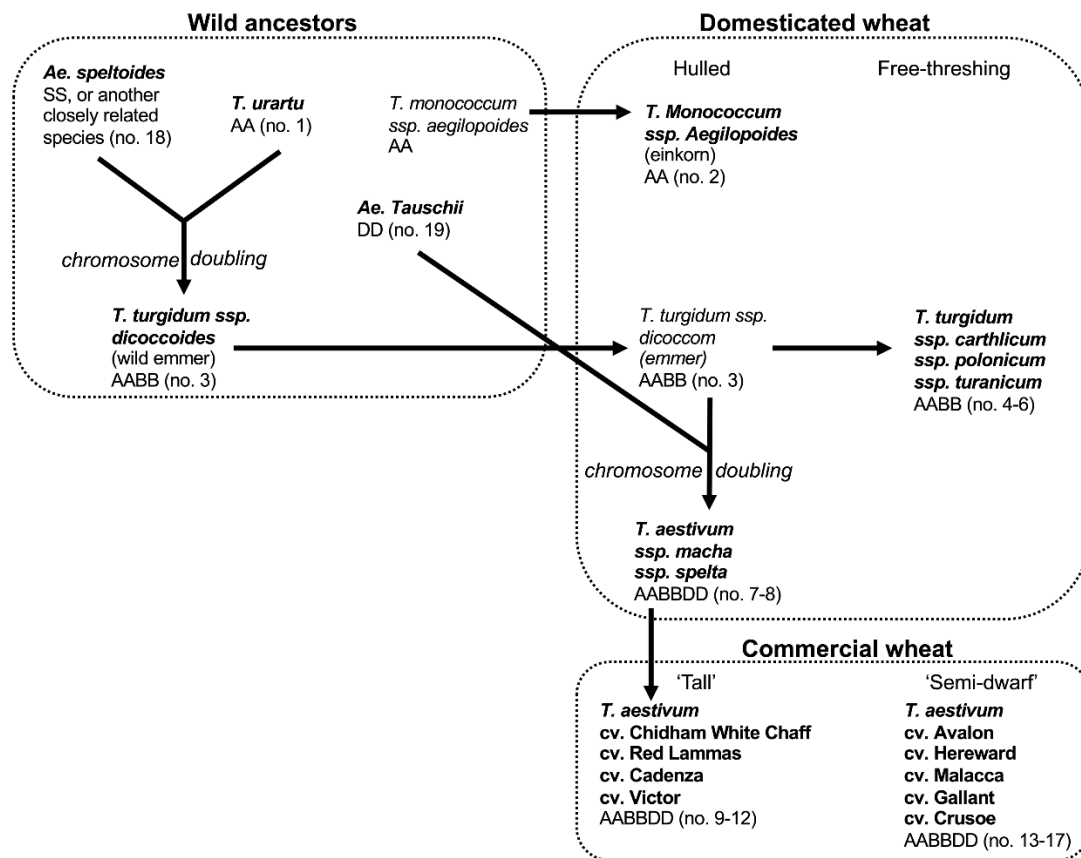


Figure 3-1: Genetic relatedness of wheat species used on this study (adapted from Tkacz et al., 2020 (21))

3.2 Materials and methods

3.2.1 Experimental design

Triticum accessions were selected to represent three plant genetic groups at three time points along an evolutionary transect of wheat domestication and breeding: two diploid accessions (*T. urartu* and *T. monococcum*, wild relatives from which wheat was domesticated), four tetraploid accessions (*T. dicoccoides*, *T. carthlicum*, *T. polonicum* and *T. turanicum*) and two hexaploid accessions (*T. spelta* and *T. macha*). These seeds were kindly provided by the Germplasm Resources Unit (a national capability supported by the BBSRC). Nine *T. aestivum* cultivars were also selected to represent modern, domesticated bread wheat (26, 27). Additionally, two wild grass species (*Aegilops tauschii* and *A. speltoides*) which are thought to be genome derivatives for *T. aestivum* were also selected (Table 3-1) (Supplementary Figure B-1).

Plants were grown in a glasshouse at Rothamsted Research, Harpenden in 9x9x10 cm pots filled with field soil from Woburn, Stackyard bare-fallow soil mine (LATLONG 52.000293N, -0.614308E), a well-draining sandy loam soil from the Rothamsted Research experimental farm at Woburn, Bedfordshire (UK). The soil is a Cottenham series (28) classified as a Cambic Arenosol (FAO), and has been maintained as a bare fallow for over 50 years. It was chosen to reduce the legacy effect of prior cropping systems. Topsoil (0-20 cm) was collected in August 2019 across three replicate plots and thoroughly mixed to produce a homogenized soil. Soil was sieved (2 mm mesh), air dried, and stored at 4 °C in polythene bags prior to use. Seeds were surface sterilized (70% ethanol, 10 min; 1.5 % active chlorine, 1 h; 5 x rinse, sterile distilled water (SDW); overnight imbibition, sterile water, 4 °C) and pre-germinated in Petri dishes for three days at room temperature in the dark. Seedlings were carefully transplanted to individual wells on a seed tray (36x23x7 cm) (1x seedling per well) and grown for two weeks (20 °C, 16 h/day light regime) before vernalization for twelve weeks (need temp. and light). Plants were then transferred to pots (9x9x10 cm) filled with ~500 g soil (1x plant per pot) with and without NPK granules (15% N, 9% P₂O₅, 11% K₂O, 2% MgO with micro-nutrients (B, Cu, Fe, Mn, Mo, Zn); Osmocote, UK) (~2.5 g per

pot) in a two-factor randomized block design with cultivar and fertilizer as factors (Supplementary Figure B-2). As a control (referred to in text and figures as bulk soil), plant-free pots were established and given the same treatments as pots with plants.

3.2.2 Soil collection and processing

All plants were harvested at the start of flowering (Zadoks growth stage 61) (29), within four weeks. Height (from soil surface to head of longest stem) was measured, then soil was gently tipped from the pot onto a fresh polythene bag. Loose soil was discarded and non-rhizospheric soil carefully removed. Roots were vigorously shaken in a bag to release tightly attached soil (i.e. rhizosphere), mixed to homogenize and split between two 5 ml screw cap vials. One vial was flash frozen in liquid nitrogen and stored at -80 °C for culture-independent work and one vial was stored at 4 °C for culture work. The root system was excised and placed in a sterile 50 ml centrifuge tube for subsequent rhizoplane work. The remainder of the plant was dried (80 °C, 24 h) and dry foliar plant biomass measured. To obtain rhizoplane soil, collected root samples were weighed and 1 ml SDW was added for every 0.1 g of root. Samples were shaken vigorously for 10 min using an orbital shaker. For culture-dependent work: 1 ml soil solution was transferred to a 2 ml cryo-tube, 1 ml glycerol (50 %) was added, tubes were vortexed, and flash frozen in liquid nitrogen before being stored at -80 °C. For culture-independent work: 4 ml soil solution was centrifuged (2 min, RT, 15,000 rpm), supernatant discarded, and remaining soil was flash frozen in liquid nitrogen and stored at -80 °C.

3.2.3 Sequencing and bioinformatics

Genomic DNA was extracted from bulk, rhizosphere, and rhizoplane soil (~0.25g) using the DNeasy PowerSoil DNS isolation kit (Qiagen, Inc.) and stored at -80 °C. Extractions were performed according to the manufacturer's instructions but with the use of the MP Biomedicals FastPrep-24 machine twice for 30 s at 5.5 m s⁻¹. The V3-V4 region of bacterial 16S rRNA genes in samples were amplified using primers 314F (5'-CCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (30). Amplicon preparation followed the

protocol of Kozich et al. (31) with the following setup: 15 µl 2x KAPA HiFi HotStart ReadyMix PCR (Roche), 3 µl of each 0.5 µM forward and reverse primer indexed solutions, 9 µl sterile water, and 3 µl DNA template (150 ng). PCR amplifications were performed on a Biorad CFX under the following conditions: 95°C for 3 min; 30 cycles of 95 °C for 20 s, 55 °C for 15 s, 72 °C for 20 s; 72 °C for 1 min; 4 °C hold. We included two negative control samples and two mock community samples. Indexed PCR amplification reactions were cleaned with Agencourt AMPure XP beads (Beckman Coulter) and normalized to equimolar concentration prior to being pooled. The final pool was quantified, and quality assessed using SYBR green quantitative PCR (qPCR) assay with primers specific to the Illumina adapters (Kappa) and diluted to 12 pM. The library was spiked with PhiX control V3 library (5% v, v⁻¹, 12.5 pM) and sequenced on the Illumina MiSeq platform using the Illumina MiSeq v3 kit (Illumina Inc.), to obtain 2 x 300 bp paired-end sequences.

3.2.4 Sequence processing

Demultiplexing of raw sequences was performed by CASAVA data analysis software (Illumina). Forward and reverse reads were merged using the `vsearch merge_pairs` function (32) which produced 6,722,536 reads from 298 samples (median 16,092 reads per sample). Paired-end reads were then filtered, de-replicated, and denoised using the DADA2 1.2 (33) pipeline with Quantitative Insights into Microbial Ecology (QIIME2) (version 2018.11.0) default parameters (34) which resulted in 5,320,078 high-quality sequences, retaining 79% of initial reads. Low quality samples with less than 100 reads were removed (n=17). The resulting ASV table retained high quality nonchimeric reads and was used to build a phylogenetic tree using the `align-to-tree-mafft-fasttree` command in QIIME2. Taxonomy was assigned using the SILVA132 database (35, 36). ASVs that were not classified as bacteria or were classified as mitochondria or chloroplasts were removed.

3.2.5 Microbial community analysis

Microbial diversity and community composition were analyzed at the ASV level in R 3.6.1 using Phyloseq (v1.34.0) (available at <https://joey711.github.io/phyloseq/>)

(37) and Microbiome (v1.12.0) (available at <http://microbiome.github.com/microbiome>) packages. Sequencing of the bacterial 16S rRNA gene amplicon library resulted in 9,936 ASVs in 281 samples. Samples with fewer than 1000 reads (n=25) and samples where only one species were observed in alpha rarefaction (n=13) were removed from downstream analysis (Supplementary Figure B-3 and Supplementary Figure B-4). Furthermore, 1,871 ASVs unclassified at kingdom level were removed from the dataset. After filtering and denoising, no bacterial features remained in the negative controls of DNA extraction and sequencing.

Bacterial alpha diversity indices pertaining to richness (chao1), evenness (Simpson), diversity (Shannon), and relatedness (Faith's Phylogenetic Diversity PD) were calculated based on rarefied ASV tables (1,200 sequences). The alpha diversity rarefaction plots confirmed that a sufficient depth of coverage was achieved at this cut-off. Box plot figures were created using the "ggboxplot" function in the "ggpubr" package. Two-way type III analysis of variance (ANOVA) was performed in GraphPad Prism version 8 for Mac (San Diego, CA: GraphPad Software, Inc) with fertilizer and ploidy level as factors, to determine the dominant factor contributing to variation in means for alpha diversity data. *Post hoc* multiple comparison tests with Šidák correction were performed to test for significance within groups. Normality was confirmed by quantile-quantile normality plots and the Shapiro-Wilk test; outlier data points preventing normality were removed prior to ANOVA analysis. Homogeneity of variances was confirmed by residuals versus fits plots and Spearman's test for heteroscedasticity.

For beta diversity analysis, amplicon sequencing data were normalized using DESeq2 to account for differences in sequencing bias (38). The resulting normalized ASV table (number of reads belonging to each ASV per sample) was used for Principal Coordinates Analysis (PCoA) and Canonical analysis of principal coordinates (CAP) which was employed on Bray-Curtis dissimilarity distance matrices using the 'ordinate' function with the 'betadisper' test to check for equal variance (R package: vegan (v2.5.6)) (39). Permutational multivariate analysis of variance (PERMANOVA) was used to detect significant changes in

the microbiota structure using pseudo-F value as a proxy for the strength of an individual factor (fertilization, niche, ancestral class, ploidy, genome, and plant species) on the bacterial community composition. PERMANOVA tests were performed using 'adonis2' with 9999 permutations and block factor (based on the randomized block experimental setup). Briefly, two PERMANOVA types were used: (1) permutation test under a reduced model, sum of squares type I (sequential) with 9999 permutations using fixed multifactorial design; (2) multifactorial, nested design PERMANOVA (type I) where plant species were nested in genome which were nested in ancestral class. Factor groupings are detailed in Supplementary Table B-1 and the raw PERMANOVA output can be found in Supplementary Table B-2 to Supplementary Table B-7. The same data and factors were used for CAP plotting.

To test the number of shared and unique ASVs across rhizosphere and rhizoplane samples between non-fertilized and fertilized wheat and between ploidy levels from non-fertilized and fertilized rhizosphere and rhizoplane samples, the "VennDiagram" package in R was used.

Two additional R packages, DeSeq2 and indicpecies, were used to perform the differential abundance and indicator species analyses respectively to identify ASVs preferentially associated with fertilization and vice versa as well as with diploid, tetraploid, and hexaploid wheat (38, 40). Extremely low prevalence ASVs, defined as those present in fewer than six samples, were removed before analyses. The Benjamini-Hochberg procedure was used to adjust p-values to account for multiple comparisons. An adjusted p-value below 0.05 was considered significant. Phylum level community composition was investigated by relative abundance of normalized data, after removal of bacterial ASVs only classified to Kingdom level and ASVs assigned as 'environmental samples' for visualization purposes.

3.2.6 Culture-dependent functional analysis for plant growth-promoting traits

The ability of rhizoplane-colonising bacteria to solubilise casein, phosphate, potassium, iron, and zinc was tested using previously established bioassays (41-

46), as previously described (47). To obtain a library of rhizoplane-colonising bacteria, rhizoplane soil previously frozen in glycerol were thawed, vortexed, serially diluted, and spread on tryptone soya agar (TSA) (Tryptone Soya Broth (TSB; 1/10th concentration) (Oxoid, Basingstoke, UK) and Bacto™-Agar) (5 replicates per agar type) and incubated (25 °C, 4 days). Colony counts were measured using ImageJ (48, 49).

Plant growth-promoting functions were tested using previously established bioassays. Individual colonies were picked and inoculated in 150 µl TSB (1/10th concentration) in 96-well plates and incubated (25 °C, 2 days) prior to functional analysis; two wells were left uninoculated as negative media controls. A sterile 96-prong inoculating manifold was used to spot individual inoculated isolates from the 96-well plate liquid cultures onto agar. Assays were incubated (25 °C, 5-7 days); positive isolates were counted per sample for each functional assay. In total, approximately 15,000 rhizobacterial isolates were isolated from 145 rhizoplane soil samples and 8 bulk soil samples (94 isolates per sample) and tested for nutrient-solubilizing traits (full details of bioassay composition is described in (47)).

Statistical differences in the frequency of positive vs. negative isolates between non-fertilized and fertilized wheat were performed in GraphPad Prism version 8 for Mac (San Diego, CA: GraphPad Software, Inc) (50). Box plots were created in R 3.6.1 (<http://www.r-project.org>). This software was also used to calculate two-way analysis of variance (ANOVA) and pair-wise t-tests with Šidák correction for bacterial abundance and absolute abundance of nutrient-solubilizing isolates. Data were first normalized by logarithmic transformation. Normality was confirmed by quantile-quantile normality plots and Shapiro-Wilk test; homogeneity of variances was confirmed by residuals versus fits plots and Spearman's test for heteroscedasticity.

Chapter 3

Plant	Line	History	Ploidy	Genome	No. of biological reps.		
Ancestral landraces					No NPK	Plus NPK	
Diploid wheat							
1	<i>T. urartu</i>	T1010038-PF-1	wild ancestor	Diploid	AA	4	3
2	<i>T. monococcum</i>	T1040005-PF-1	traditional cultivar	Diploid	AA	4	4
Tetraploid wheat							
3	<i>T. dicoccoides</i>	T1060022-PF-3	wild ancestor	Tetraploid	AABB	4	4
4	<i>T. carthlicum</i>	T1100001-PF-1	traditional cultivar	Tetraploid	AABB	4	4
5	<i>T. polonicum</i>	T1090001-PF-1	traditional cultivar	Tetraploid	AABB	4	4
6	<i>T. turanicum</i>	T1110001-PF-1	traditional cultivar	Tetraploid	AABB	4	4
Hexaploid wheat							
7	<i>T. macha</i>	T1240001-PF-1	traditional cultivar	Hexaploid	AABBDD	4	4
8	<i>T. spelta</i>	T1220037-PF-1	traditional cultivar	Hexaploid	AABBDD	4	4
Commercial wheat							
9	<i>T. aestivum</i>	Chidham White Chaff	Not recorded	Hexaploid	AABBDD	3	4
10	<i>T. aestivum</i>	Red Lammas	Not recorded	Hexaploid	AABBDD	4	4
11	<i>T. aestivum</i>	Cadenza	Axona*Tonic	Hexaploid	AABBDD	4	4
12	<i>T. aestivum</i>	Victor	(Squarehead*Red King)*Talavera	Hexaploid	AABBDD	4	4
13	<i>T. aestivum</i>	Avalon	TJB 30/148* TL 365a/34/5	Hexaploid	AABBDD	4	4
14	<i>T. aestivum</i>	Hereward	Norman(sib)*Disponent	Hexaploid	AABBDD	3	4
15	<i>T. aestivum</i>	Malacca	Riband*(Rendezvous)*Apostle	Hexaploid	AABBDD	3	4
16	<i>T. aestivum</i>	Gallant	(Malacca*Charger)*Xi-19	Hexaploid	AABBDD	4	4
17	<i>T. aestivum</i>	Crusoe	Cordiale*Gulliver	Hexaploid	AABBDD	4	4
Wild grass							
Genome donor							
18	<i>A. speltoides</i>	T2140038-PF-1	wild ancestor	Diploid	BB	3	2
19	<i>A. tauschii</i>	T2220012-PF-1	wild ancestor	Diploid	DD	4	4
	unplanted	bulk soil control				4	4

Table 3-1: Wheat species chosen for the current study

3.3 Results

3.3.1 Plant phenotypical data

Ploidy level significantly contributed to variation in plant height (19.0% variation according to two-way ANOVA: $F=16.6$, degrees of freedom (DF)=2, $p<0.0001$) whereas variation caused by fertilization and interaction were not considered significant ($p>0.05$) (A in Supplementary Figure B-5). Ploidy level and fertilization significantly contributed to variation in plant biomass (3.7% and 32.9%, respectively) (two-way ANOVA: ploidy, $F=1.7$, DF=2, $p=0.005$; fertilization, $F=98.2$, DF=1, $p<0.0001$) (B in Supplementary Figure B-5). Pairwise significance within fertilization groups between diploid, tetraploid, and hexaploid wheats, as well as between non-fertilized and fertilized plants of the same ploidy level are shown in Supplementary Figure B-5.

Plant height and biomass data for individual plant genotypes can be found in Supplementary Figure B-6.

3.3.2 Fertilization shapes the wheat root microbiome

Across all plant groups, fertilizer addition and soil compartment (niche) had the strongest effect on ASV level bacterial community alpha and beta diversity (A and B in Figure 3-2) (Supplementary Table B-2) (Supplementary Table B-5) (Supplementary Table B-8).

Bacterial richness, diversity, evenness and relatedness in the rhizosphere and rhizoplane were significantly lower in fertilized wheat (two-way ANOVA: $p<0.05$) whereas there were no statistical differences between treatments in bulk soil (A in Figure 3-2) (Supplementary Table B-8). PCoA of wheat rhizobacterial communities showed clustering of samples by fertilization along the first principal coordinate axis (PC1) and clustering by niche along the second principal coordinate axis (PC2) indicating that it is the effect of chemical fertilization status which is the major factor shaping these communities, followed by soil compartment (B in Figure 3-2). Two-way PERMANOVA indicated that both fertilizer effect and soil compartment were significant in wheat rhizobacterial communities, as well as their interaction, but also that ancestral class, ploidy,

genome, and plant species also significantly contribute a small proportion of the total variance in the structure of the bacterial communities (Supplementary Table B-2) (Supplementary Table B-5). Fertilization accounted for 18.4% of the variance ($p = 0.001$) and soil compartment accounted for 9.4% of the variance ($p = 0.001$).

Of the 5447 rhizosphere ASVs ($n=116$) and the 5976 rhizoplane ASVs ($n=103$), 36.7 % and 38.1 % were shared between non-fertilized and fertilized wheat species, respectively (C in Figure 3-2). The remaining 3449 rhizosphere ASVs and 3698 rhizoplane ASVs were unique to either non-fertilized or fertilized wheat (C in Figure 3-2). Differentially abundant genera in samples from non-fertilized wheat included *Pseudomonas*, *Bradyrhizobium*, *Mesorhizobium*, and *Azospirillum* compared to *Paraburkholderia*, *Cellulomonas*, *Arthrobacter*, *Streptomyces*, *Rhizobium* and *Paenibacillus* in fertilized wheat (D in Figure 3-2). These results reaffirm that chemical fertilization significantly alters the soil microbiota (47, 51-58).

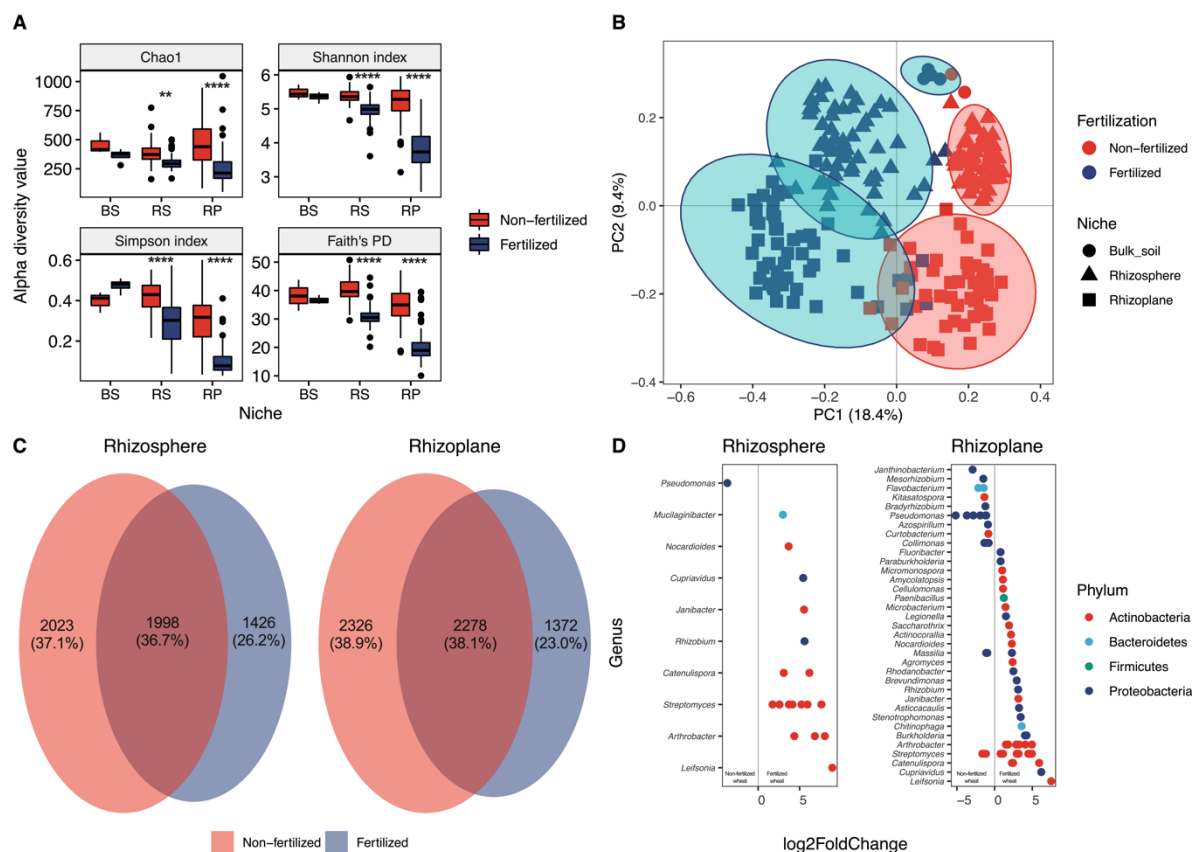


Figure 3-2: Impact of chemical fertilization on rhizobacterial (rhizosphere and rhizoplane) community composition from different wheat species.

(A) Alpha diversity estimates at ASV level for bulk soil (BS), rhizosphere (RS) and rhizoplane (RP) bacterial communities in non-fertilized and fertilized wheat; with median (line) and hinges at first and third quartiles (25th and 75th percentiles). (B) PCoA plot of bacterial composition based on Bray-Curtis distance for bacterial communities at ASV level. The percentage shown in each axis corresponds to the proportion of variation explained. (C) Venn diagrams of the bacterial microbiota from non-fertilized vs. fertilized wheat in the rhizosphere and rhizoplane showing the number and relative abundances of bacterial ASVs. (D) Differentially abundant ASVs detected by DESeq2 at a significance level of < 0.05 which were found to be enriched in rhizosphere and rhizoplane bacterial communities in non-fertilized and fertilized wheat.

3.3.3 Fertilization and ploidy level interactively affect rhizobacterial community diversity

Across all plant genome ploidy groups (diploid, tetraploid, hexaploid), fertilizer significantly lowered rhizobacterial diversity, evenness, and relatedness as measured by Shannon diversity index, Simpson index, and Faith's PD, respectively, with only the rhizosphere bacterial diversity and evenness showing no significant differences between fertilized and unfertilized tetraploid wheat (two-way ANOVA with post-hoc multiple comparison tests with Šidák correction, $p < 0.05$) (Supplementary Figure B-8) (Supplementary Table B-8). The general trend in rhizobacterial richness as measured by Chao1 was that fertilization decreased richness in both the rhizosphere and rhizoplane, though this is exacerbated in hexaploid wheat – richness was significantly lower in fertilized hexaploid wheat compared to non-fertilized hexaploid wheat (Supplementary Figure B-8). There were no statistical differences between non-fertilized and fertilized bacterial community richness, diversity, evenness, or relatedness in bulk soil with or without fertilizer addition in any other sample, further demonstrating the effect chemical fertilizer has on bacterial community diversity at the root-soil interface in hexaploid wheat. The general trend is that fertilization decreases richness in both rhizoplane and rhizosphere, though this is exacerbated in hexaploid wheat.

The influence of plant ploidy level on bacterial community alpha diversity was more pronounced in the rhizoplane compared to the rhizosphere (Supplementary Figure B-8) (Supplementary Table B-8). Post-hoc analysis shows that this variation (6.9%, 12.6%, and 5.8% influence in rhizoplane bacterial community diversity, evenness, and relatedness) is caused by differences in fertilized diploid, tetraploid and hexaploid wheat plants compared to the bacterial community of bulk soil ($p < 0.05$). There are no statistical differences between plant ploidy groups.

There is also a strong general plant effect (rhizosphere and rhizoplane) on bacterial community composition, as the rhizobacterial microbiota is clearly different from that of bulk soil (B in Figure 3-2), thus bulk soil was removed for

beta diversity analysis of individual fertilization and niche groups. The same trend in alpha diversity can be seen in beta diversity analysis when analysing the microbiota separately for each soil compartment. Fertilizer caused 24.6% and 28.6% of variation in the rhizosphere and rhizoplane respectively (Supplementary Figure B-9).

The overall influence of plant ploidy level and ancestral class on rhizobacterial community composition is similar in the rhizosphere and rhizoplane (ploidy: pseudo-F of 2.22 and 2.26 for the rhizosphere and rhizoplane, respectively, $p < 0.01$) (ancestral class: pseudo-F of 1.87 and 1.91 for the rhizosphere and rhizoplane, respectively, $p < 0.05$) (Supplementary Table B-3). The interaction between plant ploidy and fertilization significantly contributed to variation in the bacterial community in the rhizosphere (pseudo-F of 1.60, $p < 0.05$) but not in the rhizoplane but the influence of plant species is stronger in the rhizoplane (pseudo-F of 1.61, $p < 0.05$) than in the rhizosphere (Supplementary Table B-3). Identification of the relative influence of individual factors, on predefined groups of samples, e.g ploidy groups initially indicated by PCoA plot, can be further investigated by CAP analysis. The CAP plot clearly shows the importance of each factor in the rhizobacterial community (Figure 3-3).

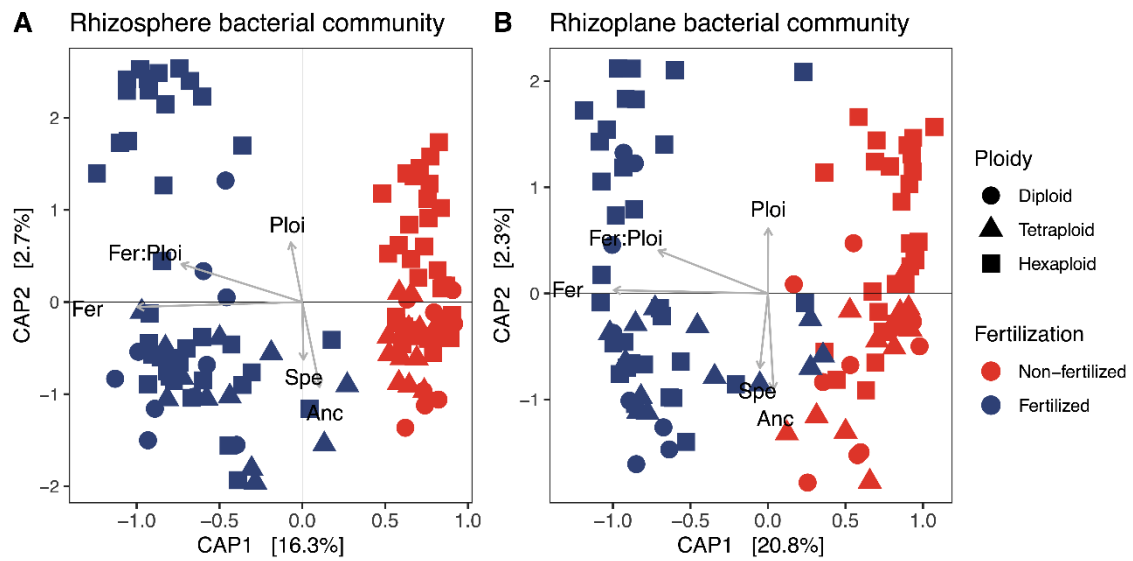


Figure 3-3: CAP analysis plots of the bacterial rhizosphere (A) and rhizoplane (B) community from non-fertilized vs. fertilized wheat.

Factors (fertilization (Fer), ploidy (Ploi), ancestry class (Anc), and plant species (Spe)) shaping the rhizobacterial community composition the interaction between them (fertilization and ploidy (Fer:Ploi)) are represented by arrows. The length of the arrow represents the strength each factor has on variation in the microbial community. Only factors that significantly contributed to rhizobacterial variation were shown (Supplementary Table B-3).

When analyzing the microbiota separately for both fertilization and niche groups ((1) non-fertilized rhizosphere microbiota, (2) non-fertilized rhizoplane microbiota, (3) fertilized rhizosphere microbiota and (4) fertilized rhizoplane microbiota), ploidy and ancestral class factors had a similar weak, yet significant influence (A in Figure 3-4) (Supplementary Table B-4). Plant species significantly influenced rhizobacterial community in all groups apart from non-fertilized rhizosphere whereas, genome factor only caused a significant effect in non-fertilized rhizoplane (Supplementary Table B-4) The trend was seen in multifactorial nested PERMANOVA tests (Supplementary Table B-7). CAP analyses clearly show the importance of each factor in the rhizosphere and rhizoplane (Supplementary Figure B-10).

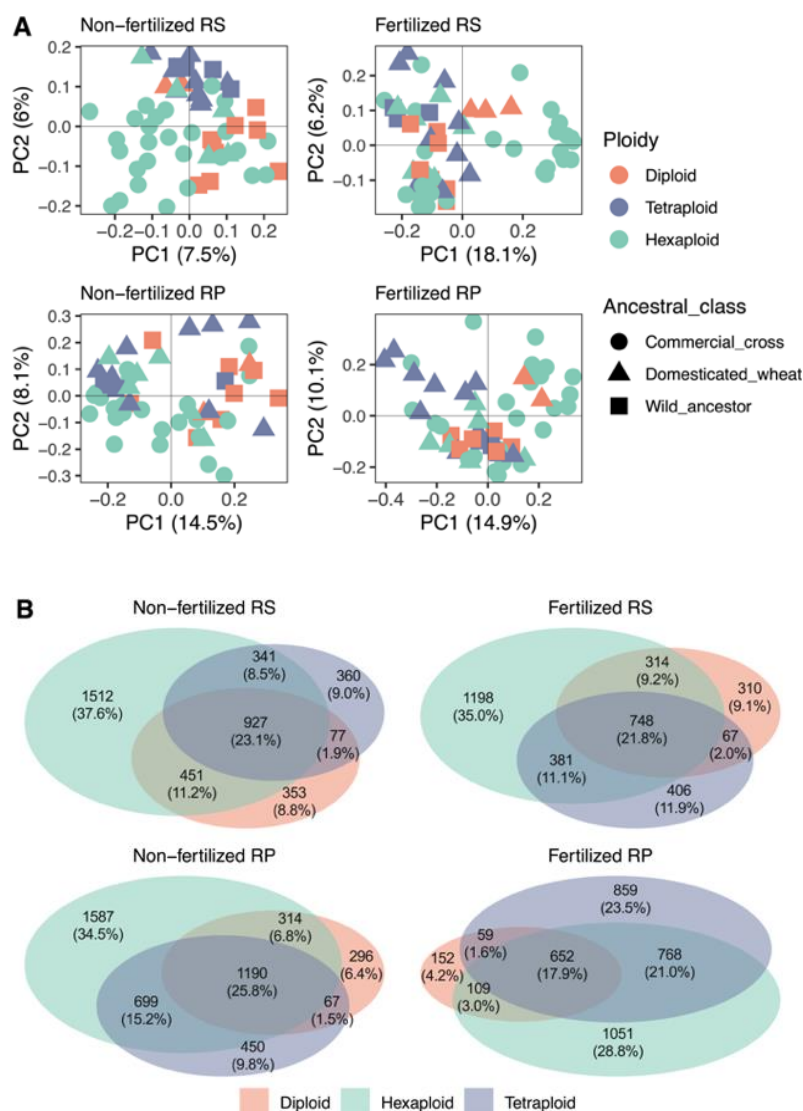


Figure 3-4: PCoA plots (A) and Venn diagrams (B) of bacterial community in non-fertilized and fertilized rhizosphere (RS) and rhizoplane (RP) samples with diploid, tetraploid and hexaploid wheat varieties.

PCoA plots of bacterial composition based on Bray-Curtis distance for bacterial communities at ASV level. The percentage shown in each axis corresponds to the proportion of variation explained. Venn diagrams of the bacterial microbiota from diploid, tetraploid, and hexaploid wheat varieties in the rhizosphere and rhizoplane showing the number and relative abundances of bacterial ASVs.

3.3.4 Domestication affected specific bacterial taxa in the rhizosphere

Bacterial communities from the root environment were mainly represented by Proteobacteria (39.4-45.5%), Bacteroidetes (3.6-19.6%), and Actinobacteria (12.3-29.9%) compared to bulk soil which were mainly represented by Proteobacteria (37.3-40.3%), Actinobacteria (19.0-30.7%), and Acidobacteria (7.4-18.5%) (Figure 3-5). Taxonomic composition shows an increase in phyla related to bulk soil with an increase in ploidy level, but only in non-fertilized wheat, with hexaploid wheat showing the highest abundance of Acidobacteria (diploid: 6.6%, tetraploid: 9.8%, hexaploid: 25.0%), Chloroflexi (1.7%, 1.2%, 3.5%), Gemmatimonadetes (0.5%, 0.3%, 2.0%), and Verrucomicrobia (1.3%, 1.7%, 3.7%). In comparison, there was little change in taxonomic composition with ploidy level in fertilized wheat with main phyla being Acidobacteria (diploid: 3.3%, tetraploid: 3.7%, hexaploid: 7.5%), Actinobacteria (25.7%, 28.7%, 24.9%), Bacteroidetes (19.6%, 14.0%, 13.7%), Firmicutes (6.7%, 6.4%, 8.6%) and Proteobacteria (43.8%, 45.5%, 43.9%).

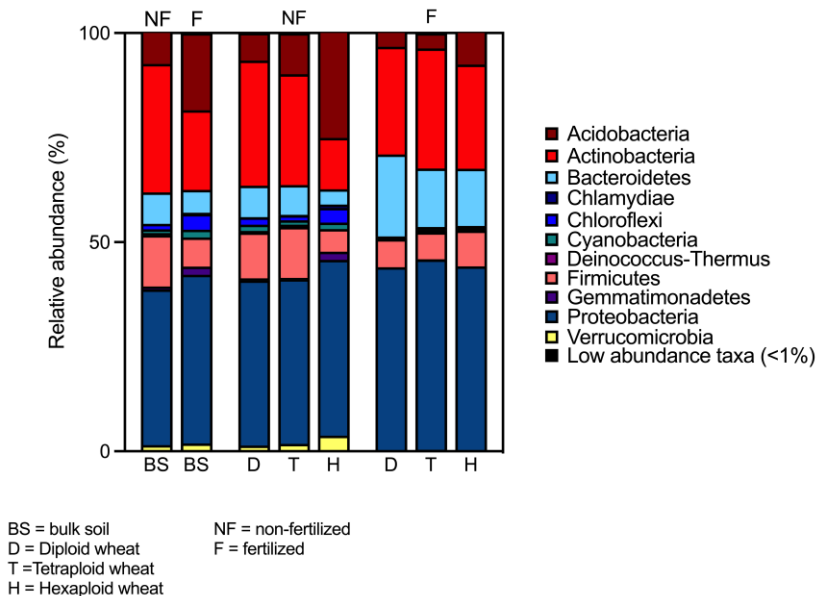


Figure 3-5: Relative abundance of phyla associated with bacterial communities in bulk soil and the root environment of diploid, tetraploid, and hexaploid wheat varieties grown under contrasting fertilization conditions.

Differential abundance analysis identified ASVs that were highly associated with non-fertilized compared to fertilized diploid, tetraploid, and hexaploid wheat varieties.

Overall, 202 ASVs (22.3% of all ASVs identified in diploid wheat), 192 ASVs (18.5% of all ASVs identified in tetraploid wheat), and 303 ASVs (19.6% of all ASVs identified in hexaploid wheat) were differentially abundant between non-fertilized and fertilized samples. Acidobacteria were enriched in non-fertilized wheat by: 17.3% (35 ASVs), 12.5% (24 ASVs), and 13.9% (42 ASVs) in diploid, tetraploid, and hexaploid varieties, respectively, compared to 0.5% (1 ASV), 1.0% (2 ASVs), and 0% in fertilized diploid, tetraploid, and hexaploid wheat, respectively (A in Figure 3-6). Whereas Actinobacteria were enriched in fertilized wheat by 18.8% (38 ASVs), 17.7% (34 ASVs), and 18.2% (55 ASVs) in diploid, tetraploid, and hexaploid varieties compared to 0.5% (1 ASV), 1.6% (3 ASVs), and 0.7% (2 ASVs) in non-fertilized diploid, tetraploid, and hexaploid wheat (A in Figure 3-6).

Eight genera (19 ASVs), ten genera (19 ASVs), and fourteen genera (35 ASVs) were enriched in fertilized diploid, tetraploid and hexaploid wheat, respectively, and they included members of the Actinobacteria (18.7% in diploid wheat, 17.7% in tetraploid wheat, and 18.2% in hexaploid wheat), Bacteroidetes (5.0% in diploid wheat), Cyanobacteria (0.66% in hexaploid wheat), Firmicutes (6.9% in diploid wheat), and the Proteobacteria (22.3% in diploid wheat, 19.3% in tetraploid wheat, and 20.1% in hexaploid wheat) (B in Figure 3-6). In comparison, two genera (4 ASVs), six genera (9 ASVs), and five genera (9 ASVs) were enriched in non-fertilized diploid, tetraploid and hexaploid wheat, respectively, and they included members of the Actinobacteria (1.6% in tetraploid wheat, and 0.66% in hexaploid wheat), Cyanobacteria (0.99% in diploid wheat, 0.05 in tetraploid wheat, and 0.66% in hexaploid wheat), and Proteobacteria (19.8% in diploid wheat, 24.5% in tetraploid wheat, and 33.2% in hexaploid wheat) (B in Figure 3-6).

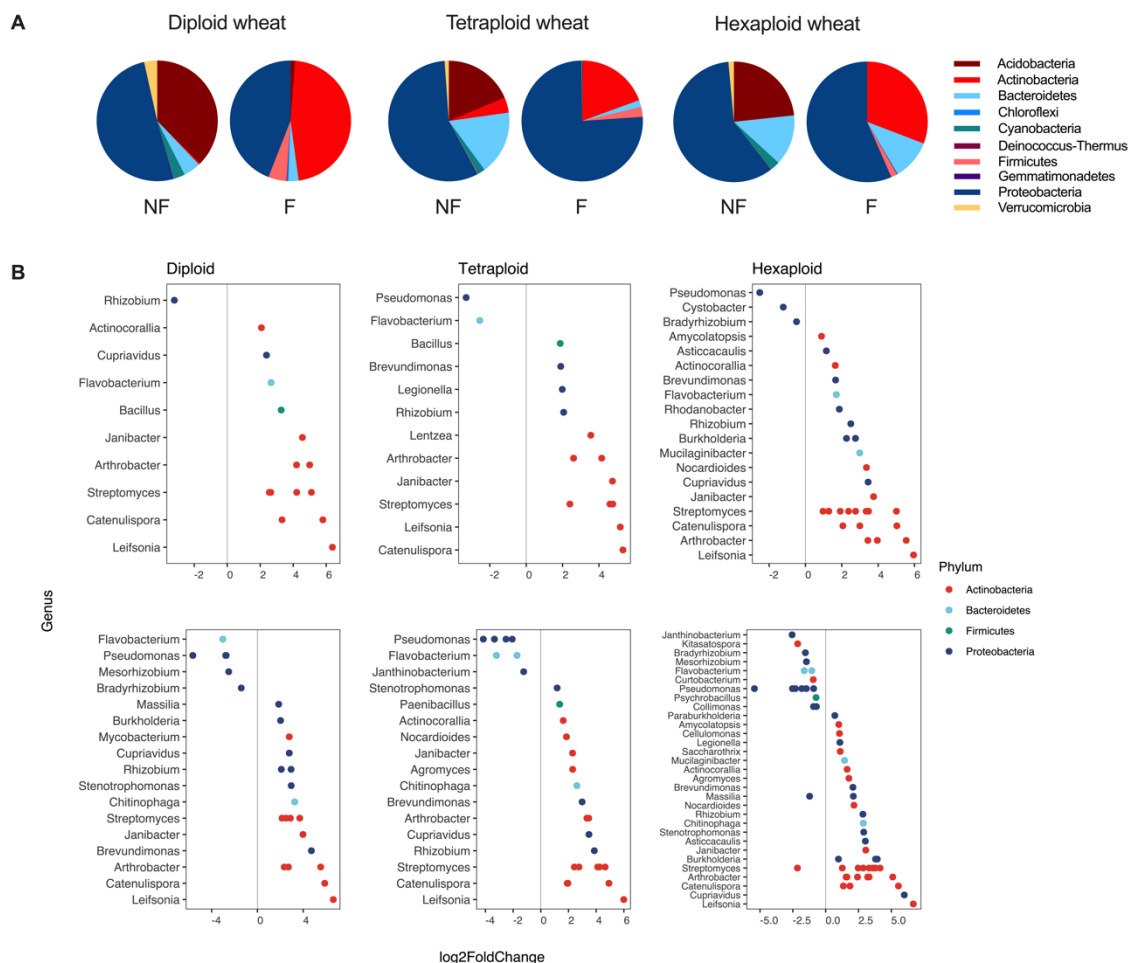


Figure 3-6: Differentially abundant bacterial ASVs detected by DESeq2 at a significance level of $p_{\text{adjusted}} < 0.05$ between non-fertilized (NF) and fertilized (F) diploid (first column), tetraploid (second column), and hexaploid (third column) wheat varieties.

(A) Percentage of differentially abundant phyla; (B) differentially abundant genera enriched in non-fertilized wheat have a negative log2FoldChange value and vice versa in fertilized wheat in rhizosphere (top row) and rhizoplane (bottom row) samples. Points represent individual ASVs and are colored based on phylum level.

Indicator species analyses identified 189 ASVs (2.3%) as indicators of non-fertilized and fertilized plant genetic groups. The most striking difference was the enrichment of ASVs classified as Actinobacteria in fertilized hexaploid wheat (9 ASVs; 38.1%) compared to diploid (non-fertilized: 3.8% (3 ASVs), fertilized: 10.1% (2 ASVs) and tetraploid (non-fertilized: 8.1% (5 ASVs), fertilized=9.8% (8 ASVs)) varieties (Figure 3-7). Additionally, there were no indicator ASVs assigned to Bacteroidetes in fertilized hexaploid wheat varieties compared to non-fertilized hexaploid (24.3% (2 ASVs)), diploid (non-fertilized: 13.9% (4 ASVs), fertilized=29.5% (8 ASVs)), and tetraploid (non-fertilized: 25.9% (12 ASVs), fertilized=9.2% (6 ASVs)) wheat varieties (Figure 3-7). Non-fertilized hexaploid wheat was the only group where indicator ASVs assigned to Cyanobacteria were identified (1 ASV (8.1%)).

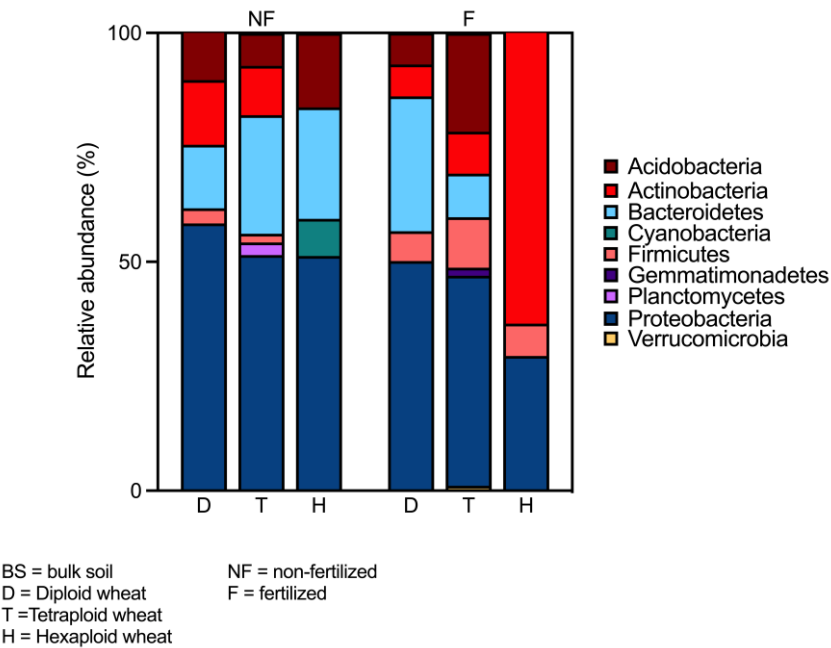


Figure 3-7: Relative abundance of phyla identified in indicator species analysis associated with each plant group in non-fertilized (NF) and fertilized (F) wheats.

3.3.5 Differences in the abundance of beneficial rhizobacteria in wheat under contrasting fertilization conditions are more pronounced with domestication

It was previously shown that the abundance of nutrient solubilizing rhizobacteria were reduced in fertilized Cadenza wheat compared to non-fertilized wheat (Chapter 2) (47). We hypothesized that the effect of fertilization would be more pronounced in hexaploid wheat varieties compared to tetraploid and diploid wheat varieties.

In general, rhizobacteria that tested positive for solubilization of plant macronutrients: organic N (casein), inorganic phosphate ($\text{Ca}_3(\text{PO}_4)_2$), potassium (potash feldspar), and plant micronutrient: iron ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) had a statistically higher relative abundance in isolate libraries cultured from non-fertilized wheat samples compared to fertilized wheat samples, from all wheat genotypes (A-D in Figure 3-8) (Supplementary Table B-10). The relative abundance of zinc (ZnO)-solubilizing isolates however was significantly higher in fertilized compared to non-fertilized hexaploid wheat; there were no statistical differences in diploid and tetraploid wheat genotypes (E in Figure 3-8) (Supplementary Table B-10). The relative abundance of total nutrient-solubilizing rhizobacteria was higher in non-fertilized diploid ($34.8 \pm 11.9\%$), tetraploid ($28.4 \pm 10.7\%$), and hexaploid ($31.9 \pm 11.9\%$) wheat varieties compared to fertilized diploid ($19.4 \pm 17.0\%$), tetraploid ($6.63 \pm 6.62\%$), and hexaploid wheat varieties ($10.1 \pm 10.6\%$) (F in Figure 3-8).

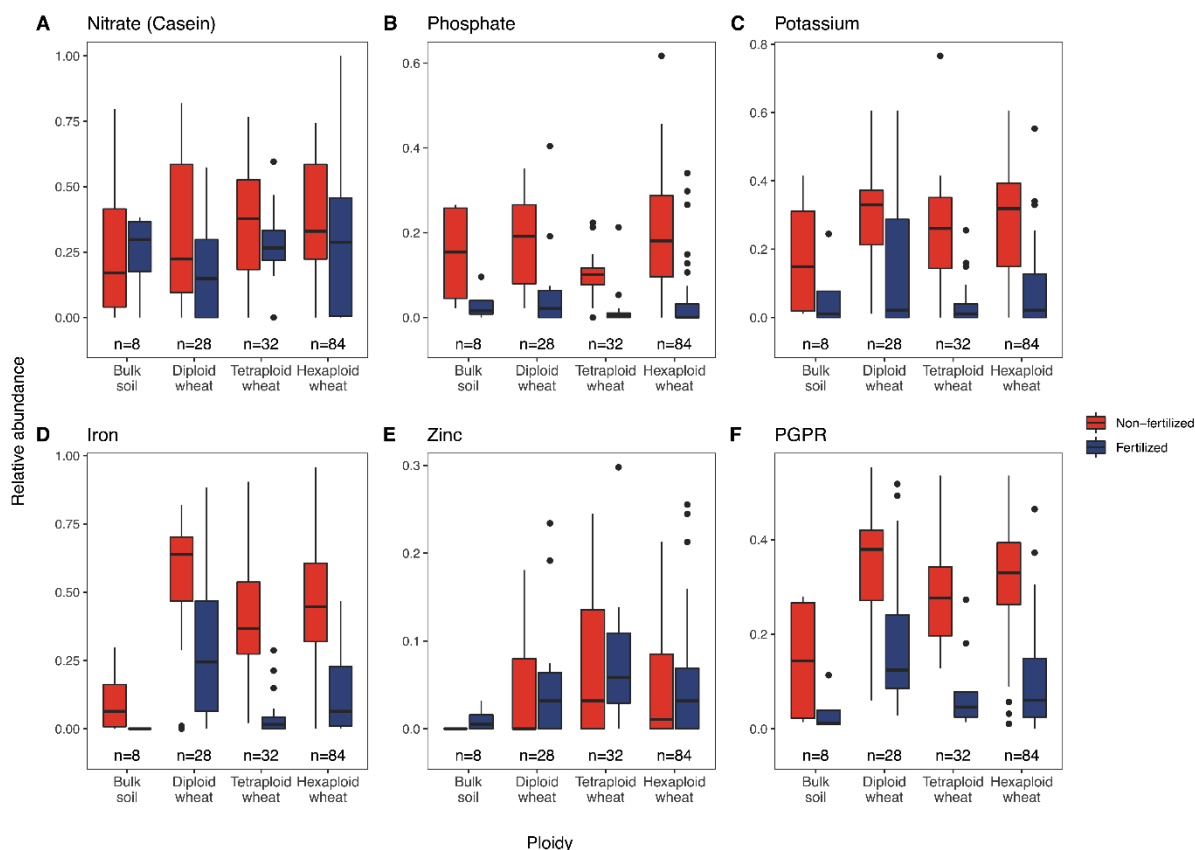


Figure 3-8: Relative abundance of culturable bacteria with nutrient acquisition traits isolated from the rhizoplane of diploid, tetraploid, and hexaploid wheats grown in soils with and without fertilizer.

(A) Nitrate-, (B) phosphate-, (C) potassium-, (D) iron-, and (E) zinc-solubilization as determined by casein, PVK, AVK, CAS, and zinc carbonate agar, respectively. Box plots show relative abundance of solubilizing isolates and relative abundance of (F) PGPR isolates, as determined by functional bioassays; with median (line) and hinges at first and third quartiles (25th and 75th percentiles). Each sample (n) represents an isolate library (n=94) derived from rhizoplane samples.

The influence of fertilization was measured by the chi-squared statistic (χ^2) to test the contribution that fertilizer has on the abundance of nutrient-solubilizing rhizobacteria in bulk soil and wheat varieties.

In general, fertilization had the greatest effect on the abundance of rhizobacteria testing positive for phosphate, potassium, and iron solubilization, in hexaploidy, followed by tetraploid, then diploid, wheat varieties (A-D in Figure 3-9) (Supplementary Table B-10). Isolate libraries cultured from bulk soil samples were the least influenced by fertilization in solubilization of all nutrients except zinc (Figure 3-9) (Supplementary Table B-10).

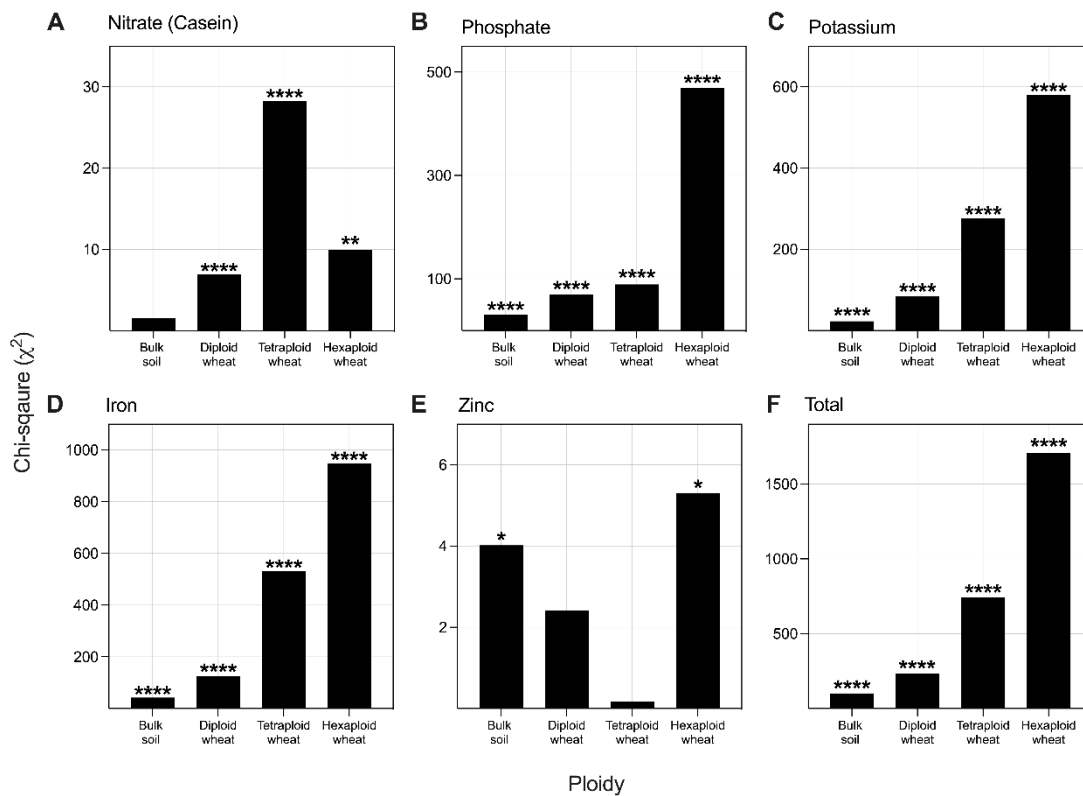


Figure 3-9: Influence of chemical fertilizer as measured by the chi-squared statistic (χ^2) on the abundance of nutrient-solubilizing bacteria isolated from bulk soil and the rhizoplane of diploid (n=28), tetraploid (n=32), and hexaploid (n=64) wheat varieties grown in low nutrient agricultural soil.

(A) Nitrate-, (B) phosphate-, (C) potassium-, (D) iron-, and (E) zinc-solubilization as determined by casein, PVK, AVK, CAS, and zinc carbonate agar, respectively, and (F) total number of PGPR.

The effects of fertilization and plant ploidy level on absolute abundance of culturable bacteria isolated from bulk soil/rhizoplane samples were tested.

Fertilization caused no statistical differences in absolute abundance of bacteria isolated from bulk soil/rhizoplane samples from diploid, tetraploid, and hexaploid wheat samples, accounting for 0.007% of variation (two-way ANOVA, $p>0.05$). Ploidy level caused 9.0% of variation (two-way ANOVA: $F=5.10$, $p=0.002$) but post-hoc multiple comparison analysis with Šidák correction revealed the differences to be attributed to bulk soil vs. the rhizoplane of diploid, tetraploid, and hexaploid wheat ($p<0.05$) (A in Figure 3-10).

In comparison, fertilization caused 20.5% of total variance of absolute abundance of nutrient-solubilizing isolated from the rhizoplane of diploid, tetraploid and hexaploid wheat varieties (two-way ANOVA: $F=24.6$, $d.f=1$, $p<0.0001$) with post-hoc tests revealing that this variation was caused by tetraploid and hexaploid wheat varieties ($p<0.0001$) whereas there were no statistical differences between absolute abundance of culturable isolates from non-fertilized vs fertilized bulk soil and diploid wheat samples ($p>0.05$) (B in Figure 3-10).

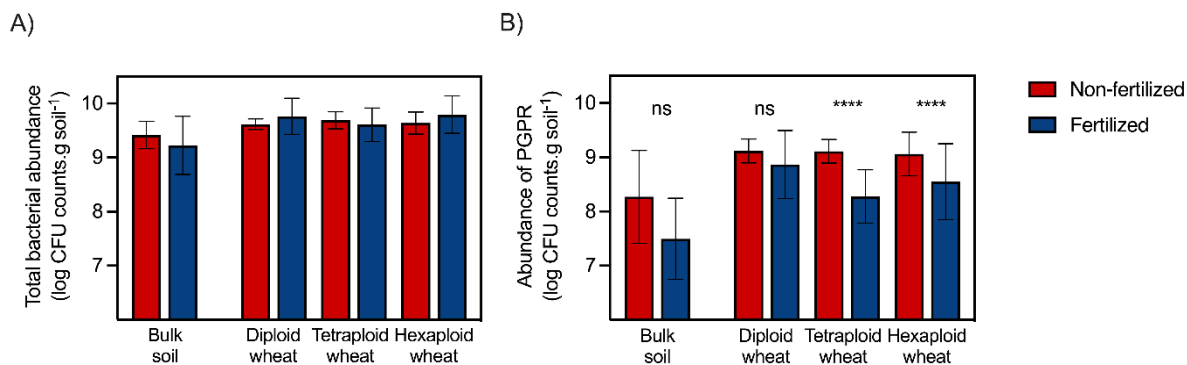


Figure 3-10: Effect of chemical fertilizer (NPK) on absolute abundance of culturable bacteria isolated from the rhizoplane of diploid, tetraploid, and hexaploid wheats grown in low nutrient soil.

(A) Absolute abundance (log CFU counts.g soil⁻¹; CFU is colony-forming units) of culturable rhizobacteria isolated from soil samples on 10TSA. (B) Absolute abundance (log CFU counts.g soil⁻¹) of nutrient solubilizing rhizobacteria as determined by functional bioassays. Significant differences as determined by post-hoc multiple comparison tests with Šidák correction are shown by 'ns' and '****' for $p > 0.05$ and $p < 0.0001$, respectively.

3.4 Discussion

We explored whether there is any unintended impact on rhizobacterial community selection and assembly, of aboveground selection for high yields during wheat domestication and modern breeding. We approached this by performing a pot experiment in soil under contrasting fertilization conditions. We aimed to establish the effect that chemical fertilizer introduction during the green revolution had on plant-microbe interactions in wheat by comparing the rhizobacterial community of wild wheat progenitors to modern varieties. This allowed us to characterize the influence of modern bread wheat lines, their wild ancestors, and domesticated species, on the structure of the rhizobacterial communities. The importance of rhizosphere microorganisms in supporting plant growth under suboptimal growth conditions has been well documented (59-63). Thus, the potential differential ability of plant accessions to select beneficial microorganisms to the rhizosphere when grown in a nutrient poor soil, relative to nutrient replete conditions, over the course of domestication and breeding, was investigated.

As expected, fertilization regime influenced rhizobacterial diversity, community structure, and taxonomic composition, with the effects being more pronounced with proximity to the root but with fertilizer exerting the dominant effect as opposed to niche in shaping the microbiome. However, for the first time this is demonstrated across a wide range of wheats as well as ancestral species with a range of ploidy numbers. Our results unambiguously show the negative impact that chemical fertilization has on rhizobacterial species richness, diversity, evenness, and phylogenetic distance, as previously demonstrated by numerous other studies (51-58). Even though this same trend has been demonstrated in bulk soil (64-70), our results show bacterial community alpha diversity to be unaffected in bulk untreated, and fertilized, soil. This could be due to the small scale of our experimental system i.e. in pots sampled at the end of one growth period. Many studies investigating the impact of chemical fertilization on bulk soil investigate large field trials over a significant length of time (64, 70). For the purpose of this study, the similarity of rhizobacterial communities in bulk soil with and without fertilizer magnifies the effect that wheat plants have over the

assembly and selection of the rhizo-microbiome and contributes to the suitability of this system for studying plant-microbe interactions.

There was no clear difference between ploidy levels in terms of rhizobacterial species richness, diversity, evenness, and phylogenetic diversity for a given fertilization regime. This has been observed previously with alpha diversity indices related to domestication (20). This was also the case for prokaryotic diversity in maize rhizosphere and rhizoplane microbial communities with there being no difference between teosinte, inbred, and modern maize (71). This was also the case for the Shannon and Simpson index in wild versus domesticated rice (72). Interestingly, Kinnunen-Grubb et al., 2020 (73) showed a similar result between diploid (*T. monococcum*), tetraploid (*T. turgidum*), and hexaploid (*T. aestivum* varieties and *T. spelta*) wheat cultivars. They found that there was no difference between rhizobacterial richness and diversity in wheat sampled at 21 weeks post germination when the first side shoot was visible. However, when sampling at an earlier growth stage (emergence of first leaf 10 weeks post germination) alpha diversity was higher in hexaploid when compared with diploid and tetraploid wheats. This suggests that the initial phase of microbial colonization is a better stage to assess differences in rhizobacterial alpha diversity between wheats when based on ploidy status. This would explain the similar rhizobacterial alpha diversity results in this study of wheats sampled at late-stage development (flowering).

In comparison, we observed three distinct rhizobacterial communities for diploid, tetraploid and hexaploid wheats in the rhizosphere and rhizoplane of non-fertilized and fertilized wheats. Similar trends have been previously shown for prokaryotic community structure of genetic groups of modern cultivars and ancestral landraces for wheat (21, 73), maize (71), and rice (72). We also found that other factors also influenced the bacterial community as well as ploidy level, such as ancestral class and plant species, in agreement with observations by Tkacz et al., 2020 (21). Furthermore, we found that plant ploidy level and fertilization regime interactively influenced the rhizobacterial community structure suggesting that microbial communities in the root environment are shaped by

interactions between agricultural management practices such as fertilization conditions, and host selection processes such as genomic content. Schmidt et al., 2019 showed that plant selective influence in maize differed in conventional (inorganic chemical fertilizer application) versus organic soil management systems to shape microbial community composition and structure (74). This shows how the rhizosphere-root environment is a complex, multi-faceted environment influenced by many factors. Reframing research priorities to better understand adaptive plant-microbe feedbacks and include roots as a significant moderating influence of management outcomes could help guide plant-oriented strategies to improve productivity and agroecosystem sustainability.

In general, the most abundant phyla across all wheat varieties were the Actinobacteria, Bacteroidetes, and Proteobacteria, indicating their importance in the wheat root microbiome, as demonstrated previously (75, 76). It was found that the Actinobacteria were more abundant in the rhizo-microbiome of fertilized wheats whereas Acidobacteria were more abundant in the unfertilized wheat rhizo-microbiome, regardless of ploidy status. The Acidobacteria are normally associated with low nutrient bulk soil condition and so it follows that they are abundant in the rhizosphere of wheat grown in low nutrient conditions. However, we did see an increased abundance of Acidobacteria in fertilized bulk soil compared to non-fertilized bulk soil which does contradict this, and interestingly Acidobacteria were most abundant in non-fertilized hexaploid wheat rhizosphere. In fact, taxonomic composition shows an increase in phyla related to bulk soil with an increase in ploidy level, but only in non-fertilized wheat, with hexaploid wheat showing the highest abundance of Acidobacteria, Chloroflexi, Gemmatimonadetes, and Verrucomicrobia. In comparison, there was little change in taxonomic composition with ploidy level in fertilized wheat with main phyla being Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. We propose that this is due to fertilization being the dominant factor over the soil microbiome and therefore causing an undifferentiated rhizobacterial community at the root-soil interface regardless of wheat genotype, as well as a reduction in selection of PGPR, as previously demonstrated. This would explain why there is a larger effect of fertilization on the abundance of

PGPR in tetraploid and hexaploid wheats as the microbiome gradually changes with an increase in ploidy level in non-fertilized wheats but not fertilized wheats. However, in the absence of fertilizer, hexaploid wheats show a similar taxonomic profile to fertilized bulk soil which could be an indication that wheat has lost the ability to select for microbes during domestication or that diversity loss in soils treated with chemical fertilizer has caused microbiome evolution to become static. Of course, we still see an increase in PGPR in culturable communities in wheat compared to bulk soil, so the difference between ploidy level may be easier to discern at a finer scale e.g. genera-specific taxa. In fact, it was shown elsewhere that ancient wheat varieties have a higher ability to interact with PGPR. Interactions of a model PGPR strain with 199 wheat genotypes revealed that PGPR colonization was higher overall on ancient compared to modern genotypes (22). It would be interesting to create an isolate database of PGPR and non-PGPR isolates from bulk soil, diploid, tetraploid, and hexaploid wheat samples to see if the culture-independent results correlate with the culture-dependent results.

This supports our hypothesis that rhizosphere microbiomes of modern hexaploid wheat varieties could have evolved a reduced ability to establish mutualistic microbiomes due to fertilization intervention. In general, Actinobacteria seem to be more associated with fertilized plants, which correlates with the results from Chapter 2 as well as Kavamura et al., 2018 (47, 54), including key genera such as *Arthrobacter*, *Catenulispora*, *Leifsonia*, and *Streptomyces* which were differentially more abundant in fertilized diploid, tetraploid, and hexaploid wheat varieties compared to non-fertilized wheat. Actinobacteria was also a key indicator phylum in fertilized hexaploid wheat. Key genera in non-fertilized wheat included *Nostoc* and *Bradyrhizobium* which were differentially more abundant in all non-fertilized wheat samples and *Pseudomonas* in all rhizoplane samples in diploid, tetraploid and hexaploid wheat. Interestingly, both the differential abundance and indicator species analysis showed Cyanobacteria to be associated with non-fertilized hexaploid wheat varieties. Cyanobacteria were also reported to be differentially abundant in *T. aestivum* bread wheat compared to wild wheat varieties (21). Cyanobacteria have previously been shown to fix

atmospheric N in rice root environments (77) which would provide an explanation for their increased abundance in low-nutrient soil, where the plant relies on gaining nutrients from other sources. Future work would be required to establish if wheat plants interact directly with this phylum, especially given their underutilized potential as biofertilization inoculants.

Overall, in terms of functional screening, isolates derived from non-fertilized plants had a greater number of PGPR than the fertilized counterparts, agreeing with the work in Chapter 2 (47). To our knowledge, this is the first study to have functionally tested a large subset of culturable isolates (approx. 15,000) from a wide range of wheat species from ancestral to modern, including wild grass *Aegilops* species. There was no clear difference in the abundance of putative PGPR between wheat genetic groups but there was a clear difference in different wheat ploidy levels on the effect of fertilization, as an increase in ploidy level resulted in a more pronounced decrease in PGPR abundance in the root environment under fertilized condition. The abundance of PGPR between fertilized and unfertilized diploid plants was not significantly different, but there was a marked decrease in fertilized tetraploid and hexaploid plants. This correlates with our previous results demonstrating an interactive effect between plant genomic content and fertilization. In fact, it has been shown that fertilization markedly affects gene expression in wheat (78). We propose that fertilization-induced wheat gene expression could play a role in the reduced abundance of PGPR in fertilized tetraploid and hexaploid wheats, which further supports our hypothesis that hexaploid wheat is more dependent on fertilization in comparison to ancestral landraces where interactions with soil microbes were crucial for holobiont fitness (79). There is still a large gap in the literature about plant signalling pathways and the genes responsible in terms of interactions with PGPR. It would be interesting to test the effect of fertilization on gene expression in ancestral wheat landraces compared to modern cultivars and correlate this with differential gene expression in plant growth-promoting rhizobacteria (12). This would provide direct targets for future plant breeding programmes to incorporate plant-microbe interactions, as well as high yields, to foster a more balanced approach to sustainable agriculture whereby plant and soil are given equal status.

Additionally, there was a significantly lower abundance of rhizobacteria with plant growth promoting traits isolated from bulk soil and no difference in abundance under contrasting fertilization conditions. This further confirms the experimental method as suitable for assessing the abundance of putative PGPR in the root environment as well as the effect of fertilization on plant selection of PGPR. Future work will determine the identification of the microbes in the screening assays in terms of PGPR versus non-PGPR isolates in diploid, tetraploid and hexaploid wheat varieties to determine the taxonomic composition of the isolates compared to function. Here we use the generalist versus specialist hypothesis to predict that plant breeding has resulted in modern varieties losing the ability to cultivate specialist microorganisms dedicated to plant growth promoting function when grown under fertilization conditions (80). It will be interesting to see if linkage can be made between culture dependent and independent datasets. If so, this could result in accurate predictions on the 'health' of cropping systems in field conditions by high throughput culture independent analyses, targeting for example specific indicator taxa informed by this study.

Taken together, this work demonstrates that the fertilization level is the predominant factor in shaping the wheat root microbiome. However, it also points clearly to the role that plant breeding has in determining the host's ability to select a beneficial microbiome. This raises the question of whether current commercial wheat has become dependent on chemical fertilizers and requires intervention for example through breeding programmes to harness the genomic potential of root microbes as a new platform for improved stress resilience of future crops and sustainable food production.

3.5 Conclusions

Understanding the evolutionary trajectories of plant-microbe interactions and an enhanced ability to capitalize on them for crop adaptation to future climatic scenarios will be crucial for sustainable agriculture (81). The study of ten *Triticum* accessions representative of a wheat domestication timeline, nine *Triticum aestivum* varieties, and two *Aegilops* species hypothesized to be evolutionary genome donors, enabled the identification of fertilization as the dominant factor

shaping the microbiome. Other factors such as niche (rhizosphere/rhizoplane), plant species, and plant genotype, contributed significantly, but were associated with less variation in microbial communities. Chemical fertilization also reduced rhizosphere bacterial species richness and diversity. Key genera differentially more abundant in non-fertilized wheats included members of the phylum Cyanobacteria (*Nostoc*) and Proteobacteria (*Bradyrhizobium*, *Pseudomonas*) compared to fertilized wheats which were richer in Actinobacteria (*Arthrobacter*, *Catenulispora*, *Leifsonia*, and *Streptomyces*). The depletion of microbes with nutrient liberation function in modern accessions suggests that the Green Revolution, which relied on chemical fertilizers, might have caused a reduced association between tetraploid and hexaploid wheat and their beneficial rhizosphere microbiota. However, the strong genotypic variation that we observed within each domestication group, indicates that there is capacity for improvement of modern wheats via introgressive hybridization of genetic material from ancestral species. This could result in improved rhizosphere-beneficial microbe associations in modern wheat, less dependent on fertilizer application.

3.6 References

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4 MICROBIOME STUDIES OF WHEAT HORMONE SIGNALLING MUTANTS

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TR, TM, IC, ST, AP and VK designed the experiments. TR and VK performed the experiments and collected the data. TR analyzed the data with input from AT-B and VK for bioinformatic analyses. TR wrote the manuscript. All co-authors edited and commented on the manuscript.

Abstract

Hormones take a crucial role in contributing to the assembly of plant microbiomes. The development of dwarf wheat cultivars through selection of *Reduced height (Rht)* semidwarfing genes which cause a limited response to the phytohormone gibberellin (GA) combined with high levels of agrochemical inputs during the green revolution resulted in high yielding cropping systems. However, changes in wheat cultivars were made without considering impacts on plant and soil microbe interactions. We studied the effect of severity in *Rht* gene-induced dwarfism on rhizobacterial diversity and composition by comparing three *Rht* gene mutant wheat cultivars (*Rht-D1b*, *Rht-D1f*, and *Rht-B1c*) to Cadenza wheat (wild-type (wt)-cadenza) under contrasting nitrogen-phosphorous-potassium (NPK) fertilization conditions in a low nutrient agricultural soil. We also investigated the effect of other hormone signalling mutants in abscisic acid (ABA), auxins, brassinosteroids, ethylene, GA biosynthetic mutants, and strigolactones. Rhizobacterial communities in *Rht* mutant wheats were distinct from those

associated with wt-cadenza wheat, with higher differential abundance in Acidobacteria, Chloroflexi and Gemmatimonadetes, phyla more commonly associated with bulk soil, in *Rht* mutant cultivars compared with a higher differential abundance of Bacteroidetes, Firmicutes and Proteobacteria, phyla more commonly associated with plant growth promotion, in wt-cadenza wheat cultivars. This suggests that *Rht* gene-mediated dwarfism in wheat introduced during the Green Revolution might have affected the ability of plants to select for beneficial rhizobacteria. Additionally, compared to wt-cadenza all the mutants apart from ABA seemed to result in reduced plant control of the microbiome under NPK fertilized conditions. There was a general trend of high rhizobacterial diversity in non-fertilized mutant and wt-cadenza plants however, when fertilizer was applied there was a reduction in diversity in wt-cadenza but this reduction does not occur in the mutants (apart from ABA). This was particularly evident in auxin, brassinosteroid, *Rht*, and strigolactone mutants, although similar trends were seen in ethylene and GA mutants. Our results suggest that hormonal signalling pathways important for structuring plant-microbe interactions are likely affected by fertilizer. We propose that hormone signalling pathways are important for plant-microbe interactions and that the addition of fertilizer results in reduce plant control of the microbiome due to a lack of selective pressure.

4.1 Introduction

Wheat domestication originated in the Near East and the crop has undergone a series of crosses and modifications leading to the hexaploid bread species, *Triticum aestivum* L (1). Wheat is a key crop for global food security, providing 20% of dietary requirements for calories and protein (2). To achieve food demand of an increasing global population, projections forecast a need to increase wheat production by 11% by 2026 with just 1.8% increase in cultivation areas (3). During the Green Revolution, Reduced height (*Rht*) dwarfing genes were introduced in modern wheat cultivars, resulting in high-yielding wheat plants, which, when combined with agrochemical management and optimal conditions, increased yields, without productivity losses caused by lodging (4, 5). Plant height is a complex trait controlled by several Quantitative Trait Loci (QTLs) and more than

20 *Rht* genes have been described, however, most of them have low potential for successful breeding programs (6). Most breeding programs take into consideration the improvement of above ground parts of plants, with little attention to below ground parts due to the inherent challenges of root analysis coupled with the influence of soil type on root traits (7, 8). As such, the effect of domestication and modern breeding on belowground traits in wheat largely remains unclear.

The introduction of semidwarfing genes (*Reduced height-1 (Rht-1)*) into wheat (*Triticum aestivum*) was a major factor in breeding higher yielding varieties during the Green Revolution (for review, see Hedden, 2003 (4)). The major contribution to global wheat production during the Green Revolution was from the introduction of the *Rht* alleles *Rht-B1b* and *Rht-D1b*, which confer dwarfism through decreased responsiveness to the phytohormone GA. This causes a reduction in stem elongation resulting in improved resistance to stem lodging and yield benefits through an increase in grain number. Six additional alleles at *Rht-B1* and *Rht-D1* have been identified, four on chromosome 4B and two on chromosome 4D, which produce dwarfs with a broad range of plant height. The *Rht-B1* and *Rht-D1* genes were shown to encode DELLA proteins, transcriptional regulators that act to repress GA-signalling (for review see Sun, 2010 (9)); their mutant alleles *Rht-B1b* and *Rht-D1b* are thought to confer dwarfism by producing more active forms of these growth repressors.

Plants produce phytohormones which regulate plant growth and development. There are six classes of plant hormones, namely, auxins, brassinosteroids, GAs, abscisic acid, ethylene, and strigolactones. Many rhizosphere bacteria can produce phytohormones such as auxins, GAs, abscisic acid, and ethylene (10). The capacity of rhizosphere bacteria to influence plant hormonal status, by bacterial production or metabolism of hormones, is considered an important mechanism by which they promote plant growth, and productivity (11). The importance of hormones in mediating such plant/microbe interactions has been highlighted by experiments showing that inoculation with PGPR causes pivotal changes in plant expression of hormone-mediated genes (12-14). As presently

known, brassinosteroids and strigolactones are not produced by bacteria and fungi.

It has recently been shown that wheat dwarfing influenced selection and composition of the wheat rhizosphere microbiome (15). It is thought that the reduced responsiveness to GA influences the composition of the root microbiome. Furthermore, microbes produce a range of phytohormones (10). It would be interesting to determine if the plant breeding process has influenced the status of other classes of plant phytohormones such as ABA, brassinosteroids, ET, auxins and strigolactones. We hypothesize that genetic changes which occurred because of plant breeding during the Green Revolution may have reduced selection for beneficial root microbes in wheat. We hypothesize that the introduction of the *Rht* gene during the green revolution impacted rhizobacterial diversity and composition. We aim to test this by growing wheat mutants with increasing severity in the *Rht* genes in a low nutrient soil and analysing rhizoplane soil samples by 16S rRNA gene amplicon sequencing. We will also apply this method to a range of wheat hormone signalling mutants in abscisic acid, auxin, brassinosteroids, ethylene, GAs, and strigolactones to test the impact that other phytohormones have on rhizobacterial diversity and composition.

4.2 Materials and methods

4.2.1 Rhizoplane sampling of wheat accessions

A variety of wheat hormone signalling mutants were selected to test the hypothesis that phytohormones influence the selection and composition of the root microbiota (Table 4-1). Wheat mutants with increasing severity in the *Rht* genes (*Rht-D1b*, *Rht-d1f*, and *Rht-B1c*) were selected to test the impact that introduction of semidwarfing genes (*Reduced height-1 (Rht-1)*) into wheat (*Triticum aestivum*) during the Green Revolution had on root microbiome selection (Supplementary Figure C-1) (16). These lines were mutagenized from Cadenza wheat which carry wild-type alleles *Rht-B1* and *Rht-D1* (16). We also included two GA biosynthetic oxidase (ox) mutants (*Ga20x2b*-KO semi-dwarf and *Ga3'ox2*-KO extreme dwarf mutant) (17, 18). An additional ten hormone signalling mutants in: auxin (*IAA1*, *iaa1*, *iaa2*), abscisic acid (ABA 8' hydroxylase mutant), brassinosteroids (*dim*), ethylene (*ETR1a*, *ETR1b*, and *ETR1c*), and strigolactones (*d14*, *spl14*) were grown (Supplementary Figure C-2) (19).

The auxin mutants (*AUX0188*, *AUX1366*, *AUX1420*) are insensitive to auxin by dominant gain-of-function mutations in IAA genes and have been demonstrated to show IAA-insensitive root phenotypes and have reduced lateral root formation. The ABA 8' hydroxylase mutant was generated by a knockout in the three homoeologous genes and is expected to have increased ABA production as these enzymes are involved in ABA inactivation. Ethylene insensitive gain-of-function mutants (*ETH1229*, *ETH1331*, *ETH1445*) block ethylene binding to the ethylene receptor (*ETR1a*, *ETR1b*, and *ETR1c*).

Plants were grown in a glasshouse at Rothamsted Research, Harpenden in 9x9x10 cm pots filled with field soil from Woburn, Stackyard bare-fallow soil mine (LATLONG 52.000293N, -0.614308E), a well-draining sandy loam soil from the Rothamsted Research experimental farm at Woburn, Bedfordshire (UK). The soil is a Cottenham series (20) classified as a Cambic Arenosol (FAO), chosen to reduce the legacy effect of prior cropping systems. Topsoil (0-20 cm) was collected in July 2020 across three replicate plots and thoroughly mixed to produce a homogenized soil. Soil was sieved (2 mm mesh), air dried, and stored

at 4 °C in polythene bags prior to use. Seeds were surface sterilized (70% ethanol, 10 min; 1.5 % active chlorine, 1 h; 5 x rinse, sterile distilled water (SDW); overnight imbibition, sterile water, 4 °C) and pre-germinated in petri dishes for three days at room temperature in the dark. Seedlings were carefully transplanted to pots filled with three parts soil and one part perlite (approx. 500 g) (1x plant per pot) with and without NPK granules (15% N, 9% P₂O₅, 11% K₂O, 2% MgO with micro-nutrients (B, Cu, Fe, Mn, Mo, Zn); Osmocote, UK) (~2.5 g per pot) in a two-factor randomized block design with mutant-id and fertilizer as factors (Table 4-1). As a control (referred to in text and figures as bulk soil), plant-free pots were established and given the same treatments as pots with plants.

4.2.2 Soil collection and processing

All plants were harvested at the start of flowering (Zadoks growth stage 61) (21), within four weeks. Height (from soil surface to head of longest stem) was measured, then soil was gently tipped from the pot onto a fresh polythene bag. Loose soil was discarded and non-rhizospheric soil carefully removed. Roots were vigorously shaken, the root system was excised and placed in a sterile 50 ml centrifuge tube for subsequent rhizoplane work. The remainder of the plant was dried (80 °C, 24 h) and dry foliar plant biomass measured. To obtain rhizoplane soil, collected root samples were weighed and 1 ml SDW was added for every 0.1 g root. Samples were shaken vigorously for 10 min using a shaker, 4 ml soil solution was centrifuged (2 min, RT, 15,000 rpm), supernatant discarded, and remaining soil was flash frozen in liquid nitrogen and stored at -80 °C. Graphs and statistical analysis were rendered in GraphPad Prism version 8 for Mac (San Diego, CA: GraphPad Software, Inc). Effect of fertilization and mutant wheat cultivars on plant height, plant biomass (dry weight of aerial plant), and root biomass (weight of excised root) were tested by two-way ANOVA with pair-wise t-tests with either Dunnett or Šidák corrections depending on recommendation. Pair-wise tests were performed within fertilization groups, whereby all mutants were compared to wt-cadenza.

4.2.3 Soil DNA Extraction and Quantitation

For each sample, total soil DNA was extracted from approximately 0.25 g homogenized soil (Qiagen DNeasy PowerSoil DNA isolation kit (Venlo, Netherlands)), according to the manufacturer's instructions using the MP Biomedicals FastPrep-24 machine twice (30 s, 5.5 m s⁻¹). DNA purity and concentrations were established by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, United States), and a Qubit 2.0 Fluorimeter using the dsDNA HS assay kit (Thermo Scientific), respectively.

4.2.4 Illumina bacterial 16S rRNA gene sequencing

Bacterial 16S rRNA gene amplicons were subjected to Illumina® sequencing using the MiSeq platform. Amplicons (~460 bp) spanning the V3-V4 hypervariable region of the 16S rRNA gene were produced using primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (22). Rhizoplane DNA was sent to Novogene UK (Cambridge, UK) for 2 x 300 bp paired-end sequencing on a MiSeq instrument.

4.2.5 Sequence processing

Demultiplexing of raw sequences was performed by CASAVA data analysis software (Illumina). Forward and reverse reads were merged using the vsearch merge_pairs function (23) which produced 6,722,536 reads from 298 samples (median 16,092 reads per samples). Paired-end reads were then filtered, de-replicated, and denoised using the DADA2 1.2 (24) pipeline with Quantitative Insights into Microbial Ecology (QIIME2) (version 2018.11.0) default parameters (25) which resulted in 5,320,078 high-quality sequences, retaining 79% of initial reads. Low quality samples with less than 100 reads were removed (n=17). The resulting amplicon sequences variant (ASV) table retained high quality nonchimeric reads and was used to build a phylogenetic tree using the align-to-tree-mafft-fasttree command in QIIME2. Taxonomy was assigned using the SILVA132 database (26, 27). ASVs that were not classified as bacteria or were classified as mitochondria or chloroplasts were removed.

4.2.6 Microbial community analysis

Microbial diversity and community composition were analyzed at the ASV level in R 3.6.1 using Phyloseq (v1.34.0) (available at <https://joey711.github.io/phyloseq/>) (28) and Microbiome (v1.12.0) (available at <http://microbiome.github.com/microbiome>) packages. Sequencing of the bacterial 16S rRNA amplicon library resulted in 9 313 ASVs in 118 samples. ASVs unclassified at kingdom level, unclassified bacterial ASVs and uncultured bacterial ASVs (2,185) were removed from the dataset.

Bacterial α -diversity indices pertaining to richness (chao1), and diversity (Shannon) were calculated based on rarefied ASV tables (22,042 sequences). The α -diversity rarefaction plots confirmed that a sufficient depth of coverage was achieved at this cut-off (Supplementary Figure C-3). Box plot figures were created using the “ggboxplot” function in the “ggpubr” package. Two-way type III analysis of variance (ANOVA) was performed in GraphPad Prism version 8 for Mac (San Diego, CA: GraphPad Software, Inc) with fertilizer and hormone mutant level as factors, to determine the dominant factor contributing to variation in means for alpha diversity data. *Post hoc* multiple comparison tests with Šidák correction were performed to test for significance within groups. Normality was confirmed by quantile-quantile normality plots and the Shapiro-Wilk test. Homogeneity of variances was confirmed by residuals versus fits plots and Spearman’s test for heteroscedasticity.

For beta diversity analysis, amplicon sequencing data were normalized using DESeq2 to account for differences in sequencing bias (29). The resulting normalized ASV table (number of reads belonging to each ASV per sample) was used for Principal Coordinates Analysis (PCoA) and Canonical analysis of principal coordinates (CAP) which was employed on Bray-Curtis dissimilarity distance matrices using the ‘ordinate’ function in the Phyloseq package. Permutational multivariate analysis of variance (PERMANOVA) was used to detect significant changes in the microbiota structure using pseudo-F value as a proxy for the strength of an individual factor (fertilization, hormone mutant) on the

bacterial community composition. PERMANOVA tests were performed using 'adonis2' with 9999 permutations and block factor.

To identify ASVs preferentially associated with each hormone mutant compared to Cadenza, the differential relative abundances (fold changes) of ASVs between the different groups were determined. Low abundance ASVs with less than 3 counts in <20% of the samples were removed. This was performed individually for each data set with the DeSeq2 package, using the Wald significance test and the Benjamini-Hochberg p value correction. Phylum level community composition was investigated by relative abundance of normalized data, after removal of bacterial ASVs only classified to Kingdom level and ASVs assigned as 'environmental samples' for visualization purposes.

Mutant-id	Hormone	Replicates-pot	Replicates-pot+NPK
<i>Rht-D1b</i>	Gibberellin	4	4
<i>Rht-D1f</i>	Gibberellin	4	4
<i>Rht-B1c</i>	Gibberellin	4	4
<i>Ga20x2b-KO</i>	Gibberellin	4	4
<i>Ga3'ox2-KO</i>	Gibberellin	4	4
<i>d14</i>	Strigolactone	4	4
<i>spl14</i>	Strigolactone	4	4
<i>dim/dwf1</i>	Brassinosteroid	4	4
AUX0188	Auxin	4	4
AUX1366	Auxin	4	4
AUX1445	Auxin	4	4
ETH1229	Ethylene	4	4
ETH1331	Ethylene	4	4
ETH1445	Ethylene	4	4
<i>ABA80H1-KO</i>	Abscisic acid	4	4
wt-cadenza	Wild type	4	4
Bulk soil	Unplanted control	4	4

Table 4-1: Wheat mutants used in this study.

4.3 Results

4.3.1 Effect of the *Reduced height (Rht)-D1b*, *Rht-D1f*, and *Rht-B1c* semidwarfing genes on plant height

Rht-1 semi-dominant dwarfing mutations cause varying degrees of GA-insensitive dwarfism: *Rht-D1b* and *Rht-D1f* alleles produce only about a 20 % reduction in plant height, while *Rht-B1c* plants are about half the size of the appropriate tall controls (16). Wild-type (*wt*) Cadenza plants had a mean height of 48.5 ± 3.3 cm and 50.5 ± 7.0 cm in non-fertilized and fertilized plants respectively, compared to *Rht-D1b*, *Rht-D1f*, and *Rht-B1c* which had a mean height of 42.8 ± 1.9 , 33.4 ± 1.0 , and 16.6 ± 1.9 cm in non-fertilized wheat plants respectively and 32.2 ± 5.3 , 28.0 ± 2.7 , 11.7 ± 6.4 cm in fertilized wheat plants respectively. The effect of mutations in the *Rht-D1* and *Rht-B1* alleles caused 89% of the variation in plant height between *wt* Cadenza and mutant wheat (two-way ANOVA, $p < 0.0001$) (Figure 4-1).

4.3.2 Effect of the *Reduced height (Rht)-D1b*, *Rht-D1f*, and *Rht-B1c* semidwarfing genes on plant and root biomass

As expected, fertilization significantly increased plant aerial biomass (fertilized plants=1.049g and non-fertilized plants=0.5585g) (two-way ANOVA: $F=25.51$, $p < 0.0001$) but post-hoc multiple comparison tests with Šidák correction showed only Cadenza wheat plants had a significantly higher plant aerial biomass compared to non-fertilized Cadenza (1.097g compared to 0.5375g in fertilized and non-fertilized wheat, respectively) ($p < 0.05$) (A in Figure 4-2). The opposite general trend was observed for root biomass with biomass being lower in fertilized wheat (non-fertilized plants=5.582g and fertilized plants=2.916g) (two-way ANOVA: $F=10.10$, $p < 0.01$) (B in Figure 4-2). Statistical differences in mean root biomass were only seen between *Rht-D1f* plants as shown with post-hoc multiple comparison tests with Šidák correction ($p < 0.05$) (B in Figure 4-2).

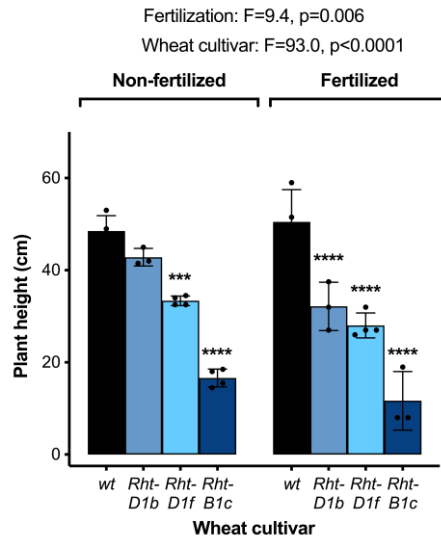


Figure 4-1: Average height (cm) of wild type (wt)-cadenza and *Rht* (*Reduced height*) mutant wheat (*Rht-D1b*, *Rht-D1f*, *Rht-B1c*) plants grown in pots under greenhouse conditions.

Plant height was measured from soil surface to head of longest stem. Two-way ANOVA F and p values are provided, and the results of pair-wise t -tests with Dunnett correction are presented as '****' and '*****' for $p<0.01$ and $p<0.0001$, respectively. Pair-wise tests were performed within fertilization groups, whereby all mutants were compared to wt-cadenza.

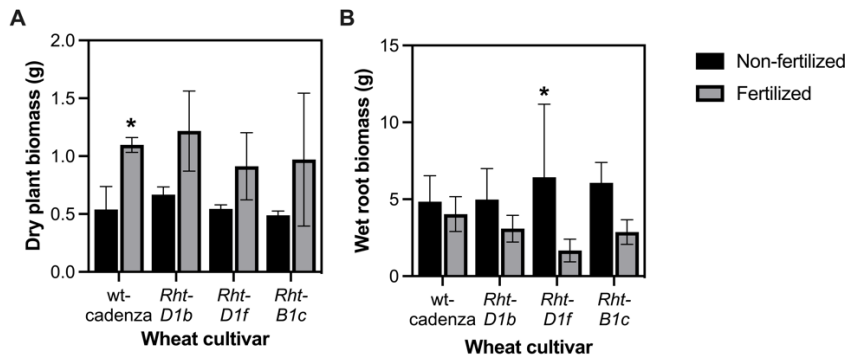


Figure 4-2: Average dry plant aerial biomass (g) (A) and wet root biomass (g) of wild type (wt)-cadenza and *Rht* (*Reduced height*) mutant wheat (*Rht-D1b*, *Rht-D1f*, *Rht-B1c*) plants grown in pots under greenhouse conditions.

Results of pair-wise t -tests with Šidák correction are presented as '*' for $p<0.05$. Pair-wise tests were performed within wheat groups, whereby non-fertilized plants were compared to fertilized plants.

4.3.3 Effect of the *Reduced height (Rht)-D1b*, *Rht-D1f*, and *Rht-B1c* semidwarfing genes on rhizobacterial diversity

Bacterial communities from the wheat rhizoplane were mainly represented by Proteobacteria (38.9-77.3%), Actinobacteria (14.5-46.0%), and Bacteroidetes (3.8-13.4%) compared to bulk soil which was mainly represented by Proteobacteria (41.9-47.7%), Actinobacteria (28.0-36.7%), and Acidobacteria (9.7-11.3%) (Figure 4-3). The PCoA plot shows bacterial communities from fertilized cultivars differ from non-fertilized cultivars (PERMANOVA, $F=2.1790$, $p=0.0001$), with the first PCoA axis corresponding to 14.8% of the variation. The effect of the hormone mutants was not considered significant (PERMANOVA, $F=1.0987$, $p=0.0887$), nor the interaction between the hormone mutants and fertilization ($F=1.0661$, $p=0.1431$) (A in Figure 4-4). Identification of the relative influence of individual factors was further investigated by CAP analysis using fertilization and hormone mutant as factors (ANOVA, $F=1.2391$, $p=0.021$) (B in Figure 4-4).

In general, the effect of the *Rht* semi-dwarfing genes on rhizobacterial diversity were more pronounced in fertilized wheat plants compared to non-fertilized plants. In fertilized plants, wheat mutants whereby the effects of the *Rht* semi-dwarfing genes were stronger (*Rht-d1f* and *Rht-B1c*) had a higher bacterial richness and diversity (based on chao1 and Shannon index) (Figure 4-5). When comparing richness and diversity indices between non-fertilized and fertilized wheats, only wt-cadenza showed statistical differences, with richness and diversity being significantly reduced in fertilized plants.

From a total of 189 significantly differentially abundant ASVs detected with DESeq2, 82.9% of ASVs belonging to the phylum Proteobacteria were significantly more abundant in the rhizoplane of wt-cadenza wheat, as opposed to 48.3% in the *Rht* mutant wheat, but only in fertilized plants (Figure 4-6). In non-fertilized plants, there was little difference between the abundance of Proteobacteria (59.3% in wt-cadenza and 53.1% in *Rht* mutant wheat) (Figure 4-6). ASVs differentially abundant and assigned to Bacteroidetes (4.27% and 6.15%), and Firmicutes (0.34% and 0.10%) were only observed in wt-cadenza

wheat (order of values in non-fertilized and fertilized wheat, respectively) and Planctomycetes (0.05%), and Verrucomicrobia (0.14%) were only observed in non-fertilized wt-cadenza wheat (Figure 4-6). On the other hand, *Rht* mutant wheats showed a larger proportion of ASVs belonging to the phyla Acidobacteria (17.5% and 15.6%), Chloroflexi (1.88% and 2.26%), and Gemmatimonadetes (6.90% and 5.87%) (order of values in non-fertilized and fertilized wheat, respectively) (Figure 4-6). A closer analysis at genus level indicates that wt-cadenza is enriched with a higher proportion of ASVs from the phylum Proteobacteria (wt-cadenza=5.8% and *Rht* mutants=1.1%) and genera from the Bacteroidetes were only observed in wt-cadenza (1.6%) compared to *Rht* mutants which were differentially more abundant in genera from the Actinobacteria (wt-cadenza=2.6% and *Rht* mutants=4.2%) (Table 4-2).

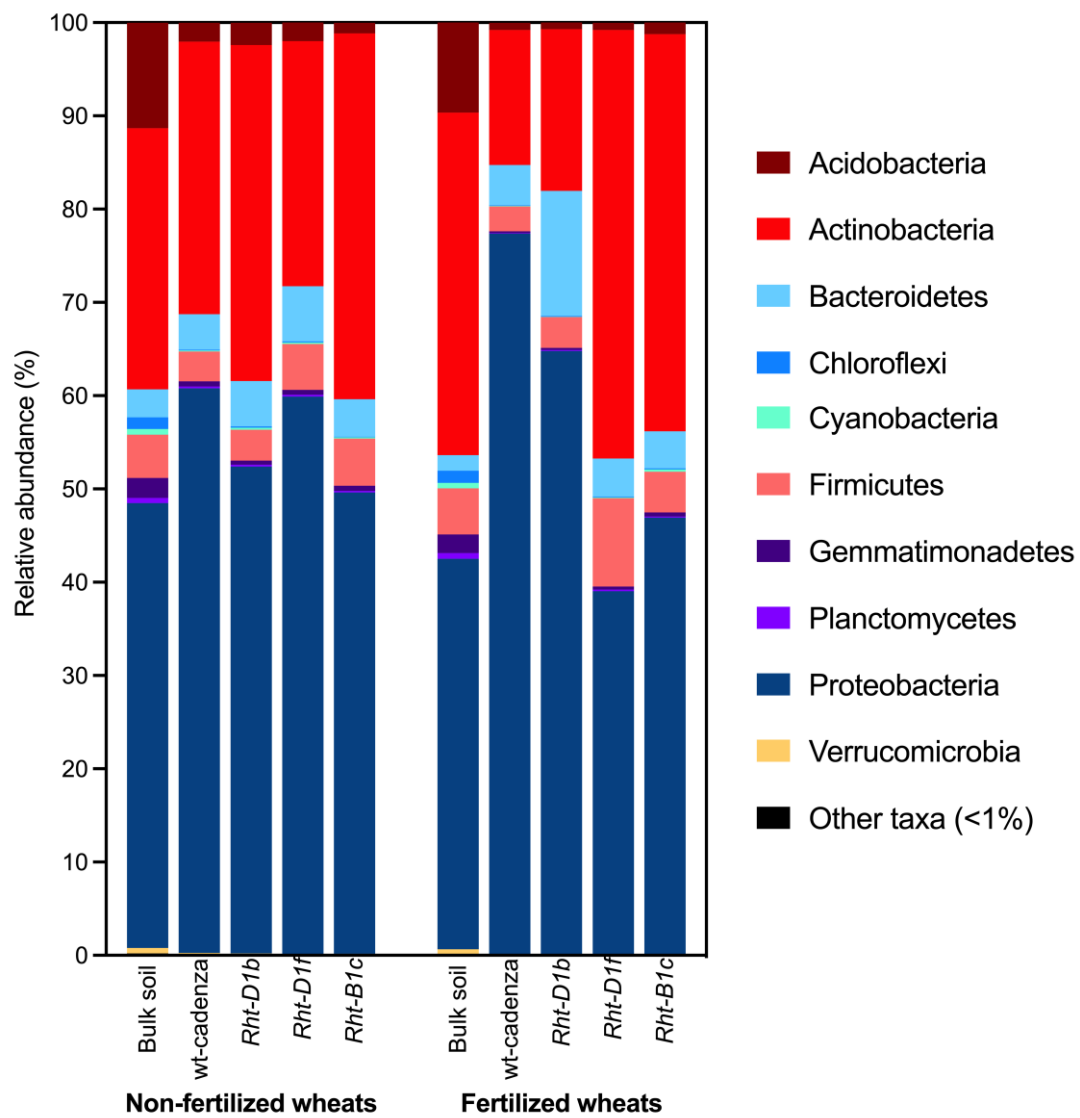


Figure 4-3: Relative abundance (%) (>1%) of 16S rRNA gene derived bacterial sequences of bulk soil and rhizoplane samples of wt-cadenza and *Rht* (*Reduced height*) gene mutants (*Rht-D1b*, *Rht-D1f*, *Rht-B1c*) classified at phylum level.

Other taxa include Chlamydiae, Deinococcus-Thermus, Nitrospirae, and Spirochaetia.

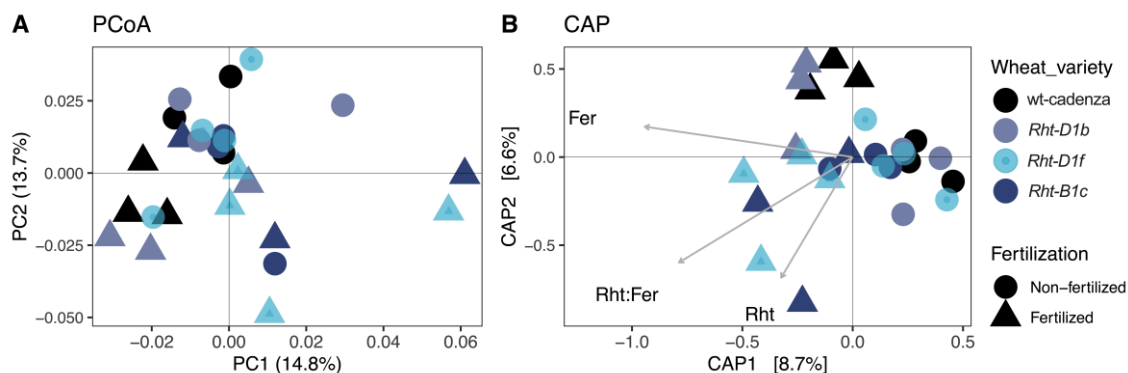


Figure 4-4: Principal coordinates of analysis (PCoA) (A) and canonical analysis of principal coordinates (CAP) (B) plots created using Bray-Curtis dissimilarity distance matrix grouped by fertilization and wheat variety.

Factors (fertilization (Fer) and wheat variety (Rht)) shaping the rhizobacterial community composition and their interaction (Fer:Rht) are represented by arrows. The length of the arrow represents the strength each factor has on variation in the microbial community.

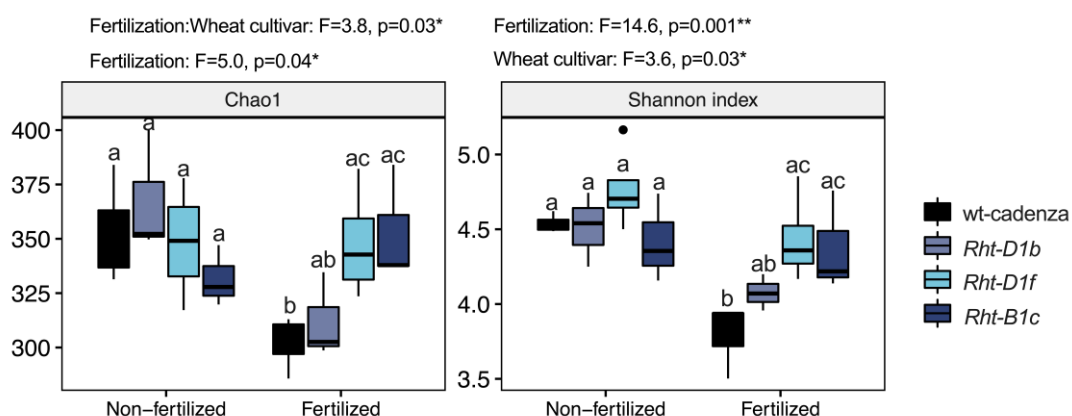


Figure 4-5: Alpha diversity metrics of 16S rRNA gene data of wheat rhizoplane obtained from wild type (wt)-cadenza and *Rht* (Reduced height) mutant (*Rht-D1b*, *Rht-D1f*, *Rht-B1c*) wheats.

Statistically significant differences were determined by two-way ANOVA; F and p values are provided, p values are presented as ‘*’ and ‘**’ for $p < 0.05$ and $p < 0.01$, respectively. and different letters denote statistically significant differences according to pair-wise t-tests with Dunnett correction ($p < 0.05$). Pair-wise tests were performed within fertilization groups, whereby all mutants were compared to wt-cadenza and between non-fertilized and fertilized plants of the same cultivar.

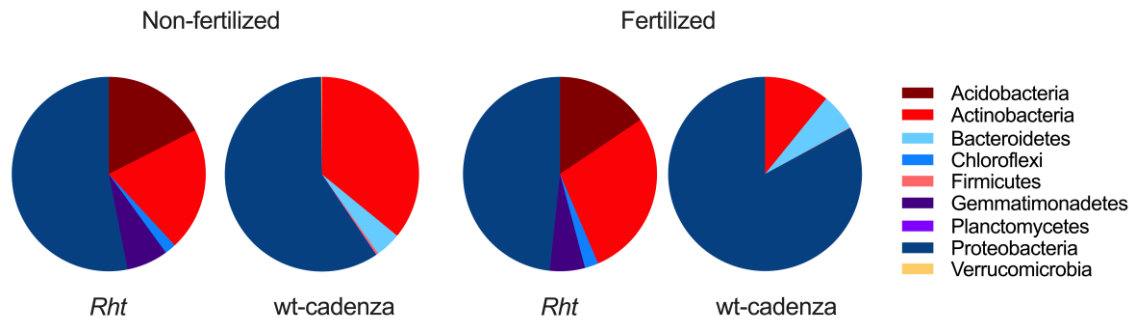


Figure 4-6: Pie charts showing differentially abundant bacterial ASVs grouped at phylum level, from a total of 189 detected among *Rht* (*Reduced height*) mutant and *wt-cadenza* wheats grown in soils with and without fertilizer addition.

Wheat variety	Phylum	Class	Genus
<i>wt-cadenza</i>	Actinobacteria	Actinobacteria	<i>Leifsonia</i> , <i>Microbacterium</i> , <i>Rhodococcus</i> , <i>Streptomyces</i>
	Bacteroidetes	Chitinophagia	<i>Chitinophaga</i>
		Sphingobacteriia	<i>Pedobacter</i>
	Proteobacteria	Alphaproteobacteria	<i>Mesorhizobium</i> , <i>Rhizobium</i>
		Betaproteobacteria	<i>Achromobacter</i> , <i>Massilia</i> , <i>Paraburkholderia</i> <i>Variovorax</i>
		Gammaproteobacteria	<i>Pseudomonas</i> , <i>Stenotrophomonas</i>
<i>Rht</i> cultivars	Actinobacteria	Actinobacteria	<i>Catenulispora</i> , <i>Kutzneria</i> , <i>Nocardiodes</i> , <i>Streptacidiphilus</i> , <i>Streptomyces</i>
	Proteobacteria	Alphaproteobacteria	<i>Bradyrhizobium</i>

Table 4-2: Genera assigned to ASVs which were found to be enriched in the rhizoplane of wild type (*wt*)-*cadenza* and *Rht* (*Reduced height*) mutant wheats.

4.3.4 Effect of mutations in wheat hormone signalling genes on plant height, plant biomass, and root biomass

Mean plant height of wheat hormone signalling mutants in abscisic acid, auxin, brassinosteroids, ethylene, GA, and strigolactone were not statistically different compared to wt-cadenza (one-way ANOVA, $p > 0.05$) (Figure 4-7). Fertilization significantly increased plant aerial biomass (fertilized plants=0.9886 and non-fertilized plants=0.5268g) (two-way ANOVA: $F=27.76$, $p < 0.0001$) and post-hoc multiple comparison tests with Šidák correction showed statistical differences in mean biomass between non-fertilized and fertilized wt-cadenza and mutant wheats in auxin, and strigolactone ($p < 0.05$) (A in Figure 4-8). The opposite general trend was observed for root biomass with biomass being lower in fertilized wheat (non-fertilized plants=4.017g and fertilized plants=2.579g) (two-way ANOVA: $F=11.53$, $p < 0.01$) (B in Figure 4-8). Statistical differences in mean root biomass were seen between ethylene and strigolactone wheat mutants as shown with post-hoc multiple comparison tests with Šidák correction ($p < 0.05$) (B in Figure 4-8).

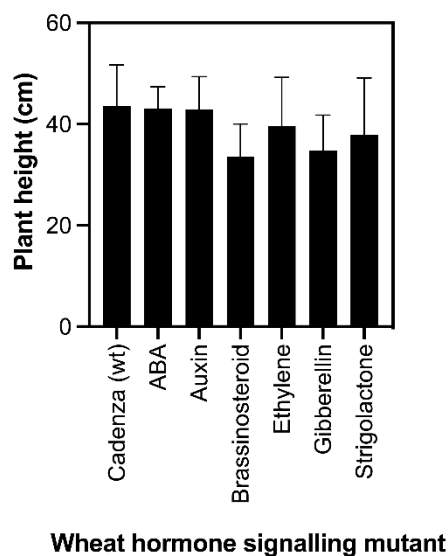


Figure 4-7: Average height (cm) of wild type (wt)-cadenza and wheat hormone signalling mutants in abscisic acid (ABA), auxin, brassinosteroid, ethylene, gibberellin, and strigolactone plants grown in pots under greenhouse conditions.

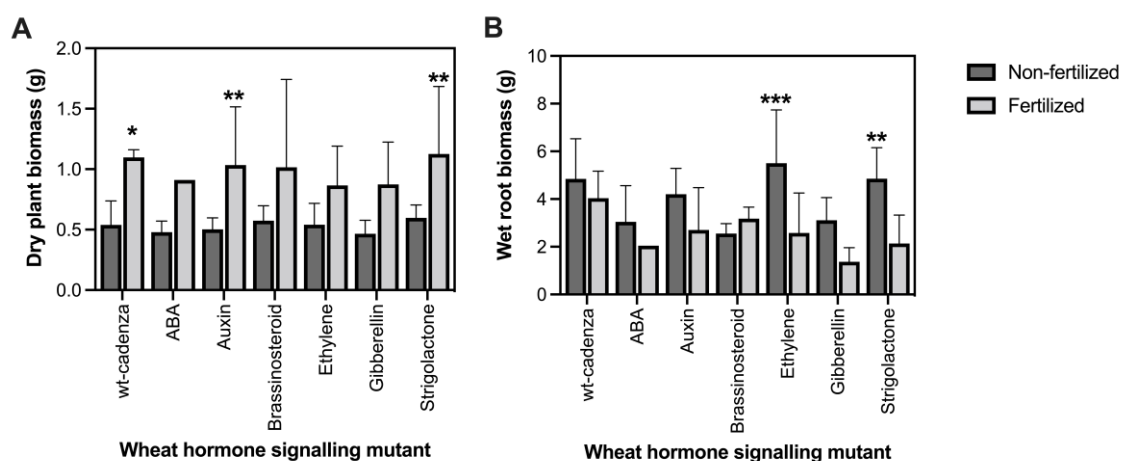


Figure 4-8: Average dry plant aerial biomass (g) (A) and wet root biomass (g) (B) of wild type (wt)-cadenza and wheat hormone signalling mutants in abscisic acid (ABA), auxin, brassinosteroid, ethylene, gibberellin, and strigolactone plants grown in pots under greenhouse conditions.

Results of pair-wise t-tests with Šidák correction are presented as ‘*’, ‘**’, and ‘***’ for $p < 0.05$, $p < 0.01$, and $p < 0.001$. Pair-wise tests were performed within wheat groups, whereby non-fertilized plants were compared to fertilized plants.

4.3.5 Effect of mutations in wheat hormone signalling genes on rhizobacterial diversity and composition

Bacterial communities from the wheat rhizoplane were mainly represented by Proteobacteria (49.5-68.9%), Actinobacteria (19.9-34.7%), and Bacteroidetes (4.1-9.7%) compared to bulk soil which was mainly represented by Proteobacteria (44.8%), Actinobacteria (32.4%), and Acidobacteria (10.5%) (Figure 4-9).

Rhizobacterial alpha diversity was significantly altered in auxin, brassinosteroid, and strigolactones wheat mutants (Figure 4-10). In general, the effect was more pronounced in fertilized plants: AUX1420 (Mean Shannon diversity index: 4.539), *dim/dw1* (4.543), and *dl4-KO* (4.489) mutant wheat plants had a significantly higher rhizobacterial diversity compared to wt-cadenza (3.794) (two-way ANOVA, $p < 0.05$) (Figure 4-10). There were no significant differences in rhizobacterial alpha diversity between wt-cadenza and wheat hormone signalling mutants in abscisic acid, ethylene, and GAs (two-way ANOVA, $p > 0.05$) (Figure 4-10). When comparing diversity indices between non-fertilized and fertilized wheats, only wt-cadenza showed statistical differences, with diversity being significantly reduced in fertilized plants.

The PCoA plots show bacterial communities from fertilized wheats differ from non-fertilized wheats for all hormone mutants (PERMANOVA, $p < 0.05$) (Table 4-3) (Supplementary Figure C-4). The effect of wheat variety was not considered significant between wt-cadenza and wheat hormone signalling mutants for all hormones (PERMANOVA, $p > 0.05$), and neither was the interaction, except for between fertilization and auxin mutants (Table 4-3). Identification of the relative influence of individual factors was further investigated by CAP analysis using fertilization and hormone mutants as factors (Figure 4-11).

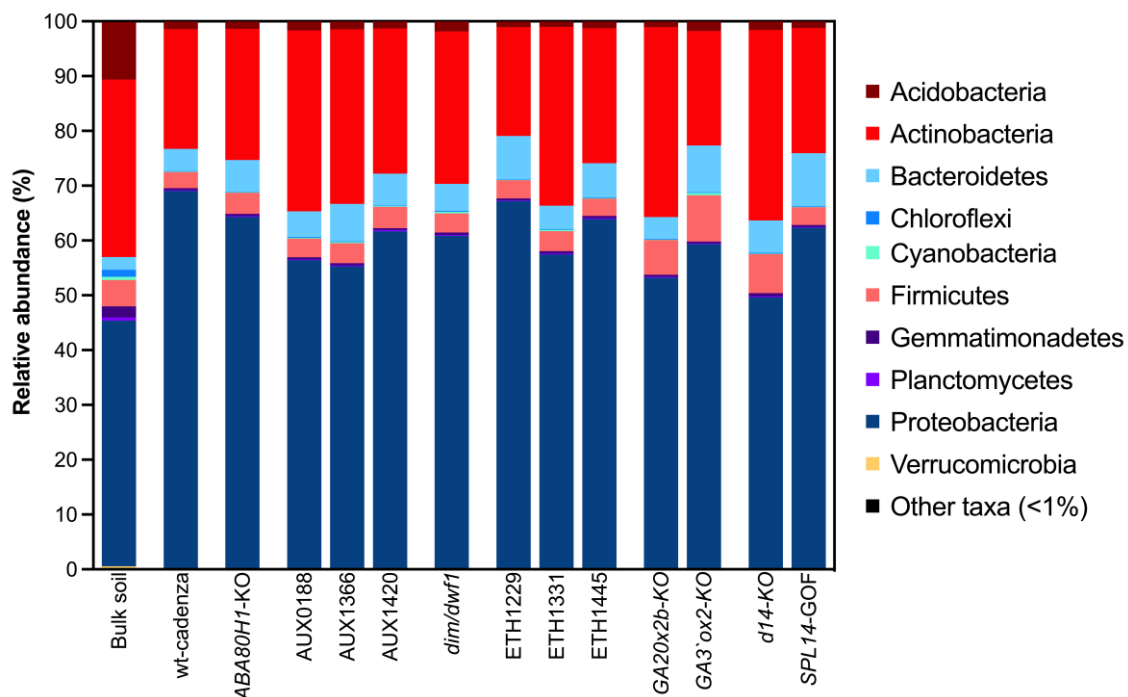


Figure 4-9: Relative abundance (%) (>1%) of 16S rRNA gene derived bacterial sequences of rhizoplane from bulk soil, wt-cadenza, and wheat hormone signalling mutants in abscisic acid (*ABA80H1-KO*), auxin (*AUX0188*, *AUX1366*, *AUX1420*), brassinosteroids (*dim/dwf1*), ethylene (*ETH1229*, *ETH1331*, *ETH1445*), gibberellin (*GA20x2b-KO*, *GA3'ox2-KO*), and strigolactones (*d14-KO*, *SPL14-GOF*) classified at phylum level.

Other taxa include Chlamydiae, Deinococcus-Thermus, Nitrospirae, and Spirochaetia.

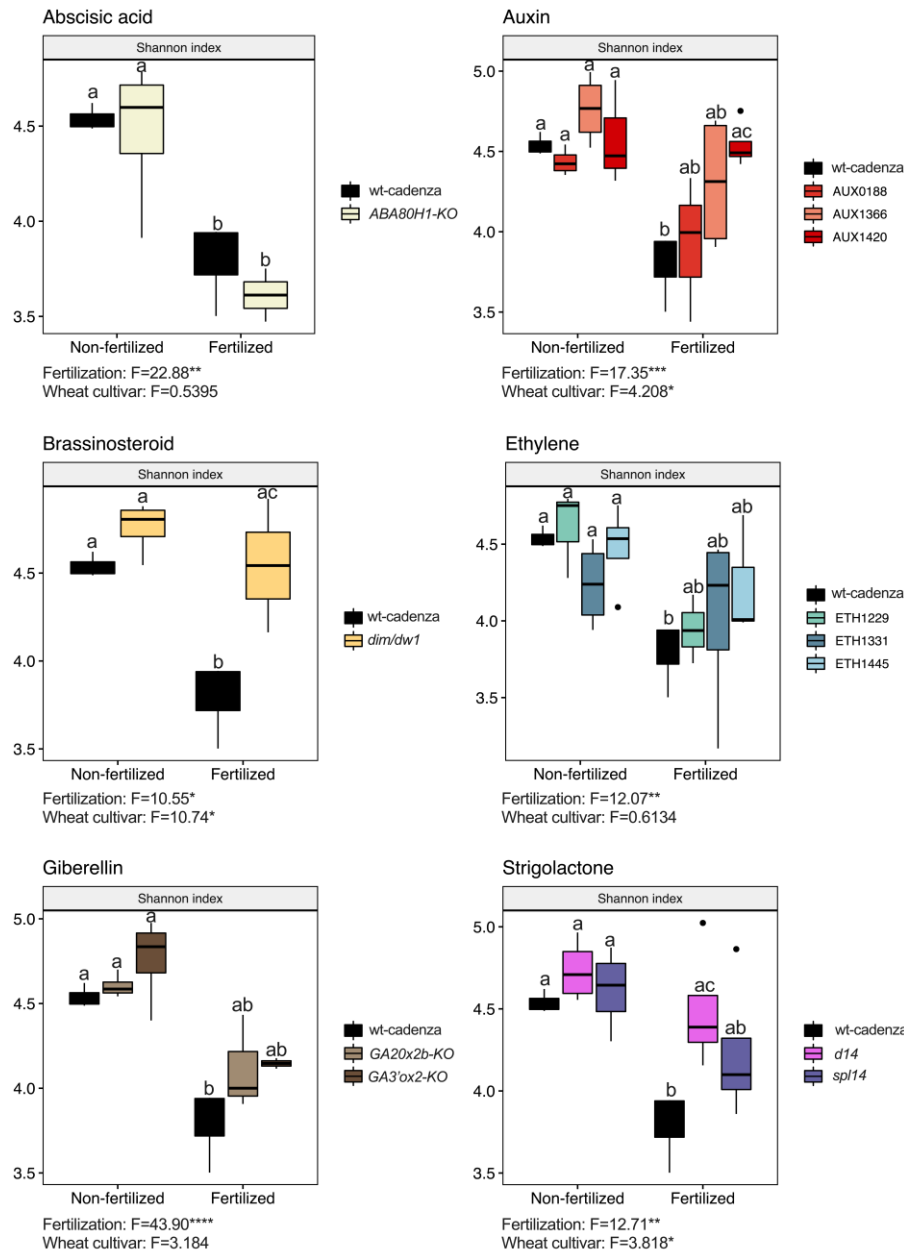


Figure 4-10: Shannon diversity metrics of 16S rRNA gene data of wheat rhizoplane obtained from wild type (wt)-cadenza and wheat hormone signalling mutants in auxin, abscisic acid, brassinosteroids, ethylene, gibberellin, and strigolactones.

Statistically significant differences were determined by two-way ANOVA; F values are provided, and p values are presented as ‘*’, ‘**’, ‘***’ and ‘****’ for $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively. Letters denote statistically significant differences according to pair-wise t-tests with Dunnett correction ($p<0.05$). Pair-wise tests were performed within fertilization groups, whereby all mutants were compared to wt-cadenza and between non-fertilized and fertilized plants of the same cultivar.

Hormone	Fertilization	Hormone	Interaction
Absciscic acid	F = 2.8994**	F = 0.8242	F = 1.2789
Auxin	F = 2.2543*	F = 1.4720	F = 1.5372*
Brassinosteroid	F = 3.0783*	F = 1.5651	F = 1.1901
Ethylene	F = 5.6856***	F = 0.7708	F = 0.5045
Gibberellin	F = 2.9673***	F = 1.2650	F = 0.7943
Strigolactone	F = 3.1542**	F = 1.4327	F = 0.7053

Table 4-3: PERMANOVA results table to assess the importance of fertilizer (no fertilizer vs. fertilizer) and hormone (wt-cadenza vs abscisic acid, auxin, brassinosteroids, ethylene, gibberellin or strigolactones) on rhizobacterial beta-diversity as represented by Bray-Curtis metric. This is a two-way PERMANOVA analysis with 9999 permutations.

p values are presented as '*', '**', and '***' for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

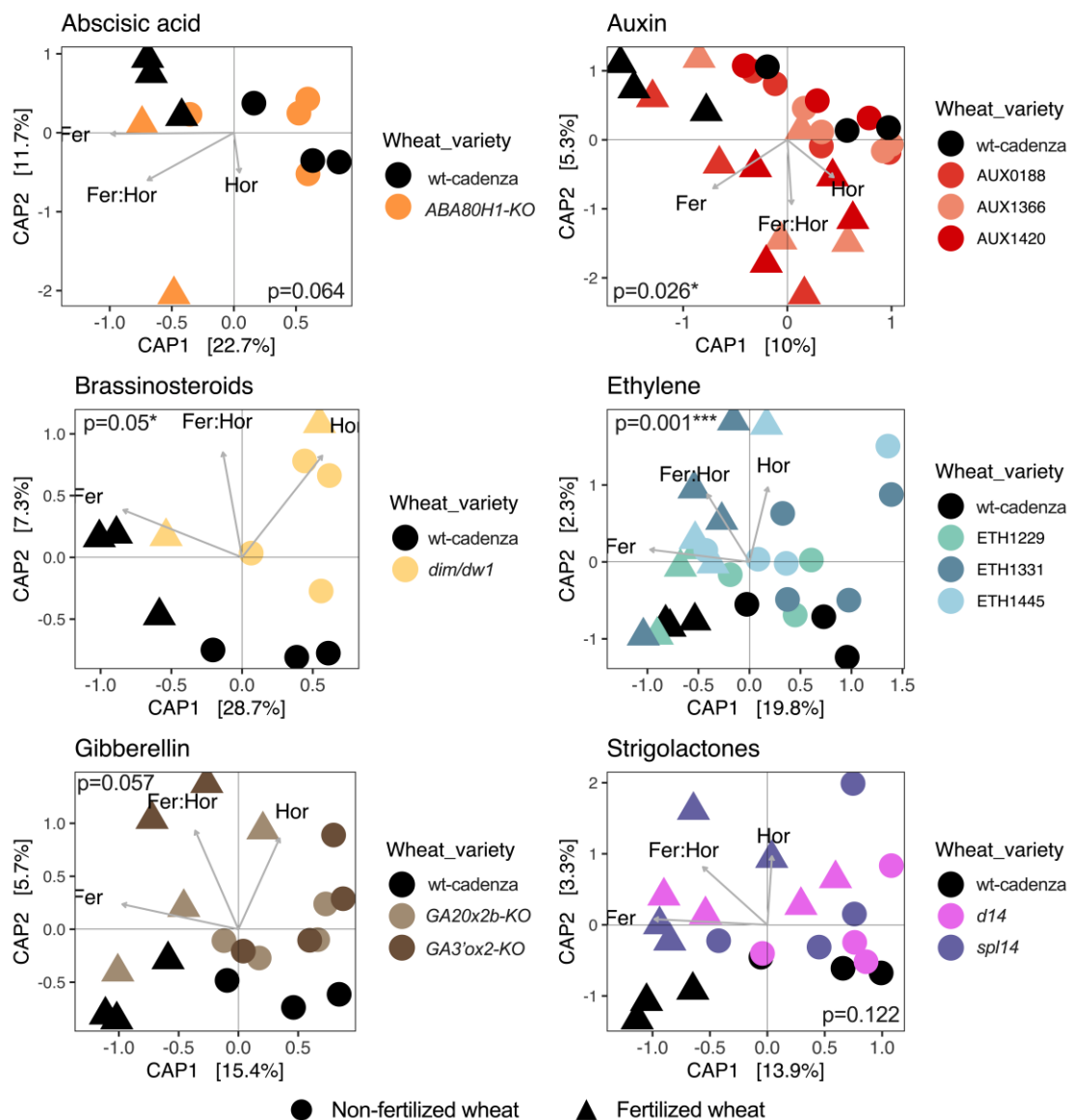


Figure 4-11: Canonical analysis of principal coordinates (CAP) plots created using Bray-Curtis dissimilarity distance matrix grouped by fertilization and hormone mutants.

Factors (fertilization (Fer) and hormone mutants (Hor)) shaping the rhizobacterial community composition and their interaction (Fer:Hor) are represented by arrows. The length of the arrow represents the strength each factor has on variation in the microbial community. ANOVA p values are presented as ‘*’, ‘**’, and ‘***’ for p < 0.05, p < 0.01 and p < 0.001, respectively.

Hormone	ASV	Phylum	Class	Order	Genus
wt-cadenza	ASV_8559	Proteobacteria	Betaproteobacteria	Burkholderiales	NA
	ASV_8170	Proteobacteria	Betaproteobacteria	Burkholderiales	NA
	ASV_8170	Proteobacteria	Betaproteobacteria	Burkholderiales	NA
	ASV_8170	Proteobacteria	Betaproteobacteria	Burkholderiales	NA
	ASV_8559	Proteobacteria	Betaproteobacteria	Burkholderiales	NA
	ASV_8170	Proteobacteria	Betaproteobacteria	Burkholderiales	NA
	ASV_8170	Proteobacteria	Betaproteobacteria	Burkholderiales	NA
ABA	ASV_7303	Acidobacteria	NA	NA	NA
	ASV_7770	Actinobacteria	Actinobacteria	Micrococcales	<i>Curtobacterium</i>
	ASV_1797	Bacteroidetes	NA	NA	NA
	ASV_6636	Bacteroidetes	NA	NA	NA
	ASV_466	Firmicutes	Bacilli	Bacillales	NA
	ASV_957	Proteobacteria	NA	NA	NA
Auxin	ASV_2552	Cyanobacteria	Nostocales	Nostocaceae	<i>Nostoc</i>
	ASV_5155	Proteobacteria	Gammaproteobacteria	Enterobacterales	NA
Brassinosteroids	ASV_7650	Proteobacteria	Alphaproteobacteria	NA	NA
	ASV_6514	Proteobacteria	Alphaproteobacteria	NA	NA
	ASV_957	Proteobacteria	NA	NA	NA
Gibberellin	ASV_3688	Firmicutes	Bacilli	NA	NA
	ASV_466	Firmicutes	Bacilli	Bacillales	NA
Strigolactone	ASV_466	Firmicutes	Bacilli	Bacillales	NA
	ASV_957	Proteobacteria	NA	NA	NA

Table 4-4: Differentially abundant ASVs which were found to be enriched in the rhizoplane of wild type (wt)-cadenza and wheat hormone signalling mutants in auxin, abscisic acid, brassinosteroids, gibberellin, and strigolactones.

4.4 Discussion

In the second half of the 20th century, semi-dwarf wheat varieties started to dominate the agricultural landscape in a period known as the Green Revolution. Underlying this short stature were the *Rht* genes which encode DELLA proteins which confer dwarfism through decreased responsiveness to the phytohormone GA which formed the cornerstone of the Green Revolution (30). We asked how selection for *Rht* genes during the Green Revolution impacted beneficial plant-microbe interactions in wheat. We also aimed to investigate the potential influence that other classes of phytohormones such as ABA, auxin, brassinosteroids, ethylene, GA, and strigolactones, had on the wheat root microbiome as there is such a strong interaction among them (31).

The rhizoplane of *Rht* mutant wheats were enriched in members of the phyla Acidobacteria, which are typically more associated with bulk soil rather than the wheat rhizosphere (32). It has previously been shown that Acidobacteria were also enriched in semi-dwarf wheat cultivars compared to tall wheat cultivars (15). A closer analysis at genus level, indicates that wt-cadenza wheat is enriched with ASVs assigned to genera normally associated with plant growth promotion, especially members of the phylum Proteobacteria, such as *Massilia* (IAA, siderophore and proteolytic enzyme production) (33), *Rhizobium* (nutrient acquisition (34), indole-3-acetic acid (IAA) production (35-37), drought tolerance (38, 39), N₂ fixation (40)), *Pseudomonas* (chitinase and B-glucanases production (41), ACC deaminase synthesis (42, 43), induction of plant stress resistance (44), antibiotic production (45), siderophore production (46), nutrient acquisition (34, 47)), *Paraburkholderia*, *Stenotrophomonas*, and *Variovorax*. Members of the phylum Actinobacteria were enriched in *Rht* wheat mutants such as *Microbacterium* and *Streptomyces*. Although some differentially recruited ASVs within the wt-cadenza rhizoplane have been assigned to genera commonly related to plant growth promotion, these abilities are normally strain-specific, thus caution must be taken when analysing the results. However, given our previous work (34) which shows that similar genera that are differentially more abundant in wt-cadenza are also functionally active, it's likely that the same applies in this study. Further work should evaluate these differences based on culture-

dependent work to identify key plant growth-promoting ASVs. Our results further suggest that selection for dwarf wheat cultivars during the Green Revolution may have inadvertently impacted selection of the plant-microbe wheat holobiont (48).

In the wheat semidwarf mutants, reduced stature is conferred through alterations in growth mediating by the GA phytohormones. The *Rht* genes confer dwarfism through decreased response to produce GA by encoding DELLA proteins which block stem elongation (16); GA biosynthetic mutants *Ga20x2b-KO* and *Ga3'ox2-KO* confer dwarfism by inactivation of key genes (*Ga20ox* and *Ga3ox*) involved in GA biosynthesis, the first step in the hormone signalling pathway (Figure 4-12) (49, 50). This explains the difference in aboveground appearance of *Rht* gene mutant wheats compared to GA biosynthetic mutant wheats (Supplementary Figure C-1 and Supplementary Figure C-2). GA is the main hormone controlling plant growth, but it is also responsible for other plant responses such as flowering and reproductive growth (9). *Rht* genes act by preventing GA-induced transcriptional changes that mediate stem elongation whereas GA biosynthetic mutants inhibit GA synthesis entirely (Figure 4-12). It was interesting that in our study, rhizobacterial diversity and taxonomic composition was impacted in *Rht* wheat mutants, particularly ones with severe plant dwarfism, but not in GA biosynthetic mutants. This suggests that it is the growth-mediated effect conferred by the *Rht* genes directly rather than the plant response to GA. So, why would *Rht*-gene induced dwarfism reduce selection of beneficial microbes but GA-induced dwarfism not? The success of *Rht*-conferred dwarfism in increasing wheat yield and doubling wheat production is immense. Thus, how can future wheat genetic breeding programmes incorporate plant-microbe interactions with sustained crop yields (51, 52)? Yields from current wheat cultivars containing *Rht-B1b* and *Rht-D1b* alleles may be approaching their limits, while a changing climate may reduce the usefulness of these genes in the future. This means genes which produce more extreme dwarf cultivars and therefore higher yields, such as *Rht-B1c*, are likely to be deployed in future wheat genetic breeding programmes. However, our study showed that the impact on rhizobacterial diversity and composition was exacerbated in wheat mutants with more severe dwarfism. Furthermore, the rhizobacterial community was only impacted in wheat

plants treated with fertilizer, which further supports our hypothesis from Chapter 3 that fertilization alters gene expression in wheat which could impact plant-microbe interactions (53), which would also suggest that fertilization and *Rht* expression interact. It would also be interesting to test in future work if cadenza without a mutant *Rht* allele (i.e. a taller cadenza – the wt-cadenza we are using here is still a mutant, it is just that this mutation is compounded with further mutations in the other *Rht* mutants in this study) to see how the microbiome structure shifts with and without fertilizer addition.

This same trend was seen for other hormone mutants, in that mutants significantly different from wt-cadenza only showed this effect with the presence of fertilizer. One possible reason for this could be that the plants grown in fertilized soils grew to be larger and healthier, however plant phenotypical data show plant biomass to only be significantly higher in fertilized auxin and strigolactone mutants compared to non-fertilized plants and root biomass was generally higher in non-fertilized mutants, especially in ethylene and strigolactone mutant plants. Plant height was similar among all hormone mutants (excluding *Rht* mutants). So, this would suggest that it is another factor other than plant health that is contributing to the difference in microbiomes only in the presence of fertilizer.

Additionally, compared to wt-cadenza all the mutants apart from ABA seem to result in reduced plant control of the microbiome under NPK fertilized conditions i.e. when there is no fertilizer there is a high diversity of microbes for both mutants and wt-cadenza, however, when fertilizer is applied there is a reduction in diversity in the wt whereas this reduction does not occur in the mutants (apart from ABA). This trend in increasing rhizobacterial diversity among fertilized plants was particularly evident in auxin, brassinosteroid, and strigolactone mutants, although similar trends were seen in ethylene and GA mutants. This could show that the hormonal signalling pathways important for structuring plant-microbe interactions are affected by fertilizer. Also, the increase in species richness under fertilized conditions in the mutant plants could be akin to a diseased root system. A similar trend was seen in Mauchline et al., with take-all, in that diseased plants

had a higher diversity of saprophytes than healthy ones and that this was driven by wheat cultivar in the first year of the crop (54).

In general, all hormone mutants seemed to have some impact on the microbiome structure. In terms of rhizobacterial alpha diversity, the most severe effects were seen in auxin, brassinosteroid, and strigolactone mutants, whereas beta diversity effects were only seen in auxin, brassinosteroid and ethylene mutants. In comparison, differential abundance analysis identified the most ASVs to be differentially abundant in ABA mutants. This is not surprising as complex interactions between hormone signalling pathways have been posited as key for dealing with and fine-tuning responses to combined biotic and abiotic stresses whilst also limiting growth trade-offs. So, it would stand that disruption in one pathway would affect some traits but not others. To comprehend hormone function in shaping microbiomes requires looking back to the origin and evolution of plant–microbe interactions (55). Plants started to colonise land at the mid-late Ordovician period (470–443 million years ago). Terrestrial colonisation involved contending with a range of stresses and nutrient acquisition without a root system. Regulatory networks of hormones – specifically auxin, ABA, cytokinins, GA, jasmonic acid (JA), salicylic acid (SA) and strigolactones – are conserved across embryophyte lineages, whose common ancestors were some of the earliest colonisers of land (56). ABA is canonically involved in abiotic stress tolerance and seed dormancy, the antagonistic relationship with GA breaking dormancy in favourable conditions (57). Auxins, such as IAA, are implicated in plant growth and development but more recently their involvement in stress tolerance has become apparent (58). ABA also supports plant–AMF interactions (59-61) and shows a positive correlation with the abundance of strigolactones. ABA and IAA can be used as the sole carbon source by some rhizobacteria, for example, a *Rhodococcus* sp. and a *Novoshingobium* sp. can use ABA (62), while *P. putida* strain 1290 can metabolize IAA (63). Secretion of ABA or IAA into the rhizosphere could be a mechanism for plants to select microbes capable of using these as a carbon source. SA, JA and ethylene are best studied for their function in plant–microbe interactions (64) however, plant hormones such as cytokinins, GA and brassinosteroids are underrepresented in microbiome research. GA and

brassinosteroids are involved in nodulation and AMF formation (65). While GA has a rather suppressive effect on arbuscule formation, brassinosteroids seem to support it (66, 67). The impact of these two hormones on nodulating rhizobia is far less clear – with promotion or inhibition being dependent on concentration and/or species – but it has been speculated that cross-talk with ethylene is, in part, responsible. In conclusion, the importance of these hormones in interactions with both beneficial and pathogenic microbes is clear, future studies using NGS techniques (e.g. metatranscriptomics with microbiome profiling and plant RNA sequencing) should help to assess their impact on the wider microbiome (55).

The correlation that the rhizoplane of severe dwarf plants with decreased GA sensitivity is associated with an increased colonisation of bacteria normally associated with bulk soil and a decrease of microbial phyla associated with plant growth promotion, suggests that GA sensitivity influences the composition of the root microbiome. However, little is known about how plant-produced GA controls selection of beneficial rhizobacteria. It has been shown that GA has a rather suppressive effect on arbuscule formation through DELLA protein induced inhibition (65). It is known that some microbes, such as plant growth-promoting members of the Rhizobiaceae family (68) as well as some *Pseudomonas* spp. (69) produce this hormone, and we find these bacteria to be differentially more abundant in the rhizoplane of wt-cadenza wheat cultivars. It could follow that plant GA insensitivity leads to a reduction in plant-microbe communication in the root environment and could reduce selection of GA-producing microbes in the root zone. However, as well as there being little known about GA-mediated plant-microbe interactions there is also little known about GA synthesis in rhizobacteria (55, 69). Given the importance of this hormone in increasing wheat yield as well as mediating interactions with beneficial microbes, future studies using culture - independent and -dependent techniques should help to assess their impact on the wider microbiome.

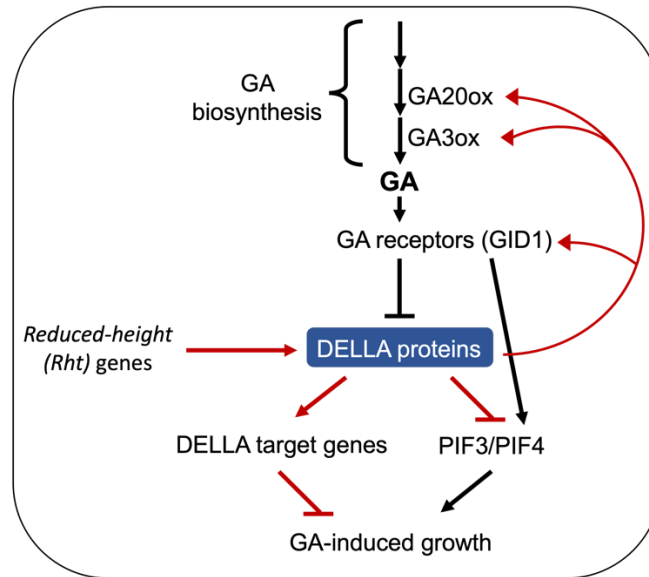


Figure 4-12: Mechanism of *Reduced height (Rht)* gene induced dwarfism

Rht gene-induced pathway: DELLA proteins block activation of Gibberellin (GA)-induced plant growth by activation of its downstream target genes (presumably encoding GA signaling repressors) and inhibition of PIF3 and PIF4 functions by direct binding to these proteins. DELLA also directly feedback induces transcript levels of GA20ox2, GA3ox1 and GID1, thus reducing the amount of GA produced. GA-induced pathway: elevated levels of active GAs lead to DELLA degradation via GID1, and PIF3 and PIF4 activate their target genes to promote hypocotyl growth. Red lines indicate *Rht* gene-induced pathways and black lines indicate GA-induced pathways. Figure adapted from Sun, 2008 (49).

4.5 Conclusions

The interaction of plants with complex microbial communities is the result of co-evolution over millions of years and contributed to plant transition and adaptation to land. Wheat is one of the most important crops in the world; however, loss of genetic variability, abiotic stress caused by variable climatic conditions, and biotic stress caused by pests and pathogens threaten future productivity. Here, a profound difference in taxonomic composition was found between the rhizoplane of *Rht* mutant wheats compared to that of wild type cadenza wheat. Furthermore, there was a general impact on microbiome structure in wheat hormone signalling mutants in ABA, auxins, brassinosteroids, ethylene, gibberellins and strigolactones. Taken together, it is clear that hormones play an essential role and despite our limited knowledge, seem to be as important for microbiome assembly and as such bilateral plant–microbe interactions. Future efforts to employ microbial communities, for instance for more sustainable and biodiversified crop production systems, require a better understanding of hormonal activities and their prevalence in plant–microbiome interactions.

4.6 References

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5 GENERAL DISCUSSION

The research presented in this thesis investigated the effects that agricultural domestication has had on beneficial plant-microbe interactions in wheat. Presented, is a combination of culture-independent and culture-dependent approaches, utilized to further understand the role of plant growth-promoting rhizobacteria (PGPR) in ancestral, modern, and mutant, wheat varieties under contrasting fertilization conditions.

A high-throughput culture-dependent methodology was developed to assess the abundance of rhizobacterial isolates displaying nutrient acquisition traits to identify putative PGPR isolates in a representative population of the culturable rhizo-microbiome (Chapter 2). Putative culturable PGPR isolates were linked to total community DNA amplicon sequence variants (ASVs) by 16S rRNA gene Sanger sequencing to create an isolate database, similar to that of the SILVA database, against which to search culture-independent amplicon sequences (Chapter 2). This method showed how PGPR populations were depleted in the root environment of wheat grown in soils with chemical fertilizer addition compared to wheats grown in soil with no fertilizer addition which, in contrast, showed a selection in favour of PGPR with increasing proximity to the root (1). This was the first study to utilize a range of functional bioassays to assess culture-dependent function and then link this to culture-independent amplicon data in the wheat rhizosphere, and so far, within six months, has been cited in three review papers (2-4) and an original research article (5). This method was developed to attempt to reduce the gap between the culturable components of a microbial ecosystem, and total community diversity (6, 7). In fact, despite the difference in the number of ASVs between culture-independent (4,879), culture-dependent (426), and isolate (96 ASVs) datasets, many of the culturable isolates were identified as proportionally dominant ASVs in total community DNA, which has been described previously in endophytic populations of *Arabidopsis* (8), and salad leaf vegetables (7). Also tested was the abundance of salt tolerant rhizobacteria, which showed no difference in abundance between non-fertilized and fertilized wheat, compared to traits in nitrogen, phosphate, potassium, and

iron solubilization, which were reduced in fertilized wheat. It is proposed that this is because the plant does not exert a selective pressure on nutrient acquiring rhizobacteria when there are ample nutrients available.

In addition, culture-dependent functional analysis was applied to ten *Triticum* accessions representative of a wheat domestication timeline, nine *Triticum aestivum* varieties, and two *Aegilops* species hypothesized to be evolutionary genome donors (Chapter 3). The culture-dependent method showed the negative effect of fertilization on nutrient acquisition traits in the rhizosphere such as nitrogen, phosphate, potassium, and iron solubilization, validating the results from Chapter 2 which were performed on one variety of wheat (*T. aestivum* cv. Cadenza). Notably, unplanted control pots (bulk soil) showed a significantly lower abundance of PGPR compared to planted pots, and no difference between fertilization conditions which indicates that the reduction of PGPR in fertilized wheat is likely due to a plant effect and not a soil effect (Chapter 3). It was therefore interesting that fertilization had no effect on the abundance of PGPR in diploid wheats and it's proposed that this is due to a loss of plant-microbe signalling pathways as the wheat genome underwent expansion.

Introduction of the *Reduced-height (Rht)* genes during the Green Revolution has been hypothesized to reduce PGPR selection due to their influence on the plant hormone gibberellin (GA). 16S rRNA gene amplicon sequencing revealed marked differences in rhizosphere microbiome in severe *Rht* dwarf mutants, which were more pronounced with fertilization (Chapter 4). There was a higher differential abundance in Acidobacteria, Chloroflexi and Gemmatimonadetes, phyla more commonly associated with bulk soil, in *Rht* mutant cultivars compared with a higher differential abundance of Bacteroidetes, Firmicutes and Proteobacteria, phyla more commonly associated with plant growth promotion, in Cadenza wheat cultivars. In particular, genera we previously identified as PGPR (*Microbacterium*, *Mesorhizobium*, *Pseudomonas*, *Rhizobium*, *Sphingomonas*, *Stenotrophomonas*, *Variovorax*) were differentially more abundant in Cadenza wheat compared to *Rht* mutant wheats.

We propose a scenario whereby domestication in wheat, through polyploidization and selection of *Rht* semidwarfing genes, combined with high levels of agrochemical inputs during the Green Revolution, have resulted in microbiome instability from reduced plant control due to excessive nutrient inputs reducing selective pressures.

5.1 Plant domestication: changes and trade-offs

The process of plant domestication involves selection, modification, and adoption of wild plant species with useful characteristics for human use, such as larger seed size, loss of seed dispersal mechanisms, and in-root architecture (9-11). Many of the changes in plant traits during domestication were accompanied by progressive changes in the environment, and management practices (12). Hence, plant domestication associated with anthropogenic interference to sustain high yields led to low self-support production systems with an increased need for external inputs such as chemical pesticides and fertilizers to overcome problems related to pests and diseases, vulnerability to abiotic stress, and nutrient depletion (13). The research presented in this thesis concludes that the transition from natural to agricultural systems has potentially neglected beneficial interactions between plants and microbes, one possibility through the loss of soil rhizobacterial diversity (Chapter 2) (Chapter 3).

5.2 Effect of plant genotype on rhizosphere microbiome assembly

Domestication led to a reduction in genetic diversity of plant cultivars, as shown for common bean (14, 15), maize (16), rice (17), and wheat (18). Genes associated with desirable phenotypes underwent a diversity loss because only the desired alleles were spread in the subsequent progenies, whilst unwanted diversity of the same allele was inadvertently suppressed (19). The two main events in wheat breeding were polyploidization and selection for the reduced height (*Rht*) genes (20). Besides fertilization and niche, the expansion of the wheat genome from diploid to allopolyploid caused the greatest variation in microbial community structure (between 7-18%) (Chapter 3). Similarly, the effect of increased severity in *Rht* gene mutations caused 14% of variation in the

microbial community structure (Chapter 4). The genotype of the host plant helps determine the physical and chemical rhizosphere environment influencing recruitment and establishment of microbial communities. Domestication of maize from teosinte to modern maize hybrids showed that plant genetic group accounted for 7.7% of variation in rhizobacterial communities (16). Similarly, in a study with different barley genotypes, it was found that the host genotype accounts for approximately 5.7% of the variance in the rhizosphere microbiome composition (21). Additionally, Shenton et al., 2016 demonstrated a 6.6% variation in rhizobacterial community due to differences in wild vs. modern rice genotypes (22). In fact, the introduction of semidwarfing genes into rice was also a major factor in breeding higher yielding varieties during the Green Revolution (23). It would be interesting to see if the microbiome in severe dwarf rice mutants grown under contrasting fertilization conditions show the same trend in rhizobacterial diversity as severe dwarf wheat mutants. Modern crops have been bred primarily under optimal cultivation conditions, selecting for traits associated with high yields in agricultural systems with high nutrient inputs. Thus, compared to older cultivars and wild relatives, modern cultivars may have lost some ability to recruit microorganisms that can support growth in low input conditions due to a lack of selective pressure (10).

5.3 Reinstating beneficial plant-microbe interactions in modern crop cultivars

In general terms, there are two approaches to enable reinstating beneficial plant-microbe interactions in modern crop cultivars: the 'top-down' method whereby plants are bred to select for PGPR already present in bulk soil and the 'bottom-up' method whereby PGPR are applied to soil. Application of individual microorganisms (bacteria or fungi) for improving plant growth, health and overall fitness is comparatively an easy task but its success in an open system is challenging (24). The application at the microbiome or core-microbiome level has shown to be more successful. However, getting to the relevant bacterial species and preparing their appropriate consortia is the main challenge here because of the complex nature of the microbe-plant interactions.

5.3.1 Application of PGPR to soils

Domestication and intensive cultivation of a single crop has provoked several qualitative issues, such as reduction in the efficiency of nutrient use, increased pest and disease susceptibility, vulnerability to abiotic stresses, etc. Domestication also could have weakened those plant traits which promoted the assembly of host-specific microbiomes which can also confer high adaptability to biotic and abiotic challenges (25, 26). These changes have necessitated high use of inorganic fertilizer, pesticides, growth hormones, etc. to maintain the required output (13), while also drastically degrading soil microbial diversity (27). Integration of plant-beneficial microorganisms such as nitrogen-fixing bacteria, phosphate solubilizing microbes, PGPR and arbuscular mycorrhizae have been included as agronomic components of crop husbandry and are envisaged as an environmentally benign alternative to supplement or even replace anthropogenic inputs. Individual inoculations, initially of either bacteria or fungi, have progressed to mixed inoculations of both, yielding favourable results in some crops grown under certain soil and environmental conditions (24). However, under some ecological conditions the microbial applications did not always fulfil expectations, even in the same host plants (28). Perhaps singular or binary microbe combinations failed to establish in the soil resulting in bioinoculant underperformance. It could be that the introduced microbes could not locate their interdependent groups and establish the necessary microbe-microbe interaction with necessary microbes such as keystone species specific to particular soil types. In the inoculated “foreign” soil, as in the native soils from which they were originally isolated; these groups synergize in sharing and exchanging critical metabolites such as amino acids and sugars, or signalling molecules particularly important for survival in challenging microenvironments. In short, microorganisms are dependent upon their co-existent partners for key metabolites, and hence on an environment possessing diverse microbial communities (29). This again highlights the fact that microorganisms work in a “network” mode which inherently offers broad microbial genomic diversity, which could feed back into plant genetic variability.

5.3.2 Breeding the holobiont

As an integral part of the plant hologenome, the plant microbiome is a tool that can be selected together with the plant genome to develop next-generation plant breeding approaches (30). In addition to the rhizosphere microbiome, the plant microbiome also includes for example, endophytic, spermosphere and phyllosphere microbiomes. Given that soils harbour rich microbial communities (31), and there are efficacy inconsistencies with applying microbes to soil and plants as inoculants, it follows that an alternative approach for incorporating microbiomes into future plant breeding strategies could be to reinstate plant-microbe interactions. As mentioned above, this could be achieved by selection of bespoke beneficial microbes from soil by the plant into its microbiome. This would have the advantage over the 'one size fits all' inoculation approach as microbes selected by the host plant will be inherently competent at colonising both the bulk soil as well as the plant. As such they are unlikely to be outcompeted by indigenous microbiota, which is often the case with the use of inoculants which are not optimised for the environment they are being applied.

Gopal and Gupta, 2016 (24) proposed an approach to 'co-propagate the co-evolved' whereby the microbiome is co-propagated with its plant offspring in the new cultivation with a starter microbiome culture of keystone plant-beneficial microbiota from target soils. This approach will provide an opportunity to the plants to easily recognize the suite of microbiota with which it had co-evolved and, therefore, preferably recruit them in the new environment. Our work provides a solid basis for this as we have characterized differential rhizobacterial communities in the rhizosphere of wheat hormonal mutants as well as ancestral and modern wheat varieties that span a timeline of domestication. Further work will for example, identify key plant genes that are crucial for the selection of beneficial microbiomes (both above and below ground) by testing modern wheat lines introgressed with ancestral species. ASVs associated with these accessions could provide potential candidates for starter beneficial microbiome cultures better connected with a given plant genotype. There is clearly a long way to go for the establishment of reduced agro-chemical microbiome assisted agriculture,

though the work in this thesis has shown that manipulation of the crop genome offers green shoots of hope in achieving this.

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6 GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 General conclusions

The main conclusions arising from the research conducted as part of this degree are summarized.

6.1.1 Development of a method for functional characterisation of microbiomes

Single colony functional bioassay methodology was developed to assess the abundance of bacterial isolates exhibiting selected macronutrient (nitrogen, phosphate, potassium solubilization) and micronutrient (iron, and zinc solubilization) acquisition traits, to identify putative PGPR isolates in a representative population of the culturable rhizo-microbiome. Putative culturable PGPR isolates were linked to ASVs from total community DNA ASVs by 16S rRNA gene Sanger sequencing to create an isolate database, against which to search culture-independent amplicon sequences and identify ASVs from single isolates. This method identified 1.52% of total community ASVs as culturable PGPR isolates. This method can be applied in different microbiome-based industries and can be modified to include different functional traits such as salt and drought tolerance, disease resistance, and other nutrient acquisition traits.

6.1.2 Chemical fertilizer is the dominant factor shaping the rhizosphere microbiome

The widely used nitrogen-phosphate-potassium (NPK) fertilizer influenced rhizobacterial diversity, community structure and taxonomic composition. The study of ten *Triticum* accessions representative of a wheat domestication timeline, nine *Triticum aestivum* varieties, and two *Aegilops* species hypothesized to be evolutionary genome donors, enabled the identification of fertilization as the dominant factor shaping the microbiome. Other factors such as niche (rhizosphere/rhizoplane), plant species, and plant genotype, contributed significantly, but were associated with less variation in microbial communities. Chemical fertilization also reduced rhizosphere bacterial species richness and diversity. Key genera differentially more abundant in non-fertilized wheats

included members of the phylum Cyanobacteria (*Nostoc*) and Proteobacteria (*Bradyrhizobium*, *Pseudomonas*) compared to fertilized wheats which were richer in Actinobacteria (*Arthrobacter*, *Catenulispora*, *Leifsonia*, and *Streptomyces*).

6.1.3 Chemical fertilization reduces selection of plant growth-promoting rhizobacteria

Fertilizer addition decreased the proportion of nutrient-solubilizing (nitrate, phosphate, potassium, iron, and zinc) bacteria isolated from rhizocompartments in wheat. In the commercial Cadenza wheat variety, bioassays identified a higher proportion of PGPR in non-fertilized wheat samples compared to fertilized wheat samples (rhizosphere: 12.2% increase and rhizoplane: 11.4% increase). This same trend was seen in the rhizoplane of four tetraploid and eleven hexaploid wheat cultivars, with fertilization causing a 9.2 and 5.7% reduction, respectively. In comparison, fertilization only caused a 2.7% reduction in the rhizoplane of diploid species. We propose that this is due to a loss of plant-microbe signalling pathways as the wheat genome underwent expansion. Key genera identified as PGPR included *Acidovorax*, *Bacillus*, *Microbacterium*, *Mesorhizobium*, *Pseudomonas*, *Rhizobium*, *Sphingomonas*, *Stenotrophomonas*, and *Variovorax*.

6.1.4 Domestication of wheat has shaped the root microbiome interactions

Besides fertilization and niche, the expansion of the wheat genome from diploid to allopolyploid is a cause of variation in root microbiome structure (between 7-18%). Furthermore, taxonomic composition shows a positive correlation between bulk soil associated phyla and ploidy level in non-fertilized wheat, with hexaploid wheat showing the highest abundance of Acidobacteria, Chloroflexi, Gemmatimonadetes, and Verrucomicrobia. In comparison, there was little change in taxonomic composition with ploidy level in fertilized wheat with the main phyla being Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. It is proposed that this is due to fertilization being an important factor in determining the soil microbiome and therefore causing an undifferentiated rhizobacterial community at the root-soil interface regardless of

wheat genotype, as well as a reduction in selection of PGPR, as previously demonstrated in Chapter 2. There is a larger effect of fertilization on the abundance of PGPR in tetraploid and hexaploid compared to diploid wheats. However, in the absence of fertilizer, hexaploid wheats have a similar taxonomic profile to fertilized bulk soil which could be an indication that highly domesticated wheat has a reduced ability to select microbes from the bulk soil reservoir.. However, as an increase in PGPR in culturable communities derived from wheat roots compared to bulk soil is observed, it may be the case that differences in microbiome function which are driven by ploidy level may be easier to discern at a finer scale i.e. genera-specific taxa. In future work, it would be interesting to create an isolate database of PGPR and non-PGPR isolates from bulk soil, diploid, tetraploid, and hexaploid wheat samples to ascertain if the culture-independent results correlate with the culture-dependent results.

6.1.5 Selection for *Reduced height (Rht)* genes during the Green Revolution has likely reduced selection for beneficial microbes

A profound difference in taxonomic composition was found between the rhizoplane of *Rht* mutant wheat compared to that of wild type Cadenza wheat. There was a higher differential abundance in Acidobacteria, Chloroflexi and Gemmatimonadetes, phyla more commonly associated with bulk soil, in *Rht* mutant cultivars compared with a higher differential abundance of Bacteroidetes, Firmicutes and Proteobacteria, phyla more commonly associated with plant growth promotion, in Cadenza wheat cultivars. In particular, genera we previously identified as PGPR (*Microbacterium*, *Mesorhizobium*, *Pseudomonas*, *Rhizobium*, *Sphingomonas*, *Stenotrophomonas*, *Variovorax*) were differentially more abundant in Cadenza wheat compared to *Rht* mutant wheats. Further work should evaluate these differences based on culture-dependent work to identify key plant growth-promoting ASVs.

6.2 Recommendations for future research

Sustainable agriculture requires the more efficient use of available resources, and the naturally occurring soil dwelling microbiome offers the potential to contribute to the sustainable intensification of agriculture. Root associated

microorganisms can benefit crop plants in several ways including improved plant nutritional status and protection against biotic and abiotic stresses.

The private sector has already begun to invest in market-orientated microbiome research, including microbial-based biofertilization strategies, microbiome-assisted agriculture, disease suppression in soil, and microbiome engineering. The goal for future work would be to advance the knowledge of complex soil microbial communities in agricultural soils in general—and in and around roots of high-yielding, disease, and drought resistant crops in particular—to accelerate the development and use of these crops.

Below are some key research questions and aims for future experiments that arose from this thesis which provide avenues for further study:

Future experimental aims:

1. Comparative genomic analyses of PGPR: identify the similarities and differences among non-PGPR and PGPR using a comparative genomic approach to clarify the mechanisms of plant growth-promoting activity.
2. Create a 16S rRNA gene ancestral vs commercial PGPR database.
3. Assess culturable beneficial rhizobacteria from hormone mutant wheat.

Future research questions:

1. Is wheat at a “tipping point”, in the sense that hexaploid wheat is becoming too dependent on fertilizers and is commercial wheat “beyond help”?
2. Does fertilization-induced gene expression alter selection of PGPR in wheat?
3. Why would *Rht*-gene induced dwarfism reduce selection of beneficial microbes but GA-induced dwarfism not?
4. How can future wheat genetic breeding programmes incorporate plant-microbe interactions with sustained crop yields?

APPENDICES

Appendix A SUPPLEMENTARY INFORMATION FOR CHAPTER 2

A.1 SUPPLEMENTARY METHODS

A.1.1 Amplicon library preparation and sequencing

All PCR reactions were conducted with the KAPA HiFi HotStart ReadyMix PCR Kit (Kapa, Wilmington, MA, USA) (0.5 μ M each primer, 3 μ l template DNA (150 ng), 30 μ l reaction volume, 96-well skirted PCR plates (StarLab)). PCR amplifications were performed with initial denaturation at 95°C (3 min) followed by 30 cycles at 95 °C (20 s), 55 °C (15 s), and 72 °C (20 s), and a final elongation step at 72 °C (1 min). Amplification reactions were verified by agarose gel electrophoresis and purified using a magnetic bead capture kit (Ampure; Agencourt). The resulting indexed amplicons were quantified on a Qubit Fluorimeter using the Qubit dsDNA High Sensitivity assay (Thermo Scientific) and normalized to equimolar concentration prior to being pooled. The final pool was quantified, and quality assessed using SYBR green quantitative PCR (qPCR) assay with primers specific to the Illumina adapters (Kappa) and diluted to 12 pM. The library was spiked with PhiX control V3 library (5% v, v⁻¹, 12.5 pM) and sequenced on the Illumina MiSeq platform using the Illumina MiSeq v3 kit (Illumina Inc.), to obtain 2 x 300 bp paired-end sequences.

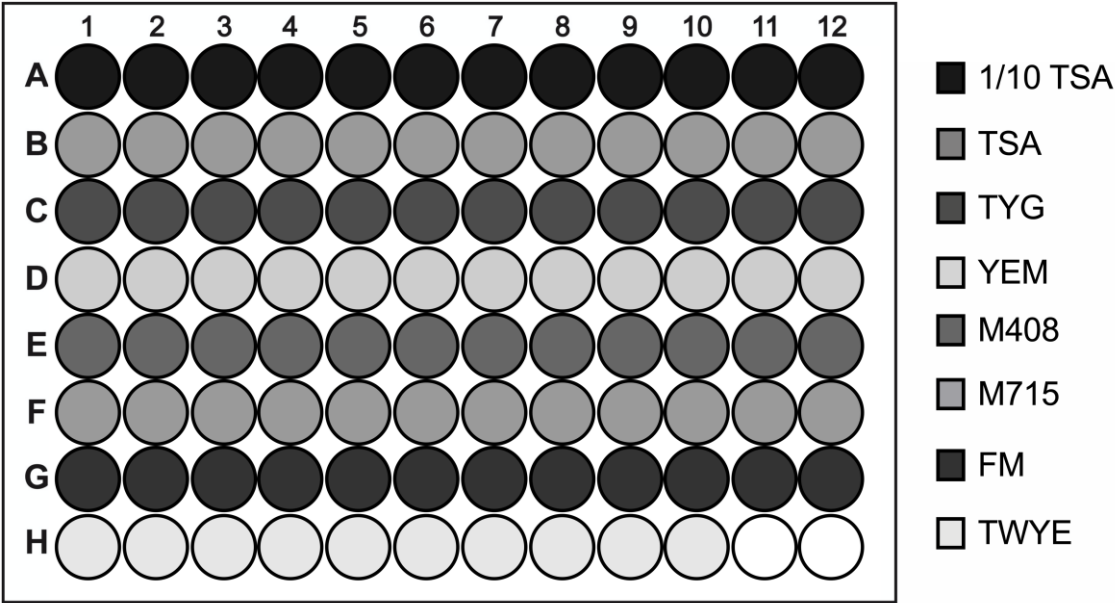
A.1.2 Colony PCR

Bacterial isolates were spot inoculated on 10TSA from frozen glycerol stocks, using a 48-prong manifold, and grown at 25 °C for 2 days. A sterile toothpick was used to inoculate a single isolate in 40 μ l lysis buffer (Tris-EDTA, 0.1% Triton) in a skirted 96-well PCR plate (StarLab, Milton Keynes, UK). Bacterial suspensions were vigorously vortexed, then boiled at 100 °C in a PCR Thermal Cycler (MJ Research PTC-200) for 2 min before being frozen at -80 °C for 30 min. An aliquot of the resulting solution was used for colony PCR. All PCR reactions were conducted with the DreamTaq Green PCR Master Mix (2x) (Thermo Scientific),

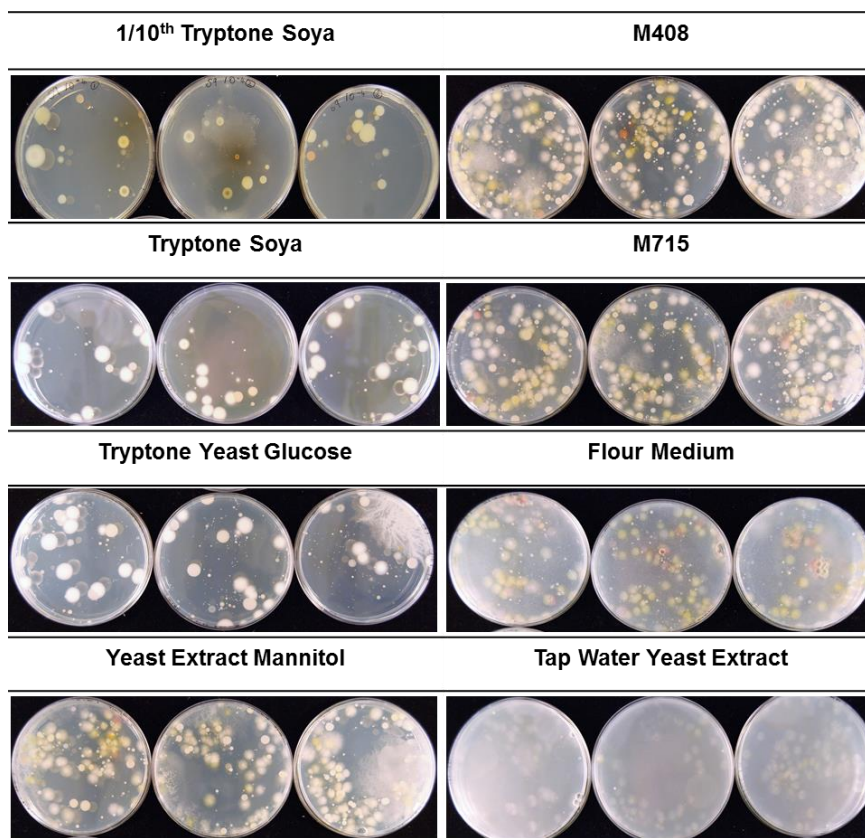
Appendix A

1 μ M of each primer, and 2.5 μ l bacterial lysis solution in a 25 μ l reaction volume in 96-well non-skirted PCR plates (StarLab). PCR amplifications were performed with an initial denaturation at 95°C (5 min) followed by 35 cycles at 95 °C (30 s), 60 °C (30 s), and 72 °C (1 min), and a final elongation step at 72 °C (5 min). Amplification reactions were verified by agarose gel electrophoresis and sent to Eurofins Genomics Germany for purification and Sanger sequencing.

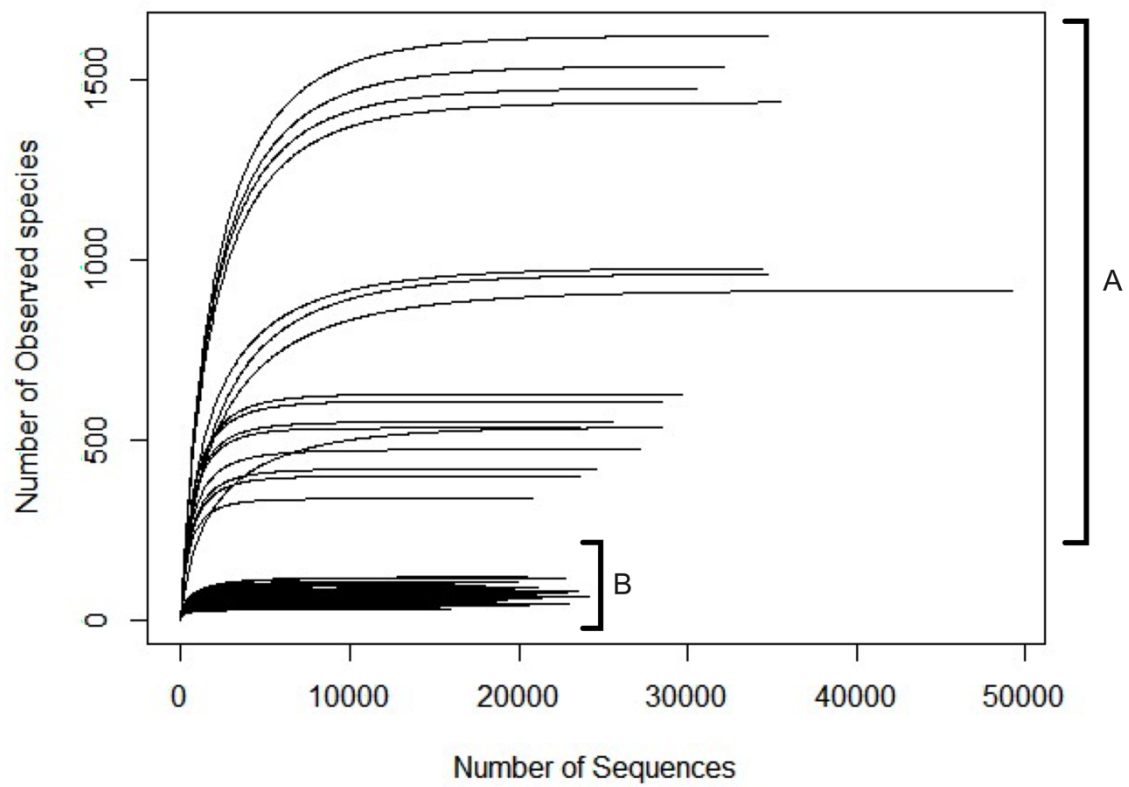
A.2 SUPPLEMENTARY FIGURES



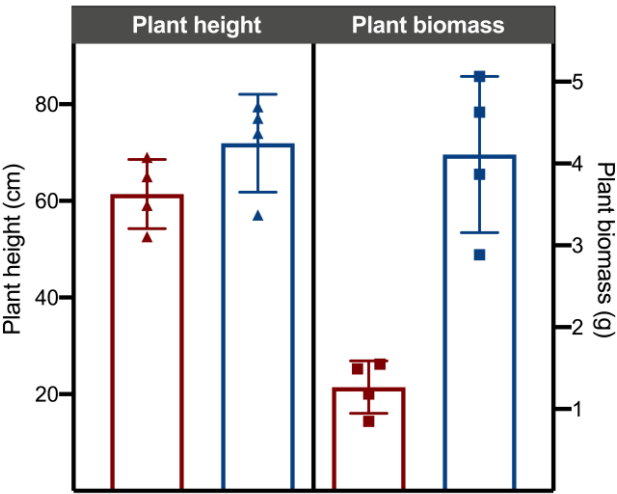
Supplementary Figure A-1 Schematic representation of 96-well plate design for isolate library. Sterile tryptone soya broth (TSB; at 1/10th conc.; 500 µl) was used as a negative control (Wells H11 and H12).



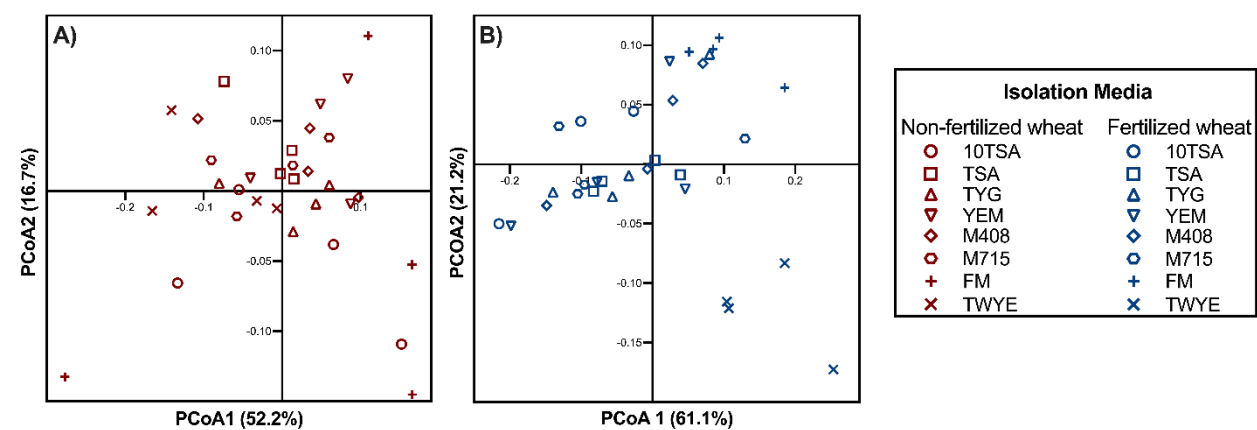
Supplementary Figure A-2. Rhizobacterial isolates cultured on agar for taxonomic analysis. Samples plated on 1/10th tryptone soya, tryptone soya, tryptone yeast glucose, yeast extract mannitol, m408 and m715 were at 10⁻³ dilution; samples plated on flour medium and tap water yeast extract were at 10⁻² dilution.



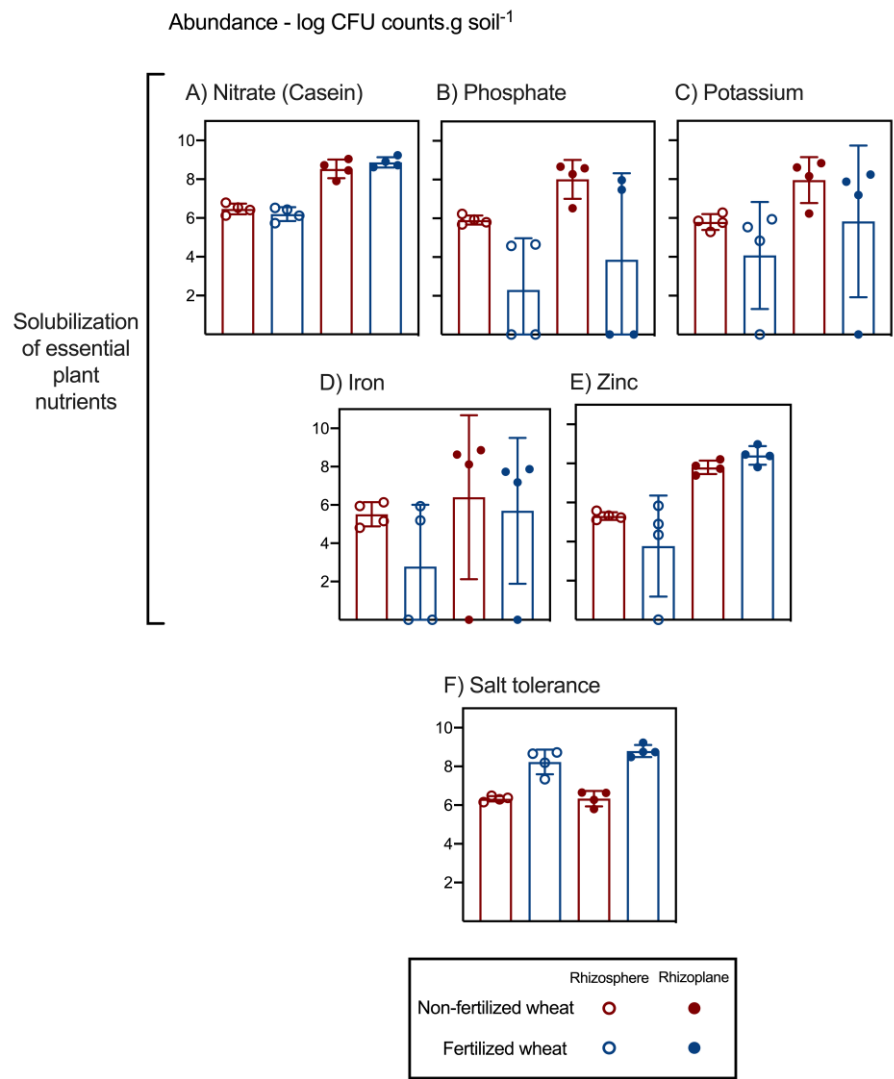
Supplementary Figure A-3. Rarefaction curve analyses of bacterial species richness as a function of sequencing depth. Culture-independent sequences (A) and culture-dependent sequences (B).



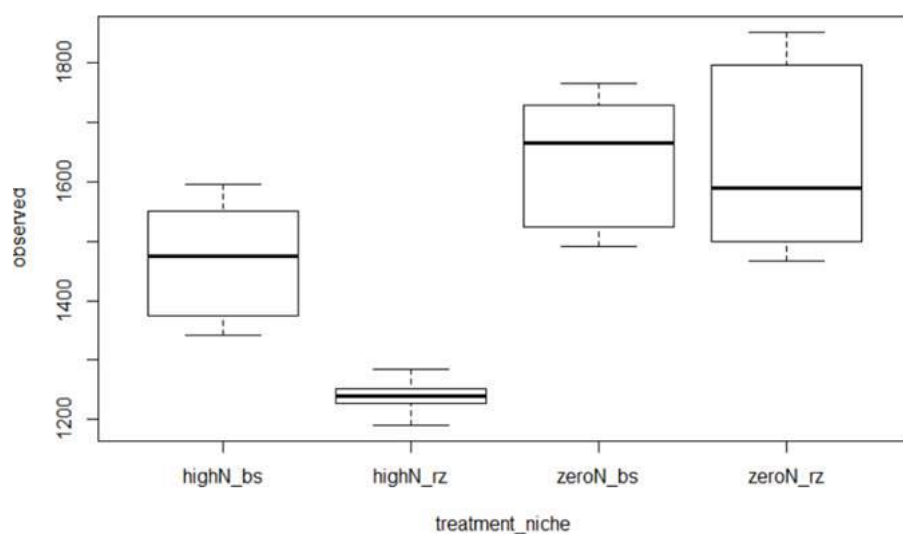
Supplementary Figure A-4. Plant height and dry biomass of wheat grown in soils with and without fertilizer addition



Supplementary Figure A-5. PCoA plots of rhizosphere bacteria cultured on a variety of agar types based on weighted UniFrac distances at ASV level, from (A) non-fertilized wheat and (B) fertilized wheat.



Supplementary Figure A-6. Absolute abundance of culturable rhizobacteria with plant growth-promoting traits isolated from wheat grown in soils with and without fertilizer.



Supplementary Figure A-7. Observed operational taxonomic units (OTUs) within bacterial 16S V4 sequences from bulk soil (bs) and wheat rhizosphere (rz) samples with (highN) and without fertilizer (zeroN) addition. Reanalyzed data from Kavamura et al., 2018 (1).

A.3 SUPPLEMENTARY TABLES

Supplementary Table A-1. Composition of bacterial growth agar used for isolation approaches

	Tryptone Soy Broth (TSA)	Tryptone Yeast Extract Glucose (TYG)	Yeast Extract Mannitol (YEM)	M408	M715	Flour Medium (FM)	Tap Water Yeast Extract (TWYE)
Compound	gram per litre (g/l) of MilliQ water						gram per litre (g l/l) of tap water
Casein (pancreatic digest)	17.0	x	x	x	x	x	x
Casein (enzymatic hydrolysate)	x	5.0	x	x	x	x	x
Soya peptone (papaic digest)	3.0	x	x	x	x	x	x
NaCl	5.0	x	x	x	x	x	x
K ₂ HPO ₄	2.5	x	x	x	x	x	0.5
Dextrose	2.5	x	x	x	x	x	x
D-glucose	x	1.0	x	x	x	x	x
Yeast extract	x	3.0	0.5	1.0	1.0	x	0.3
Mannitol	x	x	5.0	10.0	10.0	x	x
K ₂ HPO ₄	x	x	0.5	0.5	0.5	x	x
MgSO ₄ · 7H ₂ O	x	x	0.2	0.2	0.2	x	x
NaCl	x	x	0.1	0.1	0.1	x	x
CaCO ₃	x	x	x	x	1.0	0.4	x
Plain flour	x	x	x	x	x	4.0	x
Agar	20	15	20	20	20	16	18
pH	7.0	7.0	7.0	7.0	7.0	7.0	7.0

Supplementary Table A-2. Outline of technical replicates plated per soil sample on agar

Appendix A

Selective Agar	Dilution		
	x10-2	x10-3	x10-4
10TSA	NA	3x	3x
TSA	NA	6x	NA
TYG	NA	6x	NA
YEM	3x	3x	NA
M408	3x	3x	NA
M715	3x	3x	NA
FM	6x	NA	NA
TWYE	6x	NA	NA

Supplementary Table A-3. PERMANOVA of culture-independent rhizobacterial beta-diversity as represented by weighted UniFrac metric. This is a two-way PERMANOVA analysis with 999 permutations.

Sources of Variation	Degrees of Freedom	Sum of Squares	F statistic	R2	Pr(>F)	Statistical Significance
Rhizocompartment	1	0.0155	16.066	0.3597	0.001	***
Fertilizer	1	0.01233	13	0.2861	0.001	***
Fertilizer: Rhizocompartment	1	0.00369	3.825	0.0856	0.014	*
Residuals	12	0.01158				
Significance codes: 0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	0.1 ''	1	

Supplementary Table A-4. PERMANOVA of culture-dependent rhizobacterial beta-diversity as represented by weighted UniFrac metric. This is a two-way PERMANOVA analysis with 999 permutations.

Sources of Variation	Degrees of Freedom	Sum of Squares	F statistic	R2	Pr(>F)	Statistical Significance
Rhizocompartment	1	0.3188	6.223	0.2948	0.002	**
Fertilizer	1	0.1562	3.0479	0.1444	0.035	*
Fertilizer: Rhizocompartment	1	0.04282	0.8358	0.0396	0.474	
Residuals	11	0.5636				
Significance codes: 0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	0.1 ''	1	

Supplementary Table A-5. ANOVA of culture-independent rhizobacterial alpha diversity as represented by the Shannon diversity index. This is a two-way type III ANOVA.

Sources of Variation	Degrees of Freedom	Sum of Squares	F statistic	Pr(>F)	Statistical Significance
Fertilizer	1	5.074	14.62	0.0024	**
Fertilizer: Rhizocompartment	1	1.566	4.512	0.05512	.
Rhizocompartment	1	0.312	0.899	0.3618	
Significance codes: 0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	0.1 ''	1

Supplementary Table A-6. ANOVA of culture-dependent rhizobacterial alpha diversity as represented by the Shannon diversity index. This is a two-way type III ANOVA.

Sources of Variation	Degrees of Freedom	Sum of Squares	F statistic	Pr(>F)	Statistical Significance
Fertilizer	1	0.2479	3.042	0.109	
Fertilizer: Rhizocompartment	1	0.2367	3	0.116	
Rhizocompartment	1	0.0021	0.025	0.876	
Significance codes: 0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	0.1 ''	1

Supplementary Table A-7. Contingency table to assess the difference in distribution of isolates that tested positive or negative for solubilization of key plant nutrients, isolated from wheat grown in soils with and without fertilizer addition.

	Functional Bioassay	No. of isolates				Chi-squared test
		Non-fertilized wheat		Fertilized wheat		
		+ve	-ve	+ve	-ve	
Rhizosphere	Solubilization					
	Nitrate (Casein)	269	107	146	230	81****
	Phosphate	68	308	3	373	66****
	Potassium	62	314	17	359	29****
	Iron	50	326	7	369	35****
	Zinc	17	359	6	370	5.4*
	Salt tolerance	182	194	158	218	3.1
Total PGPR	306	70	163	213	116****	
Rhizoplane	Solubilization					
	Nitrate (Casein)	329	47	255	121	42****
	Phosphate	168	208	7	369	193****
	Potassium	177	199	24	352	159****
	Iron	200	176	8	368	245****
	Zinc	50	326	102	274	22****
	Salt tolerance	190	186	215	161	3.3
Total PGPR	339	37	279	97	33****	
Significance codes: 0 '****' 0.001 '***' 0.01 '**' 0.05 '.' 0.1 '' 1						

Supplementary Table A-8. Differentially abundant 16S rRNA gene ASVs which were found to be enriched in culture-independent rhizosphere samples from non-fertilized wheat and fertilized wheat. ASVs identified in both culture-independent and culture-dependent methods are indicated on bold. ASVs identified in the isolate database are indicated in red.

Treatment	ASV	Phylum	Genus	log2 Fold Change	p adjusted
Non-fertilized	ASV_540	Firmicutes	NA	22.41	2E-14
	ASV_4499	Actinobacteria	Kitasatospora	8.62	1E-03
	ASV_2669	Proteobacteria	NA	8.19	9E-05
	ASV_5446	Proteobacteria	NA	7.73	5E-04
	ASV_784	Acidobacteria	NA	7.62	3E-02
	ASV_4613	Proteobacteria	NA	7.53	9E-03
	ASV_530	Acidobacteria	NA	7.37	2E-03
	ASV_90	Acidobacteria	NA	7.34	1E-02
	ASV_2562	Proteobacteria	NA	7.33	4E-02
	ASV_6	Firmicutes	Bacillus	7.23	2E-02
	ASV_2507	Proteobacteria	NA	6.71	4E-02
Fertilized	ASV_3551	Proteobacteria	NA	-10.5	3E-07
	ASV_1216	Actinobacteria	NA	-9.70	2E-07
	ASV_3984	Actinobacteria	NA	-9.53	4E-07
	ASV_3631	Proteobacteria	NA	-9.39	5E-07
	ASV_3707	Proteobacteria	NA	-9.33	3E-03
	ASV_3908	Proteobacteria	NA	-9.28	8E-07
	ASV_2895	Proteobacteria	NA	-9.22	3E-06
	ASV_5308	Proteobacteria	NA	-9.19	1E-06
	ASV_250	Actinobacteria	NA	-9.09	4E-03
	ASV_125	Actinobacteria	NA	-9.03	4E-06
	ASV_3562	Actinobacteria	NA	-9.01	6E-06
	ASV_4070	Acidobacteria	NA	-8.94	8E-06
	ASV_3700	Actinobacteria	Catenulispora	-8.80	7E-06
	ASV_4900	Bacteroidetes	NA	-8.77	1E-03
	ASV_1975	Actinobacteria	NA	-8.60	2E-05
	ASV_1803	Proteobacteria	NA	-8.56	1E-03
	ASV_2070	Proteobacteria	NA	-8.32	1E-02
	ASV_1051	Proteobacteria	Rhizobium	-8.31	2E-03
	ASV_1088	Cyanobacteria	NA	-8.15	5E-03
	ASV_2348	Actinobacteria	NA	-8.14	2E-04

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	ASV_3722	Proteobacteria	NA	-8.01	4E-03
	ASV_469	Proteobacteria	NA	-7.77	6E-04
	ASV_5379	Actinobacteria	Arthrobacter	-7.74	3E-02
	ASV_128	Verrucomicrobia	NA	-7.68	1E-03
	ASV_4415	Actinobacteria	NA	-7.67	8E-03
	ASV_1808	Actinobacteria	Nocardioides	-7.64	1E-03
	ASV_4164	Proteobacteria	NA	-7.59	3E-02
	ASV_3136	Proteobacteria	NA	-7.58	3E-02
	ASV_3141	Proteobacteria	NA	-7.51	1E-02
	ASV_3569	Firmicutes	NA	-7.48	1E-02
	ASV_1607	Proteobacteria	NA	-7.45	1E-02
	ASV_3667	Bacteroidetes	NA	-7.42	2E-03
	ASV_4942	Proteobacteria	NA	-7.37	4E-02
	ASV_1154	Firmicutes	NA	-7.36	1E-02
	ASV_3288	Firmicutes	NA	-7.33	2E-02
	ASV_1238	Proteobacteria	NA	-7.30	2E-02
	ASV_3866	Proteobacteria	NA	-7.04	3E-02
	ASV_1288	Proteobacteria	Cupriavidus	-7.01	6E-03
	ASV_4679	Proteobacteria	NA	-6.69	4E-02
	ASV_1291	Bacteroidetes	NA	-4.59	4E-02
	ASV_2126	Actinobacteria	Catenulispora	-4.21	6E-03
	ASV_45	Proteobacteria	NA	-3.28	4E-03
	ASV_4714	Actinobacteria	Leifsonia	-2.95	6E-03

Supplementary Table A-9. Differentially abundant 16S rRNA gene ASVs which were found to be enriched in culture-independent rhizoplane samples from non-fertilized wheat and fertilized wheat. ASVs identified in both culture-independent and culture-dependent methods are indicated on bold. ASVs identified in the isolate database are indicated in red.

Treatment	ASV	Phylum	Genus	log2 Fold Change	p adjusted
Non-fertilized	ASV_3225	Proteobacteria	NA	7.62	5E-03
	ASV_5126	Proteobacteria	NA	7.58	3E-04
	ASV_5363	Proteobacteria	NA	7.07	1E-02
	ASV_3142	Proteobacteria	NA	7.04	1E-03
	ASV_150	Acidobacteria	NA	7.02	2E-03
	ASV_2870	Acidobacteria	NA	6.94	1E-02
	ASV_4403	Actinobacteria	Streptomyces	6.80	5E-02
	ASV_2108	Acidobacteria	NA	6.65	2E-02
	ASV_806	Proteobacteria	NA	6.63	3E-03
	ASV_5385	Acidobacteria	NA	6.58	2E-02
	ASV_3495	Acidobacteria	NA	6.56	2E-02
	ASV_2430	Proteobacteria	NA	6.51	4E-03
	ASV_103	Proteobacteria	NA	6.51	4E-03
	ASV_1384	Acidobacteria	NA	6.49	2E-02
	ASV_4088	Acidobacteria	NA	6.48	2E-02
	ASV_2382	Proteobacteria	NA	6.38	3E-02
	ASV_5002	Gemmatimonadetes	NA	6.35	8E-03
	ASV_930	Acidobacteria	NA	6.29	3E-02
	ASV_2658	Bacteroidetes	NA	6.26	3E-02
	ASV_935	Acidobacteria	NA	6.22	3E-02
	ASV_3485	Acidobacteria	NA	6.20	3E-02
	ASV_5172	Acidobacteria	NA	6.18	3E-02
	ASV_635	Proteobacteria	NA	6.11	9E-03
	ASV_4587	Acidobacteria	NA	6.03	4E-02
	ASV_4057	Proteobacteria	NA	5.98	1E-02
	ASV_2571	Acidobacteria	NA	5.93	1E-02
	ASV_2023	Actinobacteria	NA	5.90	5E-02
	ASV_4444	Proteobacteria	NA	5.89	5E-02
	ASV_3296	Proteobacteria	NA	5.85	5E-02
	ASV_1658	Acidobacteria	NA	5.81	2E-02

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	ASV_3115	Acidobacteria	NA	5.76	2E-02
	ASV_4562	Firmicutes	NA	5.70	2E-02
	ASV_422	Bacteroidetes	NA	5.61	3E-02
	ASV_3865	Acidobacteria	NA	5.31	4E-02
	ASV_3448	Proteobacteria	Pseudomonas	5.29	4E-02
	ASV_3917	Proteobacteria	NA	5.17	5E-02
	ASV_1772	Acidobacteria	NA	4.69	3E-02
Fertilized	ASV_2089	Proteobacteria	Lysobacter	-24.32	1E-15
	ASV_4187	Proteobacteria	Paraburkholderia	-16.01	8E-14
	ASV_1979	Proteobacteria	Rhodanobacter	-11.73	1E-05
	ASV_1818	Proteobacteria	Rhodanobacter	-11.58	3E-08
	ASV_1557	Actinobacteria	Leifsonia	-11.30	4E-08
	ASV_4616	Proteobacteria	NA	-11.14	4E-05
	ASV_4314	Proteobacteria	Burkholderia	-10.58	1E-06
	ASV_5039	Proteobacteria	NA	-10.57	4E-06
	ASV_1830	Proteobacteria	NA	-10.20	5E-07
	ASV_3329	Proteobacteria	NA	-9.92	3E-06
	ASV_1168	Proteobacteria	Burkholderia	-9.67	5E-04
	ASV_3287	Proteobacteria	NA	-9.43	4E-03
	ASV_1432	Proteobacteria	NA	-9.27	8E-04
	ASV_3385	Proteobacteria	NA	-9.16	7E-04
	ASV_1340	Proteobacteria	NA	-9.07	5E-03
	ASV_1189	Proteobacteria	NA	-8.96	7E-05
	ASV_3682	Acidobacteria	NA	-8.94	1E-03
	ASV_2671	Actinobacteria	Catenulispora	-8.81	4E-06
	ASV_2542	Proteobacteria	NA	-8.56	9E-03
	ASV_296	Proteobacteria	Burkholderia	-8.55	1E-02
	ASV_5405	Bacteroidetes	NA	-8.54	7E-05
	ASV_5248	Proteobacteria	NA	-8.49	3E-03
	ASV_1516	Proteobacteria	NA	-8.40	8E-05
	ASV_2410	Actinobacteria	NA	-8.40	4E-05
	ASV_954	Actinobacteria	Streptomyces	-8.34	1E-02
	ASV_2594	Proteobacteria	NA	-8.27	1E-02
	ASV_229	Actinobacteria	NA	-8.23	2E-04
	ASV_1963	Actinobacteria	NA	-8.23	3E-03
	ASV_4225	Firmicutes	Alicyclobacillus	-8.20	3E-03
	ASV_2941	Proteobacteria	NA	-8.15	3E-03
	ASV_2249	Proteobacteria	NA	-8.10	1E-02

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ASV_2637	Actinobacteria	Actinoplanes	-8.05	3E-04
ASV_4049	Bacteroidetes	Pedobacter	-8.03	4E-03
ASV_3790	Actinobacteria	Mycobacterium	-7.98	4E-03
ASV_4940	Actinobacteria	Catenulispora	-7.92	2E-04
ASV_2865	Actinobacteria	NA	-7.81	2E-02
ASV_1326	Actinobacteria	Arthrobacter	-7.78	2E-02
ASV_263	Actinobacteria	NA	-7.74	4E-03
ASV_4008	Proteobacteria	NA	-7.69	5E-03
ASV_2221	Proteobacteria	NA	-7.66	3E-04
ASV_4020	Proteobacteria	NA	-7.57	9E-04
ASV_2822	Actinobacteria	NA	-7.57	6E-03
ASV_5130	Actinobacteria	Arthrobacter	-7.56	2E-02
ASV_2277	Proteobacteria	NA	-7.51	8E-03
ASV_4476	Verrucomicrobia	NA	-7.46	9E-04
ASV_4373	Actinobacteria	Leifsonia	-7.41	8E-03
ASV_3514	Firmicutes	NA	-7.37	8E-03
ASV_5094	Bacteroidetes	NA	-7.33	3E-02
ASV_4095	Bacteroidetes	NA	-7.29	2E-03
ASV_5236	Actinobacteria	NA	-7.21	3E-02
ASV_805	Bacteroidetes	NA	-7.04	2E-02
ASV_5155	Proteobacteria	NA	-7.04	4E-02
ASV_4326	Acidobacteria	NA	-6.98	4E-02
ASV_2513	Proteobacteria	NA	-6.97	4E-03
ASV_616	Bacteroidetes	NA	-6.86	1E-03
ASV_3765	Actinobacteria	Pimelobacter	-6.74	5E-02
ASV_3092	Proteobacteria	NA	-6.72	2E-02
ASV_2620	Firmicutes	Bacillus	-6.53	6E-03
ASV_2405	Actinobacteria	Micromonospora	-6.42	2E-02
ASV_1437	Proteobacteria	NA	-6.31	4E-04
ASV_4630	Proteobacteria	NA	-5.57	3E-02
ASV_4783	Bacteroidetes	NA	-5.43	9E-03
ASV_5321	Bacteroidetes	NA	-5.27	2E-02
ASV_3972	Actinobacteria	NA	-5.26	4E-02
ASV_2852	Bacteroidetes	NA	-5.17	4E-03
ASV_4040	Actinobacteria	NA	-5.01	2E-02
ASV_5451	Actinobacteria	NA	-4.96	2E-02
ASV_968	Proteobacteria	NA	-4.90	3E-02
ASV_2776	Actinobacteria	NA	-4.81	3E-02

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	ASV_1703	Proteobacteria	NA	-4.44	4E-02
	ASV_3462	Proteobacteria	NA	-4.33	1E-02
	ASV_3503	Proteobacteria	NA	-3.94	2E-02
	ASV_4593	Proteobacteria	Rhizobium	-3.90	4E-02
	ASV_1593	Proteobacteria	NA	-3.49	3E-02
	ASV_2988	Actinobacteria	NA	-3.32	3E-03
	ASV_3017	Bacteroidetes	NA	-3.27	3E-02
	ASV_2197	Proteobacteria	NA	-3.22	2E-02
	ASV_5154	Proteobacteria	NA	-3.19	1E-02

Supplementary Table A-10. Differentially abundant 16S rRNA gene ASVs which were found to be enriched in culture-dependent rhizosphere and rhizoplane samples from non-fertilized wheat and fertilized wheat. ASVs identified in both rhizocompartments are indicated in bold. 22 ASVs not classified to phylum level are not shown. ASVs identified in the isolate database are indicated in red.

Rhizo-compartment	Treatment	ASV	Phylum	Genus	log2 Fold Change	p adjusted
Rhizosphere	Non-fertilized	ASV_675	Firmicutes	NA	24.4	1.14E-09
		ASV_4612	Firmicutes	NA	22.4	2.73E-08
		ASV_1126	Firmicutes	Paenibacillus	22.0	3.66E-08
		ASV_2988	Actinobacteria	NA	21.3	8.40E-08
		ASV_2569	Actinobacteria	Streptomyces	21.3	8.40E-08
		ASV_3448	Proteobacteria	Pseudomonas	10.9	0.004521
		ASV_5154	Proteobacteria	NA	10.1	0.006181
		ASV_1717	Proteobacteria	Pseudomonas	8.4	0.035455
	Fertilized	ASV_3070	Actinobacteria	NA	-25.6	1.28E-10
		ASV_1437	Proteobacteria	NA	-13.4	0.001525
		ASV_1979	Proteobacteria	Rhodanobacter	-12.9	0.000262
		ASV_1963	Actinobacteria	NA	-12.2	0.000455
		ASV_1557	Actinobacteria	Leifsonia	-11.2	0.000455
		ASV_1213	Actinobacteria	Microbacterium	-10.4	0.002577
		ASV_4616	Proteobacteria	NA	-10.3	0.002761
		ASV_229	Actinobacteria	NA	-10.0	0.024946
		ASV_5451	Actinobacteria	NA	-8.6	0.01499
		ASV_1275	Proteobacteria	Pseudomonas	27.3	7.64E-14
Rhizoplane	Non-fertilized	ASV_3331	Proteobacteria	Pseudomonas	26.7	1.44E-13
		ASV_4785	Proteobacteria	Pseudomonas	25.5	1.52E-12
		ASV_4083	Proteobacteria	Pseudomonas	25.1	3.06E-12
		ASV_4739	Proteobacteria	Pseudomonas	24.9	3.73E-12
		ASV_2155	Proteobacteria	NA	24.3	5.25E-12
		ASV_3921	Proteobacteria	NA	23.9	1.33E-11
		ASV_2852	Bacteroidetes	NA	23.6	3.10E-11

Appendix A

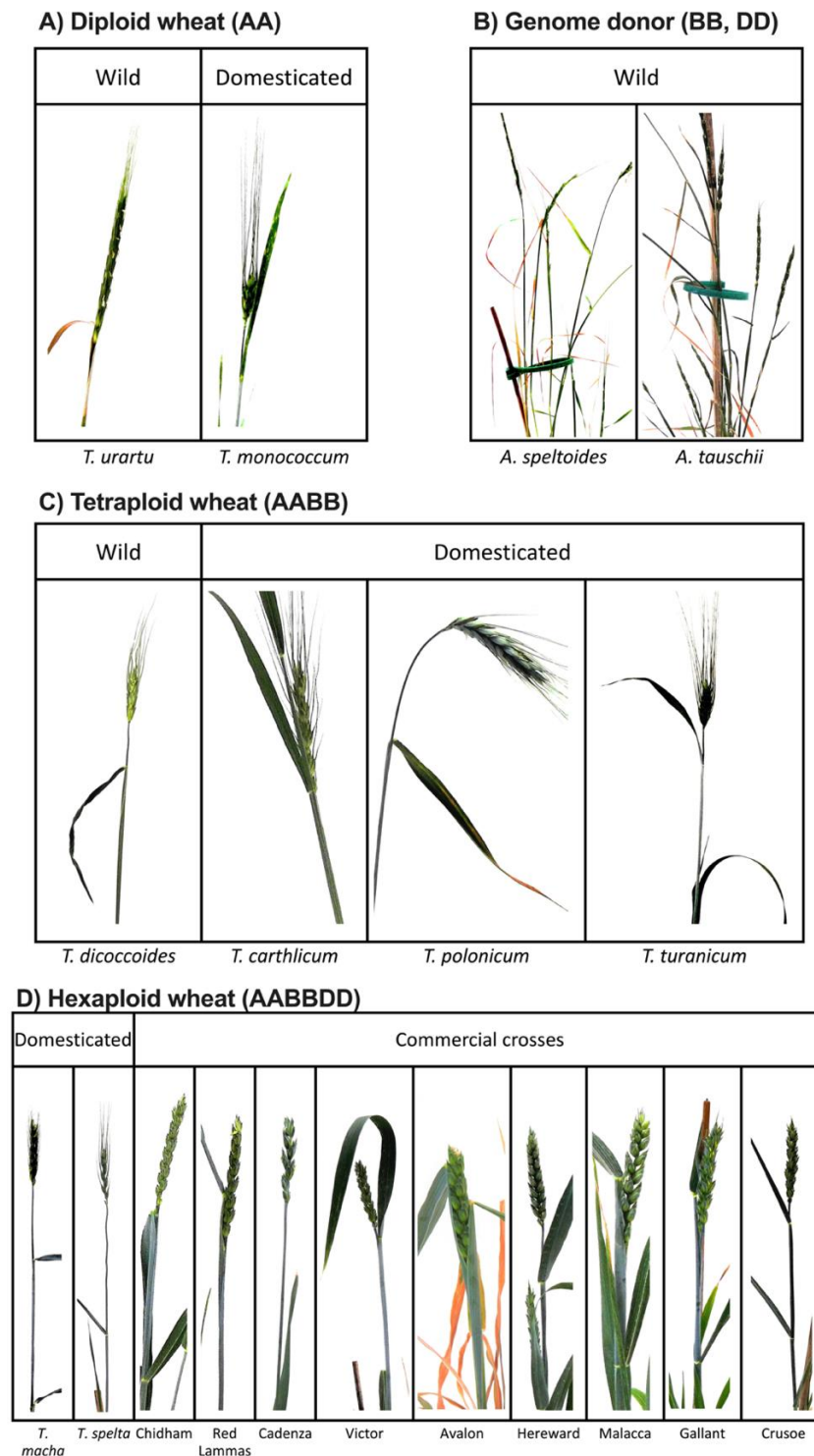
		ASV_1717	Proteobacteria	Pseudomonas	9.2	0.032949
		ASV_4593	Proteobacteria	Rhizobium	9.1	0.01636
	Fertilized	ASV_4049	Bacteroidetes	Pedobacter	- 23.4	4.66E-11
		ASV_1963	Actinobacteria	NA	-9.7	0.00347
		ASV_4187	Proteobacteria	Paraburkholderia	-9.4	0.016508
		ASV_5248	Proteobacteria	NA	-9.3	0.015383
		ASV_1979	Proteobacteria	Rhodanobacter	-8.7	0.028246
		ASV_2277	Proteobacteria	NA	-8.5	0.031228

A.4 References

1. Kavamura V. N., Hayat R., Clark I. M., Rossmann M., Mendes R., Hirsch P. R., et al., 2018. Inorganic Nitrogen Application Affects Both Taxonomical and Predicted Functional Structure of Wheat Rhizosphere Bacterial Communities. Front Microbiol, 9:1074.

Appendix B SUPPLEMENTARY INFORMATION FOR CHAPTER 3

B.1 SUPPLEMENTARY FIGURES



Supplementary Figure B-1: Wheat species chosen for this study.

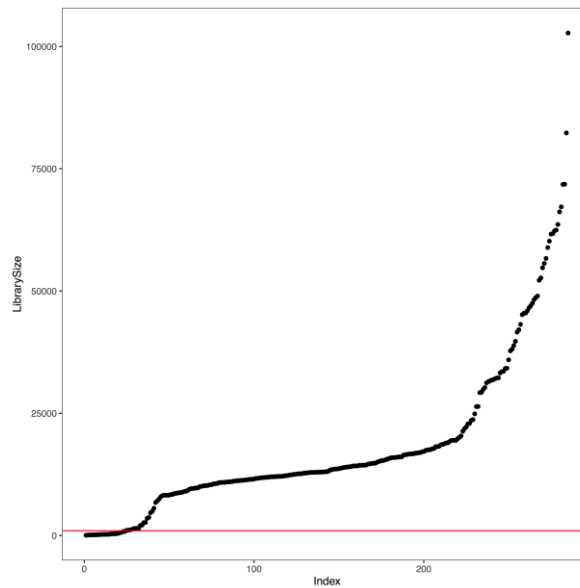
Appendix B



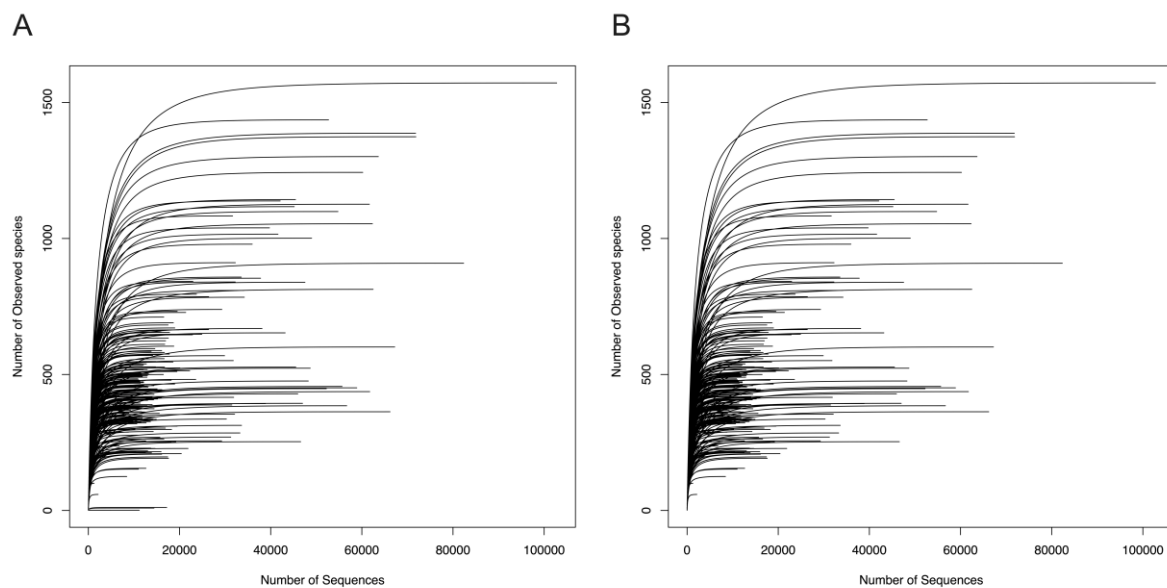
Supplementary Figure B-2: Two-factor randomized block design of experimental plant layout with fertilization (UnTreated and Treated) and plant variety (*A. speltoides*, *A. tauschii*, Bulk soil, *T. aestivum* (cv.: Avalon, Cadenza, Chidham, Crusoe, Gallant, Hereward, Malacca, Red Lamas, Victor), *T. carthlicum*, *T. dicoccoides*, *T. macha*, *T. monococcum*, *T. polonicum*, *T. spelta*, *T. turanicum*, *T. urartu*) as factors.

The design was split into four blocks based on greenhouse compartments. Each block consisted of 40 pots.

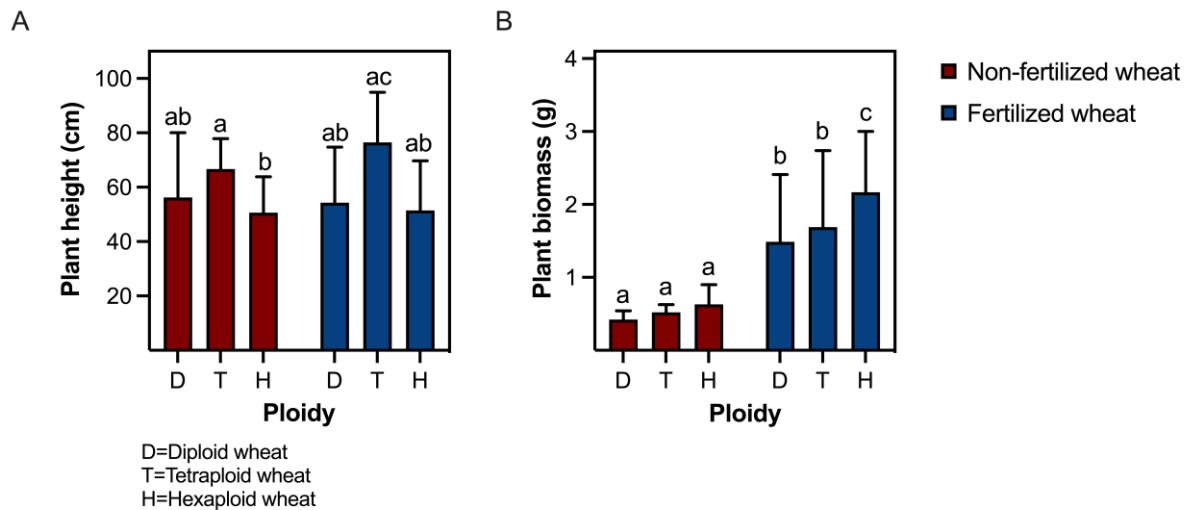
Appendix B



Supplementary Figure B-3: Overview of the bacterial 16S amplicon sequencing quality pre-processing: number of reads per sample (red line represents cut-off value for removing low quality samples and sequences).



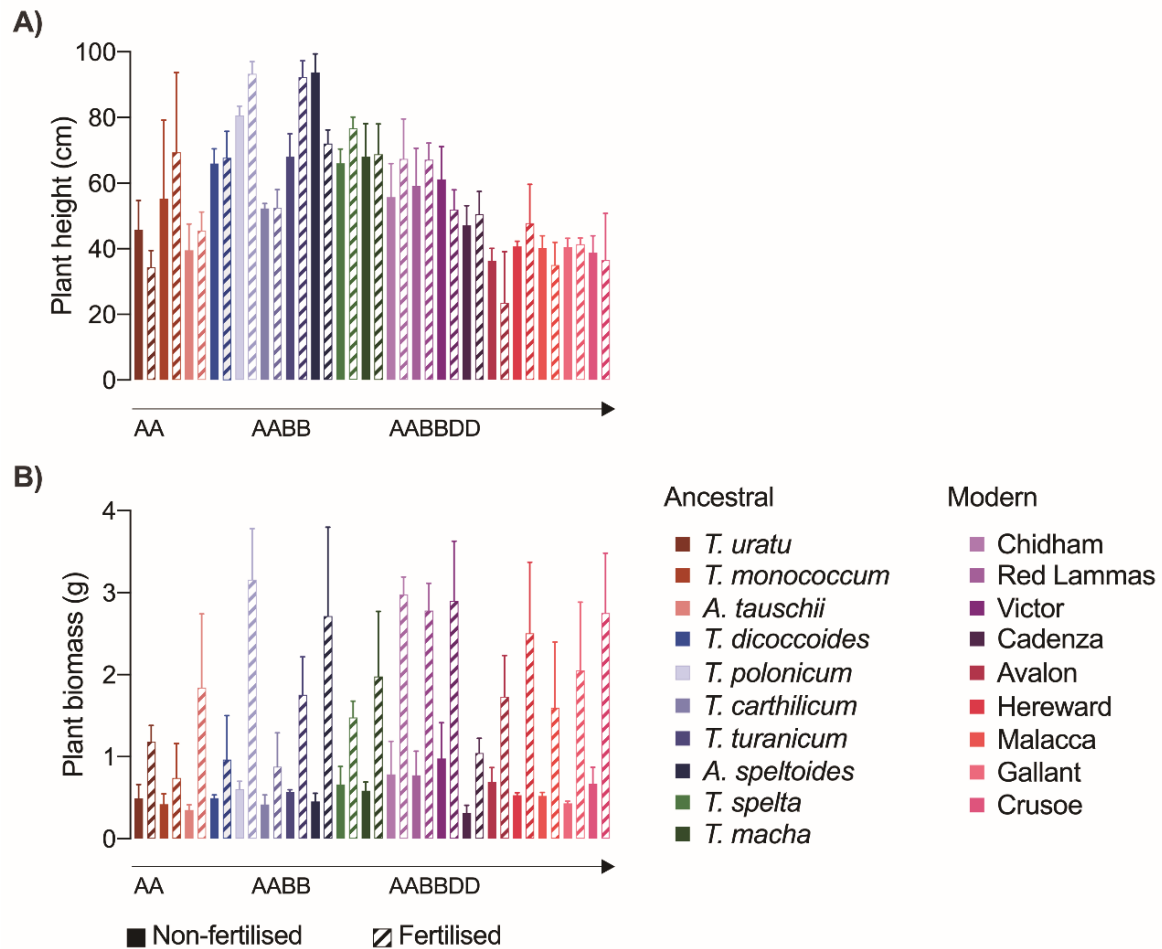
Supplementary Figure B-4: Rarefaction curve analyses of bacterial species richness as a function of sequencing depth pre-removal (A) and post-removal (B) of samples with low observed species number.



Supplementary Figure B-5: Plant height (A) and plant biomass (B) of diploid, tetraploid, and hexaploid wheat grown in nutrient-depleted agricultural soils with and without fertilizer addition.

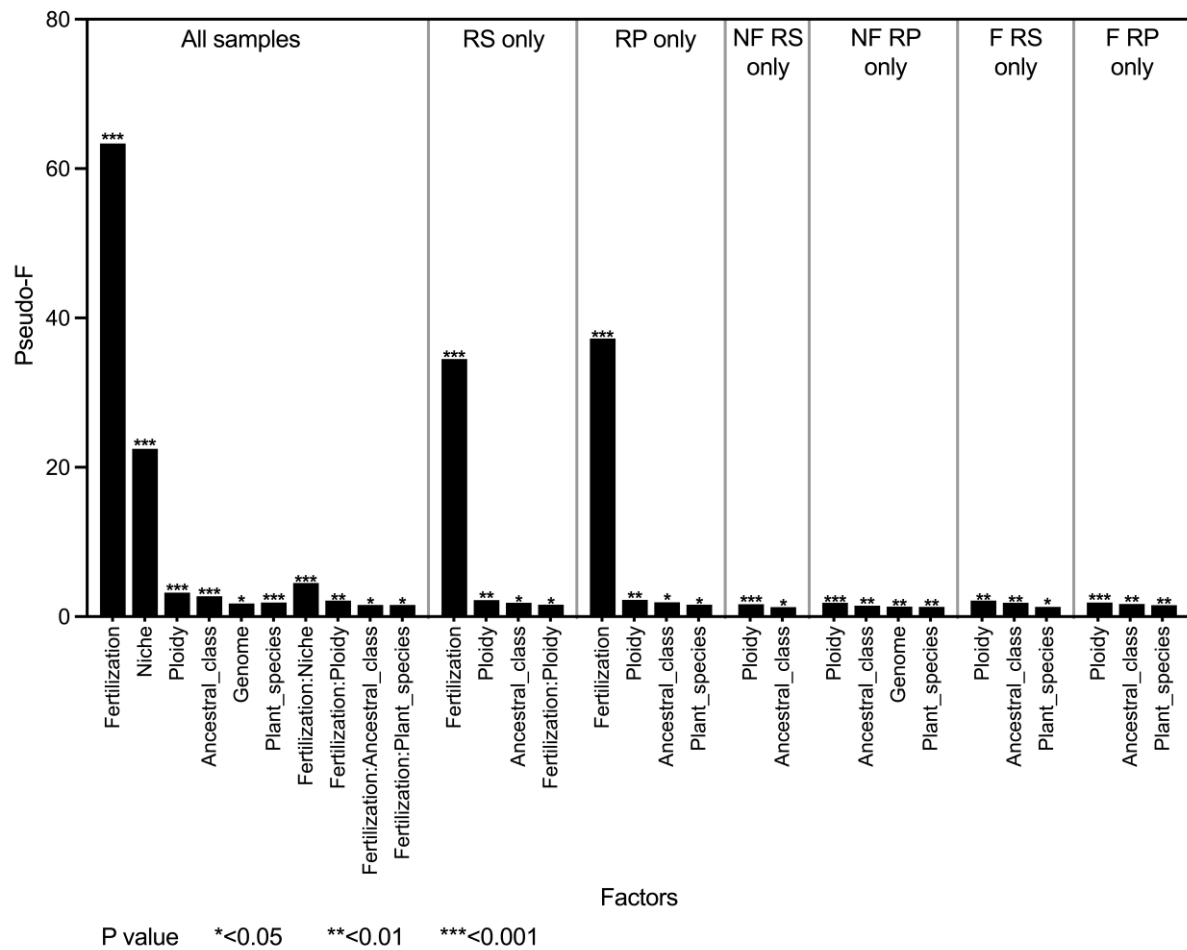
Statistically significant differences were determined by two-way ANOVA and different letters denote statistically significant differences according to pair-wise t-tests with Tukey and Šidák correction for plant height and biomass, respectively ($p < 0.05$). Plant height was measured from soil surface to head of longest stem and plant biomass was measured by dry foliar biomass. Pair-wise tests were performed within fertilization groups between diploid, tetraploid and hexaploid wheats and between non-fertilized and fertilized plants of the same cultivar.

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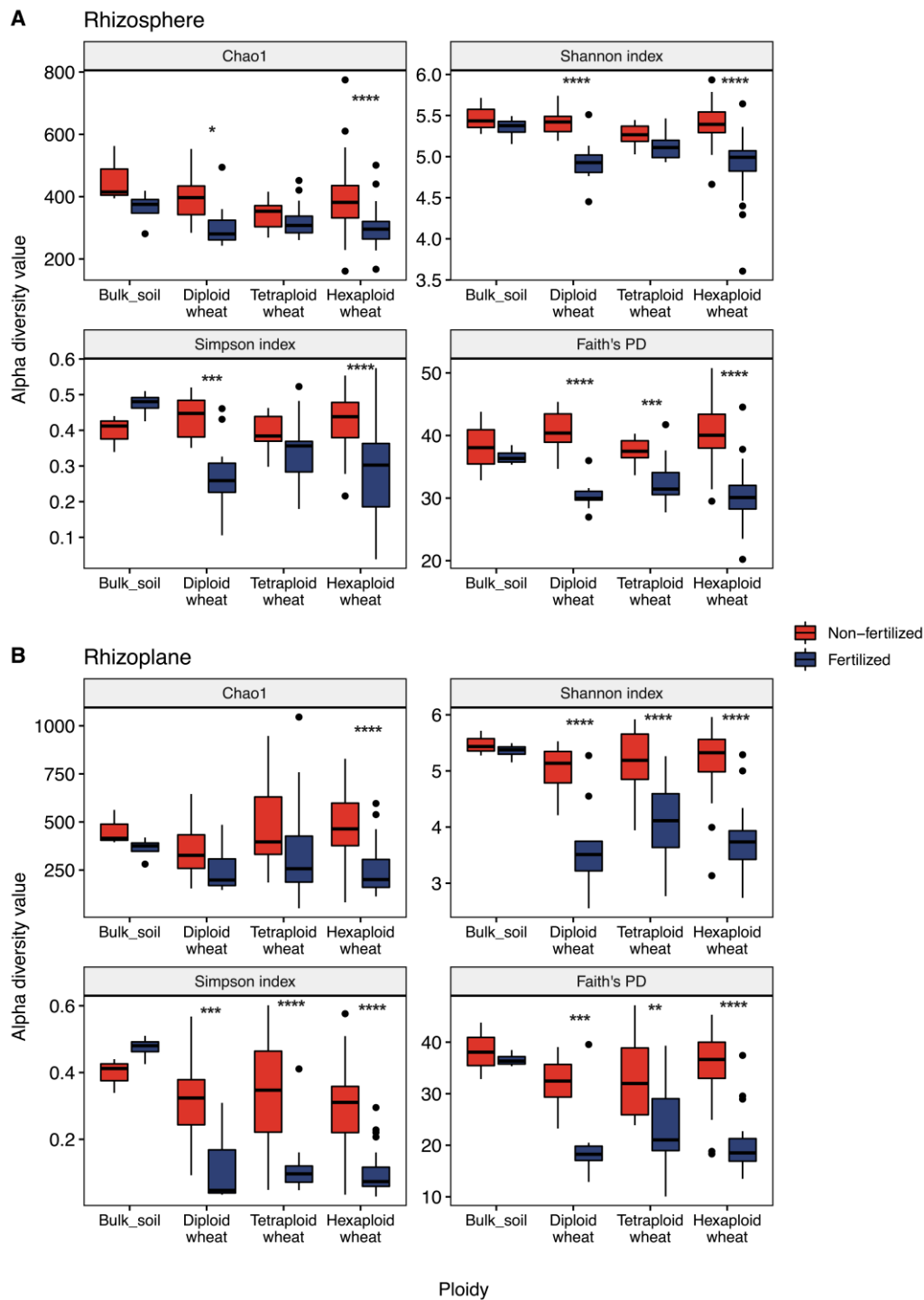
Supplementary Figure B-6: Height and dry biomass of ancestral and modern varieties of wheat grown in low nutrient soil with and without fertilizer addition. Bars show the mean A) height and B) dry plant biomass (aerial portion of plant) from 4 biological replicates; error bars show the standard deviation.

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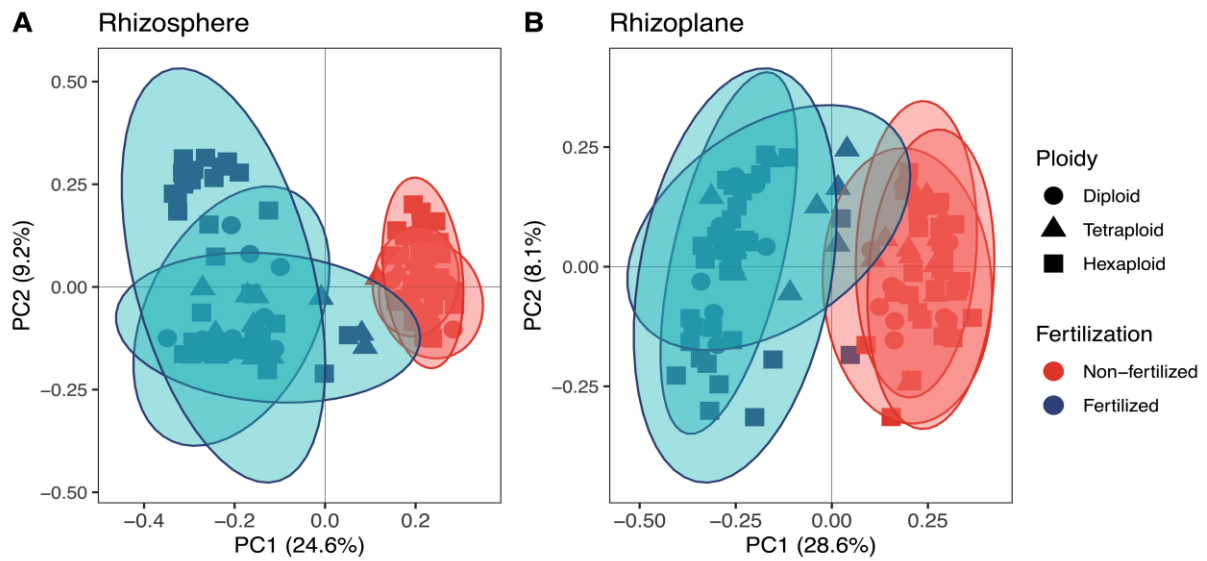


Supplementary Figure B-7: PERMANOVA Pseudo-F values for factors influencing rhizobacterial community. This is PERMANOVA type I (sequential) using Permutation of residuals under a reduced model as a permutation method using 9999 permutations. The ASV abundance has been standardized by DESeq2 median of ratios. Sample-to-sample similarity is based on Bray-Curtis similarity coefficient. This is non-nested multifactorial PERMANOVA.

Appendix B

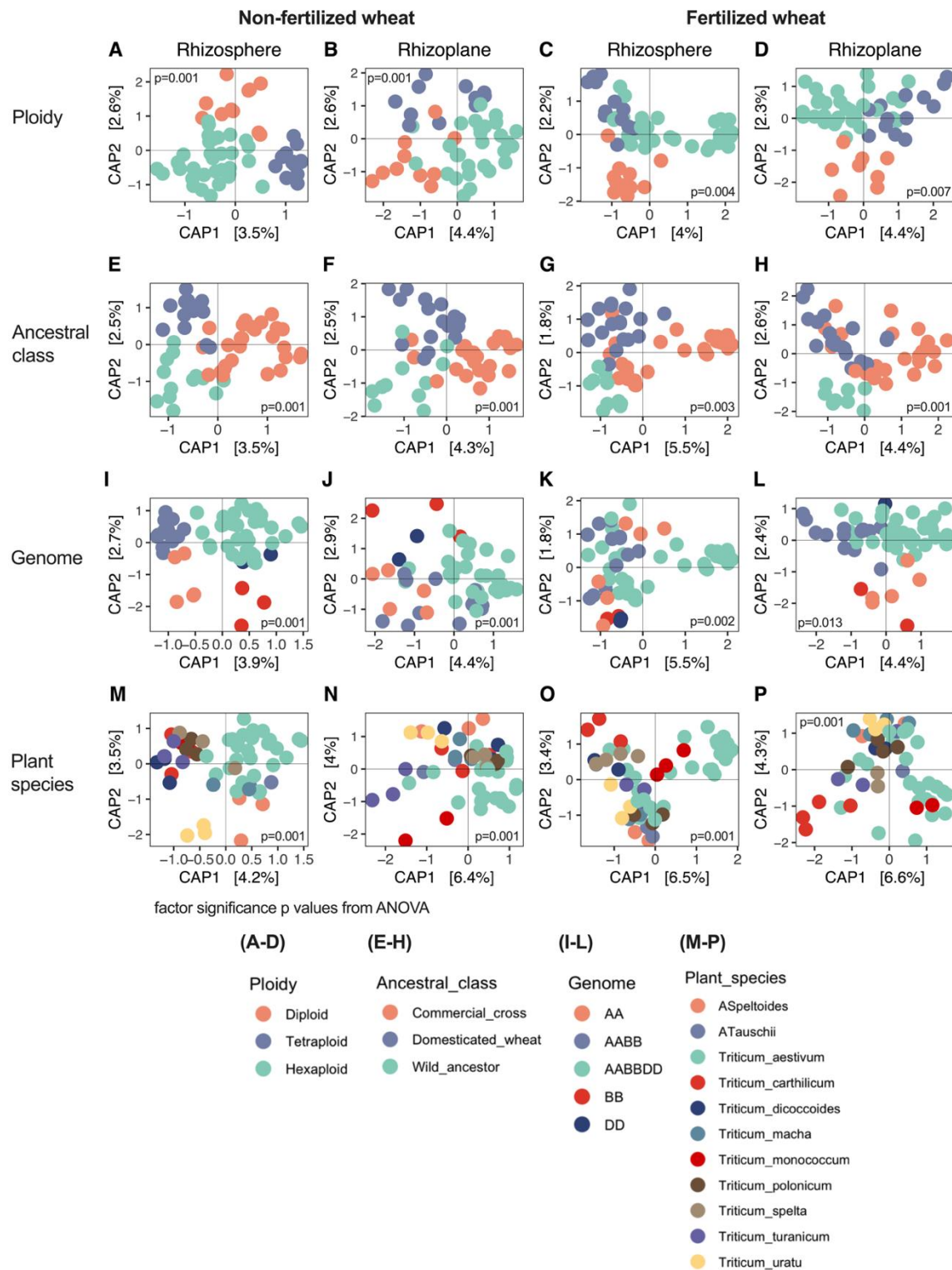


Supplementary Figure B-8: Alpha diversity estimates at ASV level for rhizosphere (A) and rhizoplane (B) bacterial communities in different wheat genotypes grown in low nutrient soil with and without the addition of fertilizer. Shown are the median (line) and hinges at first and third quartiles (25th and 75th percentiles) based on bacterial 16S-V3-V4 sequences. Significant differences as determined by two-way type III ANOVA are shown by “*”, “”, “***” and “****” for $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, between treatment groups.**

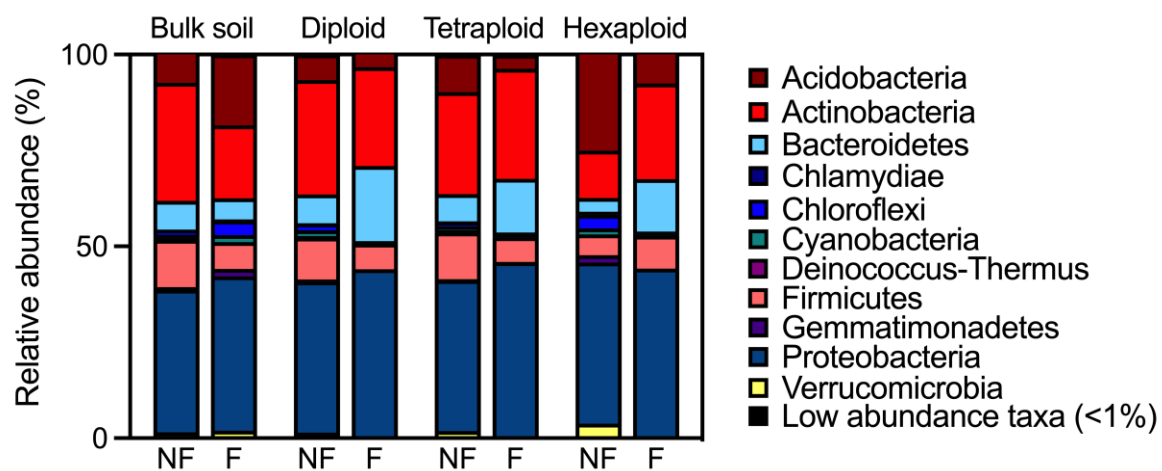


Supplementary Figure B-9: PCoA plot of bacterial composition based on Bray-Curtis distance for bacterial communities at ASV level in the (A) rhizosphere and (B) rhizoplane of diploid, tetraploid, and hexaploid wheat varieties grown under contrasting fertilization conditions. The percentage shown in each axis corresponds to the proportion of variation explained

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Supplementary Figure B-10: CAP analysis of the bacterial rhizosphere (A, E, I, and M) and rhizoplane (B, F, J, and N) community from non-fertilized wheat (first two columns) and the bacterial rhizosphere (C, G, K, and O) and rhizoplane (D, H, L, and P) community for fertilized wheat (last two columns) using factors as ploidy (A-D), ancestry class (E-H), genome (I-L), and plant species (M-P).



Supplementary Figure B-11: Relative abundance of phyla associated with bacterial communities in bulk soil and the root environment of diploid, tetraploid, and hexaploid wheat varieties grown under contrasting fertilization conditions.

B.2 SUPPLEMENTARY TABLES

Supplementary Table B-1: Factors shaping bacterial beta diversity in the wheat root microbiome.

Factors		Levels
Fertilization	<u>non-fertilized</u> , <u>fertilized</u>	2
Compartment	<u>bulk soil</u> , <u>rhizosphere</u> , <u>rhizoplane</u>	3
History	<u>wild ancestor</u> (<i>A. speltoides</i> , <i>A. tauschii</i> , <i>T. dicoccoides</i> , <i>T. urartu</i>), <u>traditional cultivar</u> (<i>T. carthlicum</i> , <i>T. macha</i> , <i>T. monococcum</i> , <i>T. polonicum</i> , <i>T. spelta</i> , <i>T. turanicum</i>), <u>commercial crosses</u> (Avalon, Cadenza, Chidham, Crusoe, Gallant, Hereward, Malacca, Red Llammas, Victor), <u>bulk soil</u>	4
Ploidy	<u>diploid</u> (<i>T. monococcum</i> , <i>T. urartu</i> , <i>A. speltoides</i> , <i>A. tauschii</i>), <u>tetraploid</u> (<i>T. carthlicum</i> , <i>T. dicoccoides</i> , <i>T. polonicum</i> , <i>T. turanicum</i>), <u>hexaploid</u> (<i>T. macha</i> , <i>T. spelta</i> , <i>T. aestivum</i> (Avalon, Cadenza, Chidham, Crusoe, Gallant, Hereward, Malacca, Red Llammas, Victor)), <u>bulk soil</u>	4
Genome	<u>AA</u> (<i>T. monococcum</i> , <i>T. urartu</i>), <u>AABB</u> (<i>T. carthlicum</i> , <i>T. dicoccoides</i> , <i>T. polonicum</i> , <i>T. turanicum</i>), <u>AABBDD</u> (<i>T. macha</i> , <i>T. spelta</i> , Avalon, Cadenza, Chidham, Crusoe, Gallant, Hereward, Malacca, Red Llammas, Victor), <u>BB</u> (<i>A. speltoides</i>), <u>DD</u> (<i>A. tauschii</i>), <u>bulk soil</u>	6
Plant species	<u><i>A. speltoides</i></u> , <u><i>A. tauschii</i></u> , <u><i>T. dicoccoides</i></u> , <u><i>T. urartu</i></u> , <u><i>T. carthlicum</i></u> , <u><i>T. monococcum</i></u> , <u><i>T. polonicum</i></u> , <u><i>T. spelta</i></u> , <u><i>T. turanicum</i></u> , <u><i>T. macha</i></u> , <u><i>T. spelta</i></u> , <u><i>T. aestivum</i></u> (Avalon, Cadenza, Chidham, Crusoe, Gallant, Hereward, Malacca, Red Llammas, Victor), <u>bulk soil</u>	11

Appendix B

PERMANOVA tables of type I, multifactorial not-nested design with 9999 permutations and blocking factor

Supplementary Table B-2: PERMANOVA – all samples

Factors	Df	SumOfSqs	R2	F	Pr(>F)	
Fertilization	1	9.484	0.1785	63.3852	0.0001	***
Niche	2	6.725	0.12657	22.4722	0.0001	***
Ploidy	2	0.967	0.0182	3.2314	0.0001	***
Ancestral_class	2	0.82	0.01543	2.7394	0.0001	***
Genome	2	0.523	0.00984	1.7467	0.0132	*
Plant_species	4	1.127	0.02122	1.8836	0.0006	***
Fertilization:Niche	2	1.346	0.02534	4.4984	0.0001	***
Fertilization:Ploidy	2	0.64	0.01204	2.1381	0.002	**
Niche:Ploidy	2	0.356	0.0067	1.1887	0.1885	
Fertilization:Ancestral_class	2	0.473	0.0089	1.5807	0.0342	*
Niche:Ancestral_class	2	0.314	0.0059	1.0483	0.3538	
Fertilization:Genome	2	0.434	0.00817	1.4512	0.0554	.
Niche:Genome	2	0.26	0.00489	0.8679	0.6343	
Fertilization:Plant_species	4	0.931	0.01752	1.5556	0.0104	*
Niche:Plant_species	4	0.606	0.01141	1.0131	0.4297	
Fertilization:Niche:Ploidy	2	0.304	0.00572	1.0157	0.3875	
Fertilization:Niche:Ancestral_class	2	0.263	0.00495	0.878	0.6348	
Fertilization:Niche:Genome	2	0.261	0.00492	0.8728	0.6166	
Fertilization:Niche:Plant_species	4	0.516	0.00972	0.8625	0.7572	
Residual	179	26.783	0.50408			
Total	224	53.132	1			

Significance codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

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Supplementary Table B-3: PERMANOVA – rhizosphere samples and rhizoplane samples

Niche	Factors	Df	SumOfSqs	R2	F	Pr(>F)	
Rhizosphere	Fertilization	1	5.1081	0.22013	34.4908	0.0001	***
	Ploidy	2	0.6573	0.02833	2.2191	0.0025	**
	Ancestral_class	2	0.5527	0.02382	1.8659	0.0128	*
	Genome	2	0.3548	0.01529	1.1979	0.1948	
	Plant_species	4	0.7336	0.03161	1.2383	0.1169	
	Fertilization:Ploidy	2	0.475	0.02047	1.6037	0.0381	*
	Fertilization:Ancestral_class	2	0.3763	0.01622	1.2705	0.1441	
	Fertilization:Genome	2	0.3491	0.01504	1.1786	0.2188	
	Fertilization:Plant_species	4	0.6767	0.02916	1.1423	0.2152	
	Residual	94	13.9214	0.59993			
	Total	115	23.205	1			
Rhizoplane	Fertilization	1	5.7312	0.25105	37.2411	0.0001	***
	Ploidy	2	0.6961	0.03049	2.2616	0.004	**
	Ancestral_class	2	0.5891	0.02581	1.9141	0.0144	*
	Genome	2	0.4079	0.01787	1.3254	0.0833	.
	Plant_species	4	0.9926	0.04348	1.6125	0.0121	*
	Fertilization:Ploidy	2	0.4583	0.02007	1.489	0.0722	.
	Fertilization:Ancestral_class	2	0.3658	0.01602	1.1884	0.2063	
	Fertilization:Genome	2	0.3534	0.01548	1.1483	0.1787	
	Fertilization:Plant_species	4	0.7694	0.0337	1.2499	0.1082	
	Residual	81	12.4653	0.54603			
	Total	102	22.8291	1			

Significance codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

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Supplementary Table B-4: PERMANOVA – non-fertilized rhizosphere, non-fertilized rhizoplane, fertilized rhizosphere, fertilized rhizoplane

Fertilization	Niche	Factors	Df	SumOfSqs	R2	F	Pr(>F)	
Non-fertilized	Rhizosphere	Ploidy	2	0.4726	0.05823	1.6728	0.0001	***
		Ancestral_class	2	0.3601	0.04436	1.2744	0.0262	*
		Genome	2	0.3318	0.04088	1.1743	0.1047	
		Plant_species	4	0.5945	0.07326	1.0522	0.313	
		Residual	45	6.3569	0.78327			
		Total	55	8.1159	1			
	Rhizoplane	Ploidy	2	0.5479	0.06842	1.8659	0.0002	***
		Ancestral_class	2	0.4186	0.05227	1.4254	0.0067	**
		Genome	2	0.3972	0.0496	1.3527	0.0094	**
		Plant_species	4	0.7715	0.09633	1.3136	0.0032	**
		Residual	40	5.8729	0.73338			
		Total	50	8.0081	1			
Fertilized	Rhizosphere	Ploidy	2	0.663	0.06642	2.1472	0.0015	**
		Ancestral_class	2	0.5703	0.05714	1.8471	0.0033	**
		Genome	2	0.3756	0.03763	1.2165	0.1683	
		Plant_species	4	0.8076	0.08091	1.3078	0.0451	*
		Residual	49	7.5645	0.75789			
		Total	59	9.981	1			
	Rhizoplane	Ploidy	2	0.6121	0.06733	1.9033	0.0005	***
		Ancestral_class	2	0.5421	0.05964	1.6857	0.0027	**
		Genome	2	0.3627	0.0399	1.1278	0.1776	
		Plant_species	4	0.9807	0.10788	1.5247	0.0019	**
		Residual	41	6.5924	0.72525			
		Total	51	9.0899	1			

Significance codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

Appendix B

PERMANOVA tables of type I, multifactorial nested design with 9999 permutations and blocking factor

Supplementary Table B-5: PERMANOVA – all samples

Factors	Df	SumOf Sqs	R2	F	Pr(>F)	
Fertilization	1	9.48	0.179	63.39	0.0001	***
Niche	2	6.73	0.127	22.47	0.0001	***
Ploidy	2	0.97	0.018	3.23	0.0001	***
Ancestral_class	2	0.82	0.015	2.74	0.0001	***
Fertilization:Niche	2	1.34	0.025	4.48	0.0001	***
Fertilization:Ploidy	2	0.65	0.012	2.15	0.0017	**
Niche:Ploidy	2	0.38	0.007	1.26	0.1389	
Ancestral_class:Genome	3	0.74	0.014	1.65	0.0076	**
Fertilization:Ancestral_class	2	0.48	0.009	1.59	0.034	*
Niche:Ancestral_class	2	0.32	0.006	1.07	0.3341	
Fertilization:Niche:Ploidy	2	0.31	0.006	1.03	0.371	
Ancestral_class:Genome:Plant_species	3	0.88	0.017	1.96	0.0006	***
Fertilization:Ancestral_class:Genome	3	0.62	0.012	1.39	0.0461	*
Niche:Ancestral_class:Genome	3	0.42	0.008	0.93	0.554	
Fertilization:Niche:Ancestral_class	2	0.27	0.005	0.90	0.6027	
Fertilization:Ancestral_class:Genome:Plant_species	3	0.74	0.014	1.64	0.0091	**
Niche:Ancestral_class:Genome:Plant_species	3	0.45	0.008	1.00	0.4474	
Fertilization:Niche:Ancestral_class:Genome	3	0.38	0.007	0.85	0.7297	
Fertilization:Niche:Ancestral_class:Genome:Plant_species	3	0.40	0.007	0.89	0.6764	
Residual	179	26.78	0.504			
Total	224	53.13	1			

Significance codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

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Supplementary Table B-6: PERMANOVA – rhizosphere samples and rhizoplane samples

Niche	Factors	Df	SumOfSqs	R2	F	Pr(>F)	
Rhizosphere	Fertilization	1	5.11	0.22	34.5	0.0001	***
	Ploidy	2	0.66	0.03	2.2	0.0033	**
	Ancestral_class	2	0.55	0.02	1.9	0.0130	*
	Fertilization:Ploidy	2	0.48	0.02	1.6	0.0328	*
	Ancestral_class:Genome	3	0.52	0.02	1.2	0.1980	
	Fertilization:Ancestral_class	2	0.38	0.02	1.3	0.1489	
	Ancestral_class:Genome:Plant_species	3	0.56	0.02	1.3	0.1214	
	Fertilization:Ancestral_class:Genome	3	0.50	0.02	1.1	0.2484	
	Fertilization:Ancestral_class:Genome:Plant_species	3	0.53	0.02	1.2	0.1850	
	Residual	94	13.92	0.60			
	Total	115	23.21	1.00			
Rhizoplane	Fertilization	1	5.73	0.25	37.2	0.0001	***
	Ploidy	2	0.70	0.03	2.3	0.0021	**
	Ancestral_class	2	0.59	0.03	1.9	0.0189	*
	Fertilization:Ploidy	2	0.47	0.02	1.5	0.0626	.
	Ancestral_class:Genome	3	0.63	0.03	1.4	0.0658	.
	Fertilization:Ancestral_class	2	0.37	0.02	1.2	0.2018	
	Ancestral_class:Genome:Plant_species	3	0.76	0.03	1.7	0.0149	*
	Fertilization:Ancestral_class:Genome	3	0.51	0.02	1.1	0.2094	
	Fertilization:Ancestral_class:Genome:Plant_species	3	0.61	0.03	1.3	0.0826	.
	Residual	81	12.47	0.55			
	Total	102	22.83	1.00			

Significance codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

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Supplementary Table B-7: PERMANOVA – non-fertilized rhizosphere, non-fertilized rhizoplane, fertilized rhizosphere, fertilized rhizoplane

Fertilization	Niche	Factors	Df	SumOfSqs	R2	F	Pr(>F)	
Non-fertilized	Rhizosphere	Ploidy	2	0.47	0.06	1.67	0.0001	***
		Ancestral_class	2	0.36	0.04	1.27	0.0274	*
		Ancestral_class:Genome	3	0.49	0.06	1.15	0.1031	
		Ancestral_class:Genome:Plant_species	3	0.44	0.05	1.04	0.3806	
		Residual	45	6.36	0.78			
		Total	55	8.12	1.00			
	Rhizoplane	Ploidy	2	0.55	0.07	1.87	0.0001	***
		Ancestral_class	2	0.42	0.05	1.43	0.0073	**
		Ancestral_class:Genome	3	0.54	0.07	1.24	0.0282	*
		Ancestral_class:Genome:Plant_species	3	0.62	0.08	1.42	0.0014	**
		Residual	40	5.87	0.73			
		Total	50	8.01	1.00			
Fertilized	Rhizosphere	Ploidy	2	0.66	0.07	2.15	0.0012	**
		Ancestral_class	2	0.57	0.06	1.85	0.0043	**
		Ancestral_class:Genome	3	0.53	0.05	1.15	0.2035	
		Ancestral_class:Genome:Plant_species	3	0.65	0.07	1.40	0.0313	*
		Residual	49	7.56	0.76			
		Total	59	9.98	1.00			
	Rhizoplane	Ploidy	2	0.61	0.07	1.90	0.0007	***
		Ancestral_class	2	0.54	0.06	1.69	0.002	**
		Ancestral_class:Genome	3	0.60	0.07	1.23	0.0805	.
		Ancestral_class:Genome:Plant_species	3	0.75	0.08	1.55	0.0009	***
		Residual	41	6.59	0.73			
		Total	51	9.09	1.00			

Significance codes: 0.0001 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

Appendix B

Supplementary Table B-8: ANOVA results table to assess the importance of fertilizer (no fertilizer vs. fertilizer) and niche (bulk soil, rhizosphere, rhizoplane) on rhizobacterial alpha diversity as represented by Chao1, Shannon diversity index, Simpson reciprocal, and Faith's Phylogenetic Diversity. This is a two-way type III ANOVA.

Value	Sources of Variation	Degrees of Freedom	Sum of Squares	F statistic	P value	Statistical Significance
Chao1	Interaction	2	196 999	5.27	0.006	**
	Niche	2	50 571	1.35	0.26	ns
	Fertilizer	1	203 218	10.9	0.001	**
	Residual	238	4x10 ⁶			
Shannon index	Interaction	2	13.4	32.8	<0.0001	****
	Niche	2	29.4	71.9	<0.0001	****
	Fertilizer	1	5.31	26	<0.0001	****
	Residual	238	48.6			
Simpson index	Interaction	2	0.17	8	0.0004	***
	Niche	2	1.6	75.2	<0.0001	****
	Fertilizer	1	0.09	8.67	0.004	**
	Residual	238	2.54			
Faith's PD	Interaction	2	458	8.31	0.0003	***
	Niche	2	3 874	70.4	<0.0001	****
	Fertilizer	1	902	32.8	<0.0001	****
	Residual	238	6 553			

Significance codes: 0.0001 '****', 0.001 '***', 0.01 '**', 0.05 '*', 0.1 '.', 1 ''

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Supplementary Table B-9: ANOVA results table to assess the importance of fertilizer (no fertilizer vs. fertilizer) and ploidy (bulk soil, diploid, tetraploid, hexaploid) on rhizobacterial alpha diversity as represented by Chao1, Shannon diversity index, Simpson reciprocal, and Faith's Phylogenetic Diversity. This is a two-way type III ANOVA.

Niche	Value	Sources of Variation	Degrees of Freedom	Sum of Squares	F statistic	P value	Statistical Significance
Rhizo-sphere	Chao1	Interaction	3	28 406	2.66	0.25	ns
		Ploidy	3	30 978	2.91	0.21	ns
		Fertilizer	1	94 681	8.88	0.0003	***
		Residual	120	811 355			
	Shannon index	Interaction	3	0.57	2.68	0.05	ns
		Ploidy	3	0.48	2.26	0.08	ns
		Fertilizer	1	1.48	20.8	<0.0001	****
		Residual	120	8.44			
	Simpson index	Interaction	3	0.10	3.88	0.01	*
		Ploidy	3	0.04	1.38	0.25	ns
		Fertilizer	1	0.08	9.20	0.003	**
		Residual	120	1.06			
	Faith's PD	Interaction	3	228	4.88	0.003	**
		Ploidy	3	29.6	0.63	0.59	ns
		Fertilizer	1	754	48.4	<0.0001	****
		Residual	120	1866			
Rhizo-plane	Chao1	Interaction	3	96 692	1.04	0.38	ns
		Ploidy	3	165 278	1.78	0.16	ns
		Fertilizer	1	347 457	11.23	0.01	**
		Residual	109	3x106			
	Shannon index	Interaction	3	3.03	2.89	0.04	*
		Ploidy	3	6.49	6.20	0.0006	***
		Fertilizer	1	16.0	45.8	<0.0001	****
		Residual	109	38.0			
	Simpson index	Interaction	3	0.04	3.20	0.03	*
		Ploidy	3	0.12	9.36	<0.0001	****
		Fertilizer	1	0.28	21.7	<0.0001	****
		Residual	109	0.01			
	Faith's PD	Interaction	3	399	3.39	0.02	*
		Ploidy	3	591	5.02	0.003	**
		Fertilizer	1	1337	34.1	<0.0001	****
		Residual	109	4 237			

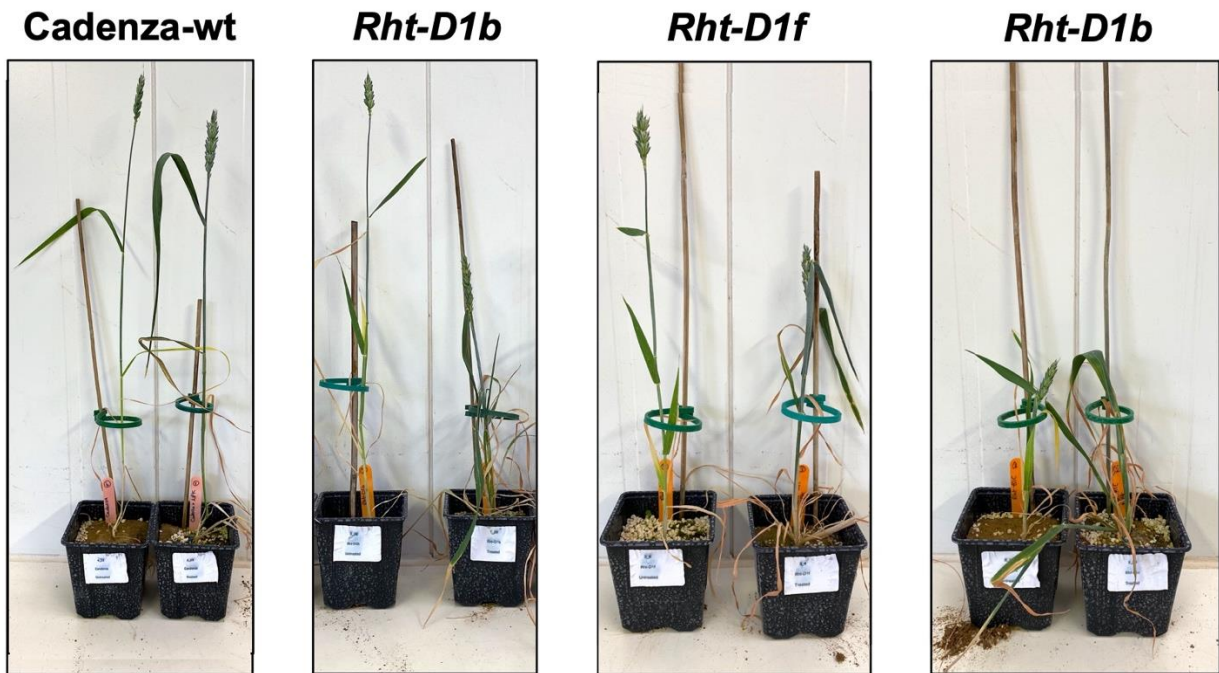
Significance codes: 0.0001 '****', 0.001 '***', 0.01 '**', 0.05 '*', 0.1 '.', 1 'ns'

Supplementary Table B-10: Number of isolates that tested positive or negative for solubilization of key plant nutrients, isolated from bulk soil and diploid, tetraploid and hexaploid wheats grown in soils with and without fertilizer addition.

	Functional Bioassay	Sample no	No. of isolates			
			Non-fertilized		Fertilized	
			(n)	+ve	-ve	+ve
Bulk soil	Solubilization					
	Nitrate (Casein)		107	269	92	284
	Phosphate		56	320	12	364
	Potassium	8	68	308	25	351
	Iron		40	336	0	376
	Zinc		0	376	4	372
Total			164	212	41	335
Diploid wheat	Solubilization					
	Nitrate (Casein)		499	817	220	1096
	Phosphate		249	1067	83	1233
	Potassium	28	410	906	174	1142
	Iron		753	563	387	929
	Zinc		59	1257	67	1249
Total			1161	155	711	605
Tetraploid wheat	Solubilization					
	Nitrate (Casein)		541	963	410	1094
	Phosphate		158	1346	32	1472
	Potassium	32	400	1104	69	1435
	Iron		613	891	80	1424
	Zinc		112	1392	118	1386
Total			1283	221	299	1205
Hexaploid wheat	Solubilization					
	Nitrate (Casein)		1371	2577	1140	2808
	Phosphate		763	3185	165	3783
	Potassium	84	1050	2898	311	3637
	Iron		1720	2228	537	3411
	Zinc		157	3791	214	3734
Total			3690	258	1227	2721

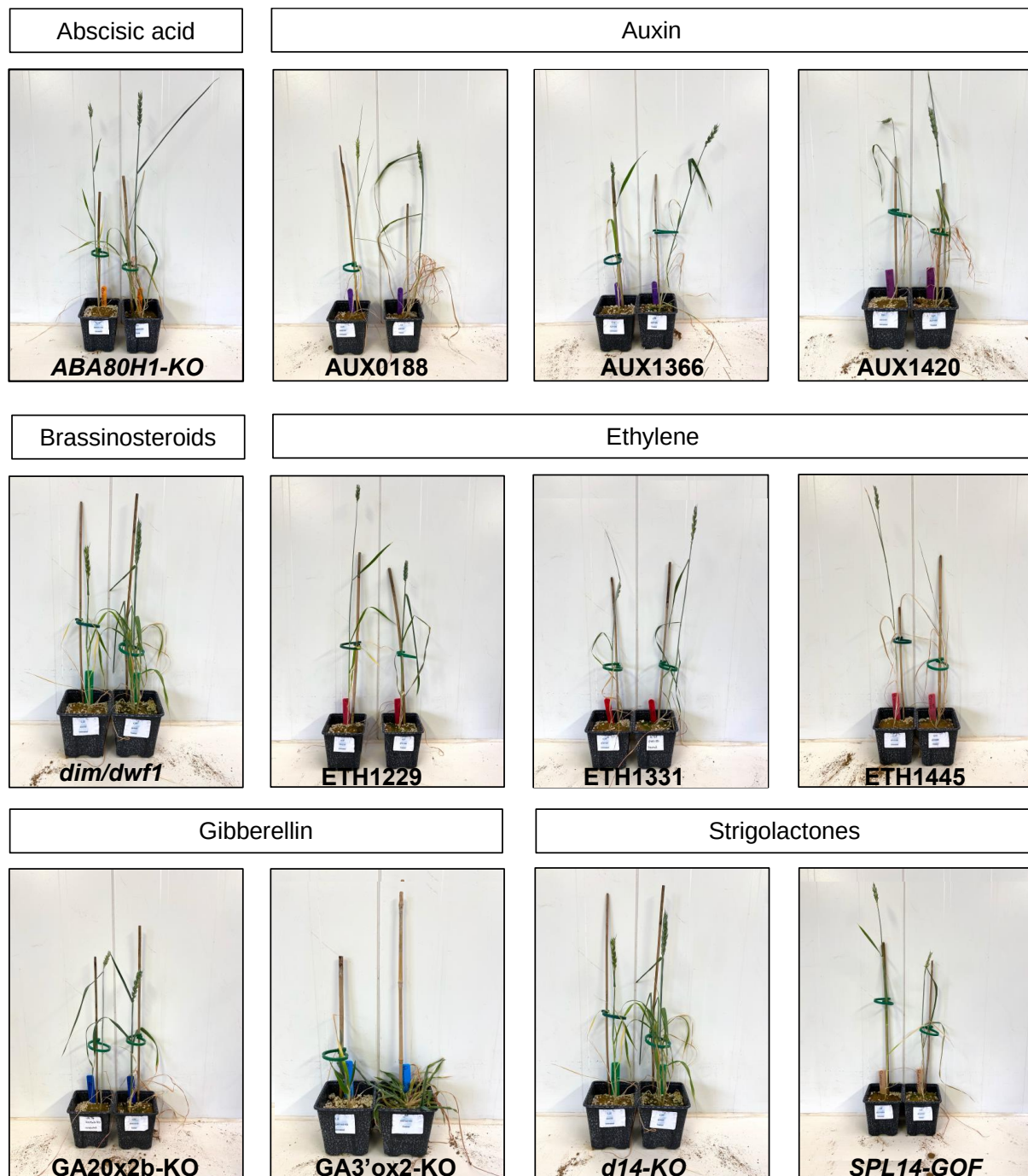
Appendix C SUPPLEMENTARY INFORMATION FOR CHAPTER 4

C.1 SUPPLEMENTARY FIGURES

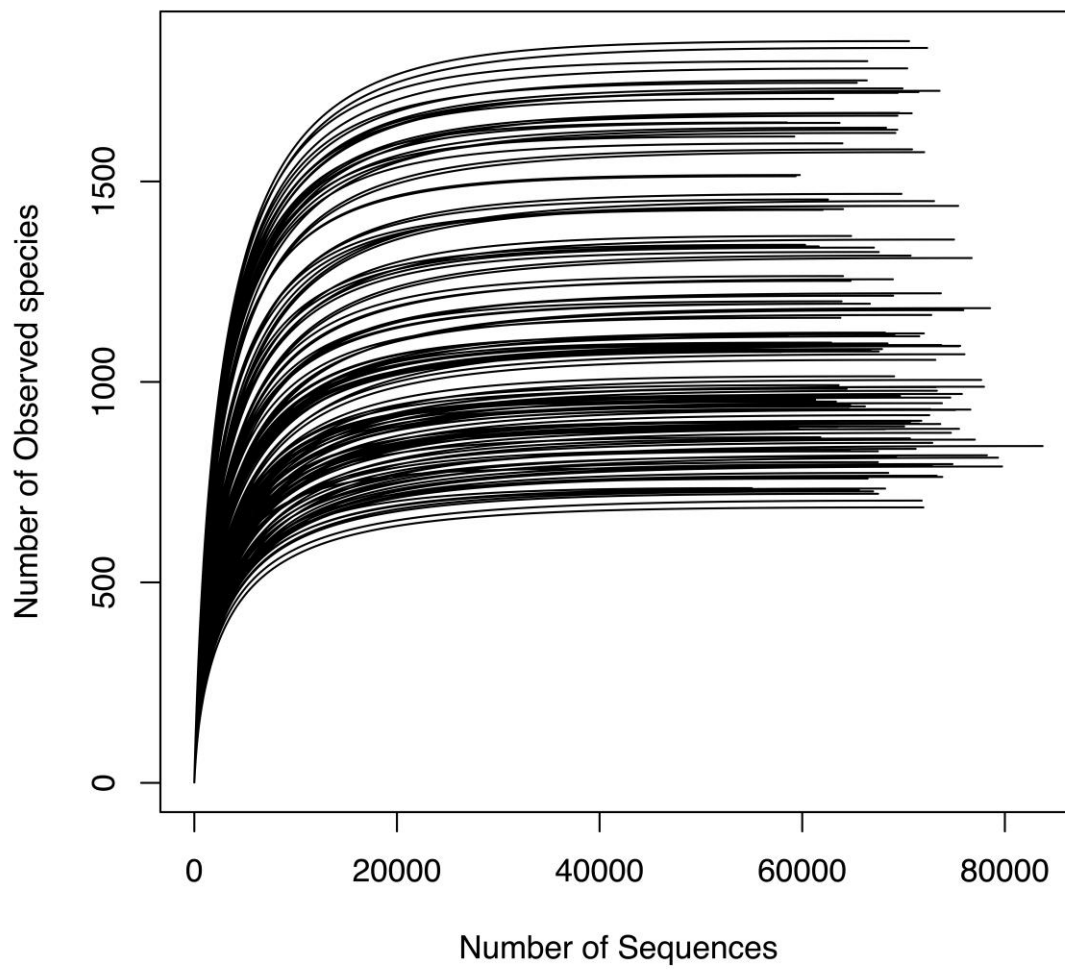


Supplementary Figure C-1: Cadenza wild-type (*wt*) and *Rht* (*Reduced-height*) mutant wheat (*Rht-D1b*, *Rht-D1f*, *Rht-B1c*) plants grown in pots with and without fertilizer addition (non-fertilized pots on the left; fertilized pots of the right of each image).

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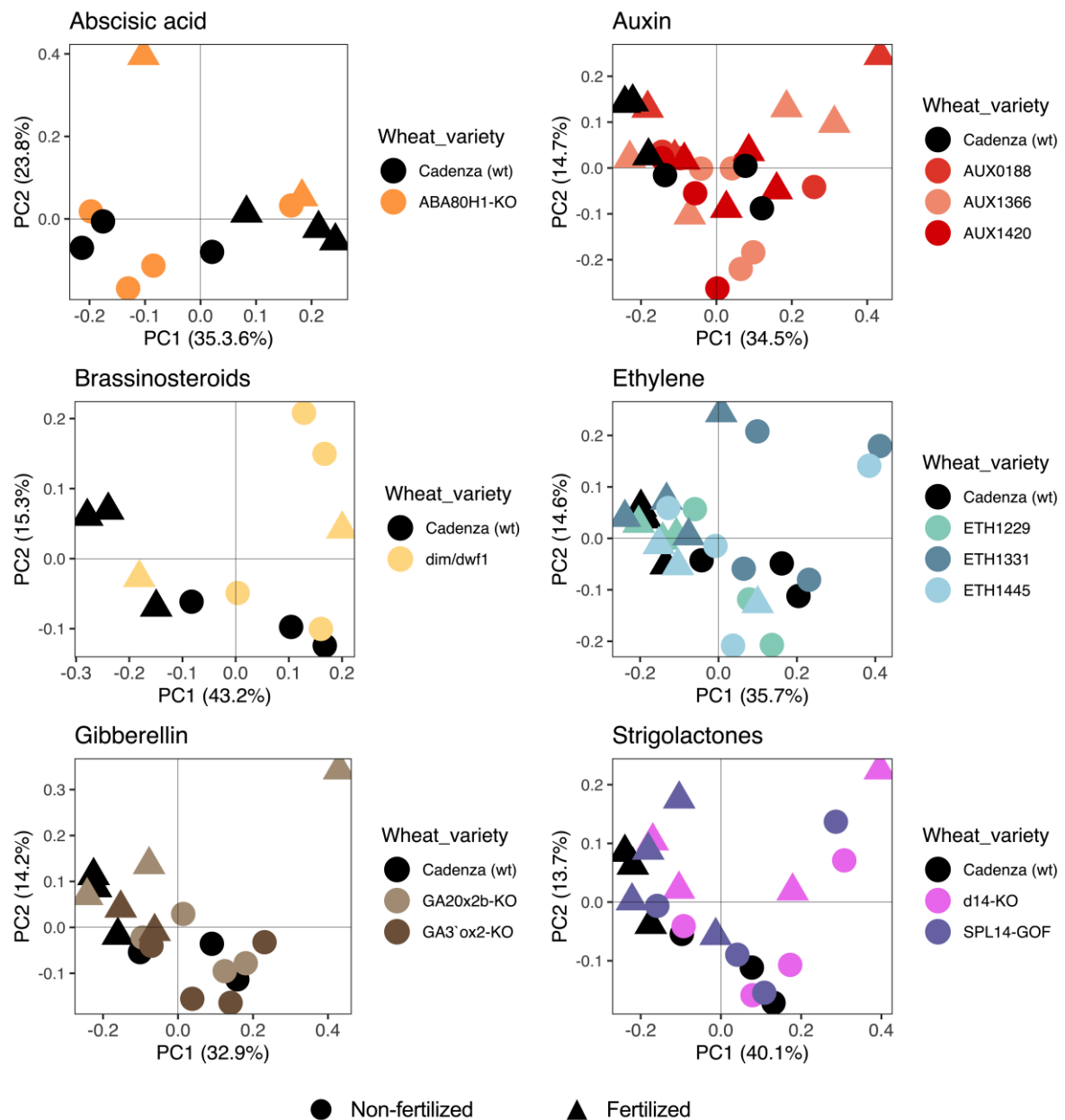


Supplementary Figure C-2: Wheat hormone signalling mutants in abscisic acid (*ABA80H1-KO*), auxin (*AUX0188*, *AUX1366*, *AUX1420*), brassinosteroids (*dim/dwf1*), ethylene (*ETH1229*, *ETH1331*, *ETH1445*), gibberellin (*GA20x2b-KO*, *GA3'ox2-KO*), and strigolactones (*d14-KO*, *SPL14-GOF*) plants grown in pots with and without fertilizer addition (non-fertilized pots on the left; fertilized pots of the right of each image).



Supplementary Figure C-3: Rarefaction curve analyses of bacterial species richness as a function of sequencing depth.

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Supplementary Figure C-4: Principal coordinates of analysis (PCoA) plots created using Bray-Curtis dissimilarity distance matrix grouped by fertilization and wheat variety for wheat hormone signalling mutants in abscisic acid, auxin, brassinosteroids, ethylene, gibberellin, and strigolactones.

Appendix D CONFERENCE ABSTRACTS, PAPERS, PRESENTATIONS

MMEG2019 Conference – Presentation and Poster

Won FEMS Microbiology Letters Poster Prize 2019

https://fems-microbiology.org/about_fems/network-and-activities/awards/poster-oral-presentation-prizes/fems-microbiology-letters-poster-prize-jess-palmer-tessa-reid/

EFFECT OF CHEMICAL FERTILIZER ON PLANT GROWTH-PROMOTING RHIZOBACTERIA IN WHEAT

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Wheat is the third most cultivated cereal in the world however, farming of commercial varieties relies on the application of chemical fertilizers to maintain yields. Here, we investigated how the addition of nitrogen-phosphorous-potassium (NPK) fertilizer affects bacterial community composition, diversity, and functionality within rhizocompartments of wheat. Rhizosphere and rhizoplane samples from wheat grown with and without NPK fertilizer were collected and their microbiomes characterized using both culture -independent and -dependent methods. We found that the addition of NPK fertilizer reduced rhizobacterial diversity and altered community composition. Furthermore, fertilizer addition decreased the number of plant growth-promoting rhizobacteria isolated from the wheat root microbiome. Functional bioassays revealed that the abundance of bacterial isolates capable of solubilising phosphate, potassium and iron were significantly reduced when inorganic fertilizer was applied. Furthermore, there was an increased selection of beneficial microbes in the rhizoplane when compared with the rhizosphere of wheat roots. The analyses of 16S rRNA genes and deduced functional profiles provide an in-depth understanding of the responses of bacterial communities to fertilizer. This knowledge will benefit the development of

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agricultural practices which are less dependent on interventions such as fertilizer application.

Effect of Chemical Fertilizer on Plant Growth-Promoting Rhizobacteria in Wheat

[ICEM 2021](#) Conference – Presentation

Won ICEM 2021: XV. award for presenting outstanding work

Tessa E. Reid, Vanessa N. Kavamura, Maïder Abadie, Adriana Torres-Ballesteros, Mark Pawlett, Ian M. Clark, Jim Harris, Tim Mauchline

Abstract—The deleterious effect of chemical fertilizer on rhizobacterial diversity has been well documented using 16S rRNA gene amplicon sequencing and predictive metagenomics. Biofertilization is a cost-effective and sustainable alternative, improving strategies depends on isolating beneficial soil microorganisms. Although culturing is widespread in biofertilization, it is unknown whether the composition of cultured isolates closely mirrors native beneficial rhizobacterial populations. This study aimed to determine the relative abundance of culturable plant growth-promoting rhizobacteria (PGPR) isolates within total soil DNA and how potential PGPR populations respond to chemical fertilization in a commercial wheat variety. It was hypothesized that PGPR will be reduced in fertilized relative to unfertilized wheat. *Triticum aestivum* cv. Cadenza seeds were sown in a nutrient depleted agricultural soil in pots treated with and without nitrogen-phosphorous-potassium (NPK) fertilizer. Rhizosphere and rhizoplane samples were collected at flowering stage (10 weeks) and analyzed by culture-independent (amplicon sequence variance (ASV) analysis of total rhizobacterial DNA) and -dependent (isolation using growth media) techniques. Rhizosphere- and rhizoplane-derived microbiota culture collections were tested for plant growth-promoting traits using functional bioassays. In general, fertilizer addition decreased the proportion of nutrient-solubilizing bacteria (nitrate, phosphate, potassium, iron and, zinc) isolated from rhizocompartments in wheat whereas salt tolerant bacteria were not affected. A PGPR database was created from isolate 16S rRNA gene sequences and searched against total soil DNA, revealing that 1.52% of total community ASVs were identified as culturable PGPR isolates. Bioassays

identified a higher proportion of PGPR in non-fertilized samples (rhizosphere (49%) and rhizoplane (91%)) compared to fertilized samples (rhizosphere (21%) and rhizoplane (19%)) which constituted approximately 1.95% and 1.25% in non-fertilized and fertilized total community DNA, respectively. The analyses of 16S rRNA genes and deduced functional profiles provide an in-depth understanding of the responses of bacterial communities to fertilizer; this study suggests that rhizobacteria which potentially benefit plants by mobilizing insoluble nutrients in soil are reduced by chemical fertilizer addition. This knowledge will benefit the development of more targeted biofertilization strategies.

Keywords—Bacteria, fertilizer, microbiome, rhizoplane, rhizosphere.

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