

Drought Tolerant Wheat Varieties Enrich Seed Microbiomes For Beneficial Microbes Under Drought Conditions

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Abstract

Climate change is predicted to increase the incidence and severity of drought conditions, posing a significant challenge for agriculture globally. Plant microbiomes have been demonstrated to aid crop species mitigate drought stress. In this study the wheat seed microbiomes from lines with contrasting drought tolerances were interrogated for microbes that alleviate drought stress. Metagenomic and culture-based methods were used to profile and characterise the seed microbiome composition of four drought tolerant and three drought susceptible wheat lines under rainfed and drought conditions. *Curtobacterium flaccumfaciens* (Cf D3-25) and *Arthrobacter* sp. (Ar sp. D4-14) were isolates enriched in drought tolerant lines under drought conditions and demonstrated the ability to promote wheat growth under drought conditions. Members of Triticeae inoculated with Cf D3-25 and Ar sp. D4-14 showed a biostimulation affect. This study indicates seed microbiomes from genetically distinct wheat lines enrich for beneficial bacteria in ways that are both line-specific and responsive to environmental stress. As such, seed from stress-phenotyped lines represents an invaluable resource for the identification of beneficial microbes with plant growth promoting activity that could improve commercial crop production, particularly under drought stress.

Introduction

Drought poses significant challenges to agricultural production in arid and semi-arid regions of the globe. Increases in mean global temperatures and decreased frequency and intensity of precipitation, as a result of climate change, are extending periods of drought and decreasing crop yields, highlighting the increasing need for abiotic stress tolerance in crop species [2]. *Triticum aestivum* (wheat) is grown on approximately 220 million hectares of land globally, producing 20% of the world's caloric requirements [3]. In Australia, drought conditions have had severe impacts on wheat production, reducing the winter harvest to 35% below the ten year average in 2019 [4, 5]. Research into the mechanisms that increase drought tolerance in crop plants focused primarily on improving crop genetics through techniques such as QTL mapping, marker assisted breeding and introgression from wild species [6-9]. The identification of drought tolerance with high heritability is confounded by complex genotype by environment interactions [7-10]. Envirotyping is a comprehensive method of measuring environmental factors with the capacity to affect phenotypic variation in plant growth designed to aid genotype by environment (GEI) and phenotype prediction [11]. Analysis of arid and drought envirotypes has identified microbiome composition as an environmental factor that affects drought tolerance in crop plants [11, 12]. As a result, there is increasing interest in interrogating the plant microbiome with the goal of identifying microbes that augment the phenotype conferred by the genome of the host plant and hence confer drought tolerance [13-17].

Plants form intimate relationships with microbes that colonise their tissues and organs, forming a single ecological unit known as a holobiont [18]. These microbiomes have been demonstrated to influence a myriad of plant traits such as germination, biomass accumulation, pathogen resistance and abiotic stress tolerance [19]. Seeds harbor the initial inoculum of microbes, playing a vital role in the transmission of microbial resources between plant generations [20]. As the initial colonisers of plant

tissue, the seed microbiome is believed to shape the overall composition of the plant microbiome and therefore have a competitive advantage over microbes recruited from soil and root [21, 22]. A comparatively limited number of bacterial genera have been detected in seed microbiomes compared to other plant microbiomes, such as root microbiome [23]. It is postulated that the seed microbiome evolves as a result of host selection for traits that complement the plant genome [18, 23]. While root microbiomes have been the focus of significant study, the seed microbiome has largely been overlooked [24].

Studies into the influence of drought on plant microbiomes have focused almost exclusively on root microbiomes [12, 16, 25, 26]. Several gram-positive rhizosphere bacteria have been demonstrated to be effective in improving drought stress tolerance in crop plants [27-29]. Timmusk et al, demonstrated that wheat treated with *Paenibacillus* and *Bacillus* species isolated from arid environments increased plant biomass by up to 78% and increased the plant survival rate five-fold under severe drought conditions [30]. Notably, *Paenibacillus* and *Bacillus* species isolated from moderate drought environments did not increase drought tolerance in wheat [30, 31]. The consensus amongst many plant microbiome studies is that drought conditions have a significant impact on the composition and diversity of the microbiome, often leading to a marked increase in *Actinobacteria* in root microbiomes [12, 16, 26]. It is clear from these studies that recruitment and enrichment of certain bacterial elements in root microbiomes were driven by host-specific metabolic and phenotypic factors [12, 26]. Further to this, microbiomes have also shown cultivar-specific enrichment in root microbiomes of rapeseed, cannabis and wheat [14, 17, 32]. We hypothesise that seed microbiomes from plants exposed to abiotic stress enrich for bacteria that are more tolerant of abiotic stress, and that the composition of seed microbiomes differ between drought susceptible and drought tolerant wheat lines.

In this study, we present the first analysis of seed microbiomes from contrasting drought tolerant and drought susceptible wheat lines under drought and rainfed conditions, with the aim of identifying microbes that are capable of increasing drought tolerance in a host plant. By interrogating the seed microbiome *in silico*, endophytes can be identified and isolated that have the potential to increase the drought tolerance of crop species at a commercial level.

Methods

Seed source

Seeds from eleven lines of *Triticum aestivum* were sourced from the Grains Innovation Park (DJPR) in Horsham, Victoria. These seeds were harvested in 2017 from a field trial where 43 wheat lines were examined for drought response. Each line was subjected to rainfed and drought conditions, simulated using rainout shelters. The drought susceptibility index (DSI) was calculated for each line, using yield differences under drought and rainfed conditions. The lines were subsequently classed as either drought tolerant, or drought susceptible.

Culturing the seed microbiome

Microbial isolation

For microbial isolation, a subset of six lines was chosen from the eleven lines that had been profiled. Bacteria were isolated from the seeds of four drought tolerant wheat lines (lines 1, 2, 3 and 4) and two drought susceptible wheat lines (lines 10 and 11). Ten seeds from each treatment were plated onto stacked pieces of sterile filter paper soaked in Nystatin (50mg L^{-1}). These seeds were germinated in the dark for two days, then grown for a further four days under light conditions. The seedlings were harvested and seed husks discarded. Plant tissues from the pooled seedlings were then immersed in phosphate buffered saline (PBS) and ground using a QIAGEN Mixermill for up to one minute at 30 Hertz. A $10\mu\text{L}$ aliquot of the resulting macerate was added to $90\mu\text{L}$ of PBS. Further 1:10 dilutions were performed to generate 10^{-3} and 10^{-4} solutions. Reasoners 2 Agar (R2A, Oxoid, Australia) agar plates were then inoculated with 10^{-3} and 10^{-4} solutions from each treatment and allowed to grow for 24-48 hours. Individual bacterial colonies were then streaked onto single R2A plates to isolate and purify single bacterial strains. A total of 438 bacterial strains were isolated from the four wheat lines and stored in 20% glycerol at $-80\text{ }^{\circ}\text{C}$.

Microbe identification using MALDI-TOF

The microbial isolates were putatively identified using the Bruker MALDI Biotyper system. The bacterial strains were taken from the $-80\text{ }^{\circ}\text{C}$ glycerol stock, plated onto R2A and grown for 48 h. Single colonies were taken from the isolate plates and prepared for analysis using the manufacturer's Extended Direct Transfer method. The plate was analysed using the Bruker MALDI-TOF ultrafleXtreme in accordance with the manufacturer's instructions. *Escherichia coli* strain ATCC 25922 was used as a quality control and as an internal standard. The resulting protein spectra were processed using the MALDI BioTyper automation 2.0 software at default settings. The microbes were then assigned a preliminary identification by comparing raw protein spectra against known spectra in the MALDI BioTyper library, which contained 2,750 species from 471 genera as of January 2019. The Biotyper library was supplemented with an in-house database generated from previous endophyte studies of this research group (D. Auer, pers comm.) The protein spectra were processed using an in-house Refiner pipeline (GeneData 13.5). A hierarchical clustering algorithm in Analyst (GeneData 13.5) was used to create a phenogram that grouped bacterial strains based on the similarity of protein profiles.

Microbiome profiling

16S rRNA library preparation and sequencing

The microbiomes of the four drought tolerant lines (line 1, 2, 3, 4) and three drought susceptible lines (line 9, 10, 11) were profiled. Ten seeds from each line were plated onto filter paper soaked with sterile water and germinated in the dark at room temperature for two days. The seedlings were grown for a further four days under light conditions. Seedlings of approximately equal size were harvested and the seed husks discarded. Ten replicates, consisting of the plant tissues from five pooled seedlings, were used for each

line. Each replicate was snap frozen in liquid nitrogen. DNA was then extracted from each sample. Minor modifications were made to the QIAGEN MagAttract 96 DNA Plant Core Kit during DNA extraction to allow for use of the Biomek FX liquid handling station. The V4 hyper variable region of the 16S rRNA gene was targeted for microbiome profiling using the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Methods S2) in conjunction with PNA PCR blockers to reduce amplification from plant organelles [33]. Paired end sequencing was performed on an Illumina HiSeq3000⁰ using the 2 x 150 bp v3 chemistry cartridge.

Bioinformatics

Microbiome bioinformatics were performed with a combination of Pandaseq and QIIME2 2019.10. The paired-end Illumina reads were trimmed and assembled using PANDaseq [34]. Using QIIME2 (v 2019.10), the 16S sequences were filtered and denoised using Deblur (via q2-deblur) [35, 36]. There was a total of 18,220,959 quality-filtered reads. There were 182 total samples with a minimum of 489 and a maximum of 695,151 reads per sample. Taxonomy was assigned to ASVs using SILVA132 99% v4 (via q2-feature-classifier) [37, 38]. All amplicon sequence variants (ASVs) were aligned using MAFFT (via q2-alignment) and a phylogeny was created using fasttree (via q2-phylogeny) [39, 40]. Alpha-diversity metrics such as observed features and Faith's phylogenetic diversity and beta-diversity metrics such as RPCA [41, 42] The rarefaction curve reached an asymptote, indicating that sampling had captured the diversity of the seed microbiomes. In order to capture the diversity of the microbiomes, a sampling depth of 15,000 reads was applied across samples. Analysis and visualisation were performed using QIIME2 pipelines. The QIIME2 feature table was exported using in biom formatted and ANOVA analysis performed on individual ASVs [43].

Plant growth promotion effects of the cultured microbiome

Evaluation of microbiome bacteria for biostimulation of Triticeae

A representative isolate of two cultured genera that demonstrated significant differences between the contrasting genotypes of wheat seed microbiomes were selected and assessed for a biostimulation effect in *Triticeae*. Bacterial strains *Cf* D3-25 and *Ar.* sp D4-14 were cultured in Lysogeny Broth (Oxoid, Australia) overnight at 26°C. After 24 hours (h), seeds of *Triticum aestivum* (cv Bob White Red Haplotype) were sterilised by soaking in 80% ethanol for 3 minutes (min), then washed five times in sterile distilled water. To determine the CFU ml⁻¹, plate dilutions combined with OD (optical density) readings were performed. The cultures were centrifuged and washed in PBS twice before being resuspended in their original volume of overnight culture. These cultures were then diluted step-wise with PBS to concentrations of 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ respectively. Seeds were soaked in undiluted, or diluted solutions for four h at 26°C in a shaking incubator. As a control, seeds were soaked in PBS without bacteria under the same conditions. Fifteen inoculated seeds were then placed on moist sterile filter paper in sterile petri plates and allowed to grow for seven days at room temperature. There were four replicates per treatment. The same assay was performed using ryecorn, barley (cv Hindmarsh), oat, and spelt using

the methods described above. Data was statistically analysed using a one-way ANOVA and Tukey test to determine any significant difference ($p \leq 0.05$) between treatments using OriginPro 2018 (v b9.5.1.195).

Greenhouse drought pot trial

The wheat seeds (cv Bob White Red Haplotype) were sterilised according to the in vitro method described in section 'Evaluation of microbiome bacteria for biostimulation of Triticeae'. The seeds were then soaked in overnight cultures of either *Cf* D3-25, or *Ar*. Sp for four hours at 26°C in a shaking incubator. For control seedlings, seeds were soaked in sterile LB for four hours at 26°C in a shaking incubator. These seeds were planted in a glasshouse in a potting medium containing a mixture of 25% Biogrow potting mix, 37.5% Fertool vermiculite and 37.5% Fertool perlite. For each treatment, eight seeds were planted at a depth of one centimetre (cm) around the edge of each pot for a total of 12 pots. The endophyte-treated seeds were subjected to one of three watering conditions. Pots under the well-watered, mild drought, or severe drought condition received 300mL, 150mL, or 50mL of water respectively, every 48 h. After the first week of growth, seeds that had not germinated were removed, reducing the total number of plants per pot to four. After six weeks of growth, the plants were photographed and measured (shoot and root length in cm). Aerial and root tissue were separated then weighed. This data was analysed using OriginPro 2018 (Version b9.5.1.195) as described in "Evaluation of microbiome bacteria for biostimulation of Triticeae.

Results

Culturing the seed microbiome

A total of 438 bacteria were isolated from the seed microbiomes of six wheat lines. At a phylum level, the isolates were identified as either *Gammaproteobacteria* (42.92%), *Actinobacteria* (27.16%), or *Firmicutes* (0.23%). At a genera level, the isolates belonged overwhelmingly to four genera, *Pantoea* (25.34%), *Pseudomonas* (17.35%), *Arthrobacter* (12.78%) and *Curtobacterium* (12.55%), while minor genera consisted of *Rathayibacter* (0.46%), *Clavibacter* (1.14%), *Erwinia* (0.23%) and *Paenibacillus* (0.23%). Unidentified isolates comprised 29.90% (Supplementary Table 1).

Pantoea and *Pseudomonas* were identified in both drought susceptible lines (DSL) and drought tolerant lines (DTL). *Pantoea* and *Pseudomonas* were the dominant genera in DSL isolates comprising 32.56% and 22.79% respectively, as opposed to 27.56% and 2.67% of DTL isolates. *Pantoea* and *Pseudomonas* were more prevalent under rainfed conditions (RC) comprising 43.8% and 26.8%, respectively, rather than 12.59% and 9.54% of all isolates from drought conditions (DC).

Arthrobacter and *Curtobacterium* isolates were exclusive to DTL and were the dominant genera accounting for 24.00% and 25.78% of isolates, respectively (Figure 1). *Arthrobacter* isolates were also exclusive to DC, while *Curtobacterium* isolates dominated under DC (22%) as opposed to RC (1.68%). Furthermore, *Curtobacterium* was the only bacteria isolated from seeds of DT line 3 under DC, while *Arthrobacter* was the only bacteria isolated from DT line 4 under DC (Appendix 3).

Clavibacter and *Rathayibacter* were exclusively isolated from DTL under DC but only accounted for 2.22% and 0.89%, respectively (Figure 1). *Erwinia* and *Paenibacillus* were exclusive to DSL under RC, but only accounted for 0.47% and 0.47% of the population, respectively. Bacteria that were unable to be identified using MALDI-TOF were isolated from both DTL and DSL, being 16.5% and 43.7% of all isolates, respectively. Unknown isolates were also identified from DC and RC, at 33.2% and 24.7%, respectively.

Microbiome profiling

Variation in diversity

Samples were collected from the seeds of wheat cultivars of differing levels of drought tolerance that had been subject to drought and rainfed conditions. The microbiomes of these seeds were characterised using 16S rRNA amplicon sequencing. To characterise the diversity of the wheat seed microbiomes, alpha and beta diversity analyses were performed. Shannon index comparison of DC microbiomes (8.813) was significantly more diverse than RC microbiomes (7.035) ($q = 2.49e-18$, $H=76.35$, Kruskal-Wallis test). However, the number of observed OTUs and Shannon's index of DTL microbiomes and DSL microbiomes, when compared there was no significant difference ($p>0.05$, Kruskal-Wallis test). Robust Aitchison PCA (RPCA) was performed on the wheat seed microbiomes, using the QIIME2 DEICODE module [42]. The microbiome profiles of DTL and DSL showed significant differences (Figure 2a, PERMANOVA pseudo- $F = 20.95$, $p = 0.001$). There was also a significant difference between the microbiomes of wheat seeds subjected to either DC and RC (Figure 2b, PERMANOVA pseudo- $F = 8.76$, $p = 0.001$).

Bacterial Taxonomic Composition

The composition of seed microbiomes was influenced by environmental conditions and wheat lines (see Figure 3). DC reduced the abundance of the three most dominant families under RC. In DTL under RC, the most dominant families were *Enterobacteriaceae*, *Pseudomonadaceae* and *Burkholderiaceae*, which represented 31.6%, 13.6% and 6.0% of all isolates, respectively, as opposed to 3.5%, 8.8% and 9.0% in DTL under DC. In DSL under RC, the most dominant families were *Enterobacteriaceae*, *Pseudomonadaceae* and *Burkholderiaceae*, which represented 23.3%, 19.7% and 6.0%, as opposed to 10.7%, 5.7% and 8.5% in DSL under DC. In DTL under DC, the three most abundant families were *Burkholderia*, *Pseudomonadaceae* and *Chitinophagaceae*. In DSL under DC, the three most abundant families were *Enterobacteriaceae*, *Burkholderia* and *Chitinophagaceae*.

Certain OTUs were enriched in seed microbiomes under drought conditions. ANOVA analysis of the microbiomes under DC and microbiomes under RC was performed (Supplementary Table 2). There were 1069 OTUs that were significant ($p > 0.05$). The ten most significant OTUs were belonged to the genera *Pseudomonas* ($p=9.04E-18$), Unknown (ANOVA $p = 2.11E-15$, $p = 1.72E-12$, $p = 1.57E-11$), *Flavobacterium* (ANOVA $p = 7.74E-13$), *Amycolatopsis* (ANOVA $p = 2.99E-12$), *Bradyrhizobium* (ANOVA $p = 3.68E-11$), *Pantoea* (ANOVA $p = 5.80E-11$), *Skermenella* (ANOVA $p = 5.96E-11$) and *Rubrobacter* (ANOVA $p = 7.45E-$

11). There were multiple significant OTUs identified that belonged to the genera isolated from wheat seeds, including *Pseudomonas*, *Pantoea* and *Arthrobacter* that appeared in the top 0.2% of significant OTUs. In DT Line 3 and DT Line 4, *Arthrobacter* was enriched 1.5-fold (Tukey test, $p=2.2E-4$) and 1.9-fold (Tukey test, $p=3.4E-6$), respectively. Approximately a third of OTUs identified were either unknown at the genus level or uncultured at the genus level. Of the top 50 OTUs, 17 belonged to unknown or uncultured genera.

The microbiomes of DTL showed enrichment of certain microbes when compared with DSL microbiomes, under both DC and RC (Figure 3). At a family level, *Enterbacteriaceae* and *Microbacteriaceae* were highlighted. DTL had a higher abundance of *Microbacteriaceae* (1.045%) compared to DSL (0.359%), under DC. DTL also had higher abundance of *Microbacteriaceae* (0.514%) compared to DSL (0.274%), under RC. When ranking the relative abundance of OTUs at a family level, *Microbacteriaceae* were ranked at 25 for DTL under DC and 27 for DTL under RC (Figure 4). Comparatively, *Microbacteriaceae* were ranked at 50 for DSL under DC and 48 for DSL under RC. Genera from the order *Microbacteriaceae* were significant in an ANOVA of the microbiome of DTL and DSL under DC, namely *Curtobacterium* ($p=3.29E-3$), *Agromyces* ($p=0.032$) and an unknown genera ($p=0.048$) (Supplementary Table 3).

Plant growth promotion effects of the cultured microbiome

Evaluation of microbiome bacteria for biostimulation of Triticeae

To assess the growth promotion effect of *Curtobacterium flaccumfaciens* novel strain *Cf*D3-25 and *Arthrobacter* sp. novel strains *Ar.* sp D4-14 on plants, a seedling assay was established with members of the tribe *Triticeae* (wheat – *Triticum aestivum*; spelt - *Triticum spelta*, durum - *Triticum durum*; ryecorn - *Secale cereale*; oats - *Avena sativa*; barley - *Hordeum vulgare*). Wheat seeds inoculated with different concentrations of *Cf*D3-25 or *Ar.* sp D4-14 were germinated and allowed to grow for seven days to evaluate potential biostimulation activity. There was a root lengthening effect observed in microbe-treated wheat seedlings (see Figure 5). In early wheat seedlings inoculated in *Cf*D3-25 solutions diluted to 10^0 , 10^{-2} and 10^{-3} (containing 7×10^8 , 7×10^6 and 7×10^5 CFU mL⁻¹, respectively), the root lengthening was significant compared to the uninoculated control, increasing root length by 7.94%, 9.05% and 7.95%. Similarly, in wheat seedlings inoculated with *Ar.* sp D4-14 solutions diluted to 10^{-3} and 10^{-4} (containing 1.13×10^6 and 1.13×10^5 CFU mL⁻¹), respectively root lengthening was significant compared to the uninoculated control, increasing root length by 21.93% and 21.78%. Oat seedlings inoculated in *Cf*D3-25 solutions of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} (8.19×10^7 , 8.19×10^6 , 8.19×10^5 , 8.19×10^4 CFU mL⁻¹, respectively) had significantly longer roots than the control, with a percentage increase of 90.81%, 101.55%, 63.85% and 104.68% respectively. Similarly, oat seedlings inoculated with *Ar.* sp D4-14 solutions of 10^{-3} and 10^{-4} (5.81×10^5 and 5.81×10^4 CFU mL⁻¹, respectively) had significantly longer roots than the control, with a percentage increase of 63.85% and 80.14% respectively. There was no significant observable root lengthening effect in barley, spelt, or ryecorn. High concentrations of *Ar.* sp D4-14 inhibited root growth in

both barley and oats. There were no significant observable shoot effects in any *Triticeae* (see Appendix 5).

Greenhouse drought pot trial

To assess the ability of *Curtobacterium flaccumfaciens* D3-25 and *Arthrobacter* sp. D4-14 to aid drought tolerance, an *in planta* assay was established in seed inoculated wheat exposed to varying levels of drought. At the end of six-weeks of growth, the wheat plants were harvested and the wet shoot and root weight were measured. Wheat plants inoculated with *Cf* D3-25 and *Ar.* sp D4-14 had significant increases in shoot and root weight, compared to control, across a range of conditions. Shoot weight of *Cf* D3-25-inoculated wheat plants, under well-watered conditions, increased by 46.82% compared to the control (Tukey test, $p=0.003$, Figure 6a). Under drought conditions, there was a 7.71% increase in shoot length of wheat plants inoculated with *Cf* D3-25 under mild drought conditions, but this increase was not significant. The shoot weight of *Ar.* sp D4-14-inoculated wheat plants did not significantly increase, compared to control. Root weight was significantly increased in six-week old wheat plants inoculated with *Cf* D3-25 under mild and severe drought conditions, compared to *Ar.* sp D4-14-inoculated and control plants (Figure 6b). Under mild conditions, *Cf* D3-25-inoculated plants had a 26.00% increase in wet root weight (Tukey test, $p=0.045$). Under severe conditions, *Cf* D3-25-inoculated plants had a 27.61% increase in wet root weight (Tukey test, $p=0.035$). Wet root weight increased 15.07% under well-watered conditions, although the difference was not significant (Tukey test, $p=0.05$). Wheat plants inoculated with *Ar.* sp D4-14 had no significant increase in wet root weight, compared to the control.

After six weeks of growth, the shoot length and number of leaves on each plant were measured. In the well-watered condition, the endophyte-treated plants had significantly more fully-expanded leaves than the control (Figure 6c). Plants that had been inoculated with either *Cf* D3-25 or *Ar.* sp D4-14 had 35% and 27% more fully-expanded leaves, respectively, than the control plants (Tukey test, $p=0.001$ and $p=0.02$). *Ar.* sp D4-14-treated plants exhibited significantly more full-expanded leaves under severe drought. Wheat plants inoculated with *Cf* D3-25 had an 8.47% increase in shoot length under well-watered conditions, compared to the control (Tukey test, $p=0.001$, Figure 6d). Similarly, wheat plants inoculated with *Ar.* sp D4-14-inoculated had significantly longer shoot lengths, under well-watered condition, with a 6.68% increase compared to the control (Tukey test, $p=0.016$). However, this effect was not evident under moderate or severe drought conditions.

Discussion

A combination of metagenomic and culture methods were used to characterize the seed microbiome composition of four drought tolerant and two drought susceptible wheat lines under rainfed and drought conditions. Water availability and line-specific selection were responsible for significant variation in both diversity and abundance of microbes. A collection of microbes was cultured from the seeds of six wheat lines. From the collection, genera that had been enriched in microbiomes under drought conditions in

drought tolerant lines were selected and screened for biostimulation effects and screened for effects on drought tolerance.

Variation of seed microbial diversity is influenced by drought and wheat cultivar

The collection of microbes cultured from the seeds of six wheat lines showed considerable differences in genera diversity and abundance under drought stress. Genera belonging to *Gammaproteobacteria*, namely, *Pantoea* and *Pseudomonas* dominated the seed microbiomes of drought susceptible lines (DSL), but were depleted in drought tolerant lines (DTL). *Pseudomonas*, in particular, was depleted significantly in DTL compared to DSL. A similar trend was also observed between seed microbiomes under rainfed conditions and drought conditions (DC), where *Pantoea* and *Pseudomonas* were depleted under drought stress. In contrast, it was clear that genera belonging to *Actinobacteria*, had undergone line-dependent selection by drought tolerant wheat lines.

The microbiomes of these seeds were profiled using 16S rRNA amplicon sequencing and analysed using QIIME2. As observed in the cultured portion of the six wheat seed microbiomes, drought stress and wheat lines had significant effects the abundance of *Gammaproteobacteria* in microbiome profiling. Under drought conditions, all lines showed a marked depletion of *Gammaproteobacteria* families. In DTL, *Enterobacteriaceae* and *Pseudomonadaceae* the two most dominant families in RC were significantly depleted under DC. This depletion was not observed to the same extent in DSL under DC. Interestingly, previous studies of the wheat microbiome under drought stress have not observed the marked depletion in *Gammaproteobacteria* observed across wheat lines in this study. In a study by Naylor et al, there was no detectable change to the abundance of *Gammaproteobacteria* under drought stress [12]. It is possible that the depletion of *Gammaproteobacteria* under drought conditions is a phenomenon that is specific to the seed microbiome. *Actinobacteria* families were selected for and enriched by DTL lines under DC. Under rainfed conditions, the *Microbacteriaceae* family made up 1.045% of DTL and only 0.274% of DSL. Under drought stress, *Microbacteriaceae* was enriched in both DTL and in DSL, though the enrichment was more pronounced in DTL. The enrichment of *Actinobacteria* in the microbiomes of wheat and other crop species has been consistently observed in multiple drought studies [12, 16, 25, 44, 45]. A study of 30 genetically divergent plant species, subjected to drought stress, observed an increase in relative abundance of *Actinobacteria* six-fold, although results varied by plant species [44]. Similarly, studies of microbiomes under drought conditions have highlighted the enrichment of *Actinobacteria* by 3.1 fold in wheat as well as a significant increase in agave [12, 45]. The enrichment of *Actinobacteria* is particularly prevalent in the root microbiome of wheat plants undergoing the early stages of drought stress [12]. It has been proposed that the enrichment of *Actinobacteria* genera under drought could be driven by one or more conserved properties of the *Actinobacterial* lineage [46].

Seed microbiomes of DTL demonstrated higher levels of *Actinobacteria* enrichment under DC, compared to the seed microbiomes of DSL. Cultivar-specific enrichment of specific genera (and occasionally specific strains) has been demonstrated in potato, common bean, cannabis, sorghum and wheat [47-51]. Wheat cultivars Lewjain, Penawawa and Symphony have been demonstrated to select species

fluorescent pseudomonads from soil microbiomes [52-54]. In root microbiomes, striga-resistant sorghum cultivars enriched for *Acidobacteria* GP1, *Burkholderia*, *Cupriavidus* (*Burkholderiaceae*), *Acidovorax* and *Albidiferax* (*Comamonadaceae*) OTUs, when grown in unfertilised soil [51]. In fact, the relationship between strain and cultivar can be so specific that a bacterial strain that is beneficial in one cultivar can be detrimental in another. In *Brassica napus*, *Paenibacillus polymyxa* Sb3-1 increased growth in the cv. Avata but caused leaf yellowing in the cv. Traviata [32]. In DT Line 3 and DT Line 4, *Arthrobacter* was enriched 1.5 and 1.9-fold, respectively. This was not observed in drought susceptible lines, suggesting that the enrichment of *Arthrobacter* is dependent on cultivar genetics. Previous studies have demonstrated cultivar-specific selection of an *Arthrobacter* OTU in the rhizosphere of wheat cultivar PI561725 and enrichment of the genera in the presence of 2,4-diacetylphloroglucinol producing species [50]. This study suggests that drought tolerant lines select and enrich for *Actinobacteria* genera when they recruit the seed microbiome of the next generation.

The seed microbiomes under DC had a higher Shannon's index than their rainfed counterparts, indicating higher levels of diversity. There is conflicting data on the effects of drought on microbial diversity. In a study by Jochum et al, multiple generations of drought stress on wheat microbiomes lead to an increase in alpha and beta diversity [25]. However, in other studies the microbiomes of sorghum and wheat exhibited a marked decrease in diversity [26, 55]. It has been suggested that increased diversity could be explained by selective enrichment for functionality, rather than taxonomy [25]. It is worth noting that an increase in diversity under drought could be due to the depletion of dominant taxa, such as *Gammaproteobacteria*, allowing a greater number of OTUs with low abundance to be captured by sequencing.

Seed microbiomes enrich for microbes that promote root growth and drought tolerance

To determine if bacteria enriched in DTL lines under drought stress had a biostimulation effect, members of the tribe *Triticeae* were inoculated with representative isolates from the two cultured *Actinobacteria* genera. Wheat and oat seeds inoculated with different concentrations of *Cf* D3-25 and *Ar. sp* D4-14 had significantly longer roots, compared to control plants. In wheat, *Cf* D3-25-treated seedlings had an increase in root length between 7.94-9.05% and *Ar. sp* D4-14-treated seedlings had a 21% increase in root length. In oat, *Cf* D3-25-treated seedlings solutions had an increase in root length between 63.85-104.68 and *Ar. sp* D4-14-treated plants had between 63.85-80.14% increase in root length. The inoculation concentration of *Cf* D3-25 did not appear to significantly impact the root lengthening effect seen in oat and wheat. High concentrations of *Ar. sp* D4-14 inhibited the growth of barley and oats. *Curtobacterium* has been found to associate with roots and promote plant growth in *Arabidopsis*, lettuce, basil, red clover and cucumber [56-58].

Representative isolates from *Curtobacterium* and *Arthrobacter* were inoculated into wheat seedlings which were then exposed to varying levels of drought to determine if they could increase drought tolerance. *Cf* D3-25-treated wheat plants showed significant increases in root weight under both mild (26%) and severe drought conditions (27%), compared to the control. Wheat plants inoculated with *Ar. sp*

D4-14 had no significant increase in wet root weight. *Cf* D3-25 treated wheat plants also showed a significant increase in wet shoot weight (47%) under well-watered conditions, although this effect was not evident under either drought conditions. Both *Cf* D3-25 and *Ar. sp* D4-14 had significantly more fully expanded leaves than the control under well-watered conditions, showing an increase of 35% and 27%, respectively. There was no significant difference in the number of fully expanded leaves in isolate-treated wheat plants under drought conditions. *Ar. sp* D4-14-treated wheat plants did not show a clear drought response in the cv. Bob White. However, as the action of these isolates may be cultivar-specific, it may be prudent to investigate the effects of *Ar. sp* D4-14-inoculation on other wheat cultivars under drought conditions.

There are a number of microbial traits that are associated with increasing drought tolerance in host plants, specifically the ability to produce 1-aminocyclopropane-1-carboxylate deaminase (ACCd), indole-3-acetic acid (IAA) and siderophores [12]. ACCd regulates ethylene, preventing it from reaching inhibitory levels and thus allowing normal root growth under drought conditions [59]. Auxin analogue IAA enhances shoot and root growth under drought conditions by regulating stomatal aperture [60]. Under drought conditions, siderophore synthesis allows for nutrient cycling [61]. In lettuce under drought conditions treatment, *Curtobacterium herbarum* strain CAH5 increased shoot length, root length and wet biomass by 1.54, 1.23 and 3.84 fold, respectively, compared to control plants [62]. *C. herbarum* CAH5 demonstrated the ability to produce IAA, ACC deaminase, siderophores and solubilise phosphate [62]. *C. flaccumfaciens* colonizes xylem system of the host plants, where it can be either pathogenic (causing vascular wilt in bean) or endophytic [63]. It has been suggested that the prominence of Actinobacteria in plant microbiomes could also be due to their method of infection [63]. Colonisation by xylem-limited bacteria increases resistance to water flow, causing stomatal closure and lowering transpiration [64]. Inoculation of *A. nitroguajacolicus*, the species of *Arthrobacter* whose 16S rRNA gene profile is the most closely related to *Ar. sp* D4-14, into wheat increased the total dry weight by more than 200% in conditions of high salinity, a condition that often occurs in tandem with drought [65]. *A. nitroguajacolicus* demonstrated the ability to produce auxin, ACC deaminase, siderophores and solubilize phosphate [65]. Another *A. pokkali* strain P3B162^T has been observed to grow in 10% and 20% polyethylene glycol (PEG) solutions, indicating the strain is drought tolerant [66]. In previous studies, *Curtobacterium* and *Arthrobacter* isolates have shown a number of traits that increase abiotic stress tolerance i.e. drought tolerance under a range of conditions.

Final remarks

This study indicates seed microbiomes from genetically distinct wheat lines enrich for beneficial bacteria in ways that are both line-specific and responsive to environmental stress. As such, they represent an invaluable resource for the further identification of beneficial microbes with plant growth promoting activity and hence improving commercial crop production. The composition of the seed microbiomes differed significantly between lines and environmental conditions. It was clear that drought tolerant lines selected for and enriched certain microbial families when exposed to either rainfed or drought conditions. Both *Cf* D3-25 and *Ar. sp* D4-14, cultured microbes belonging to taxa enriched by drought tolerant lines

under drought conditions, demonstrated the ability to promote plant growth. *Cf* D3-25 increased drought tolerance in wheat. Microbe profiling suggested the enrichment of microbes was line-specific, therefore, it would be worthwhile to assay key microbes in other wheat cultivars. Combinations of genetically tolerant and susceptible lines under different stresses could be exploited in a similar manner to find other useful bacteria e.g. in highlighting N efficient microbes in microbiomes subjected to environment low in nitrogen.

Declarations

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Author contributions

H.H. wrote the main manuscript text. H.H., G.Y., R.M. and T.S. designed the experiment. H.H. and G.Y. isolated microbes from the wheat seed microbiome. G.Y. and J.K. performed DNA extraction and sequencing. H.H., T.L, and I.T. performed bioinformatic analyses of sequencing data and all statistical analyses. H.H. performed seedling growth assays and greenhouse drought pot trial. H.H. created figures 1, 2, 5 and 6. H.H. and T.S created figures 4 and 5. R.M., T.S. and G.S. supervised the work. G.S. contributed to funding acquisition. R.M. and T.S. corrected the final version of the manuscript. All authors have read and agreed to the submitted version of the manuscript.

Additional information

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

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Figures

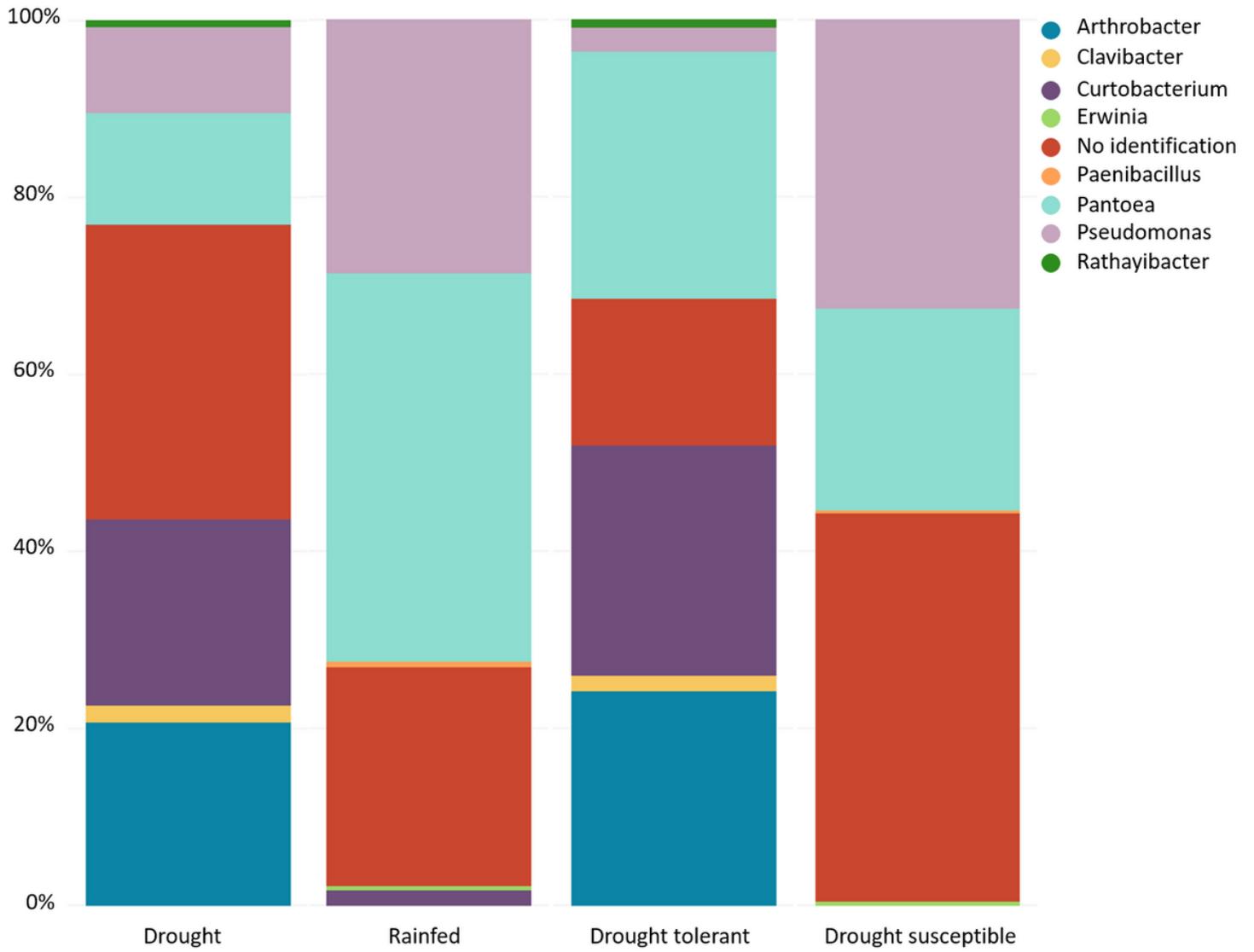


Figure 1

Microbes isolated from the seeds a) of drought tolerant lines (DTL) and drought susceptible lines (DSL) and from wheat under either drought (DC) and rainfed conditions (RC).

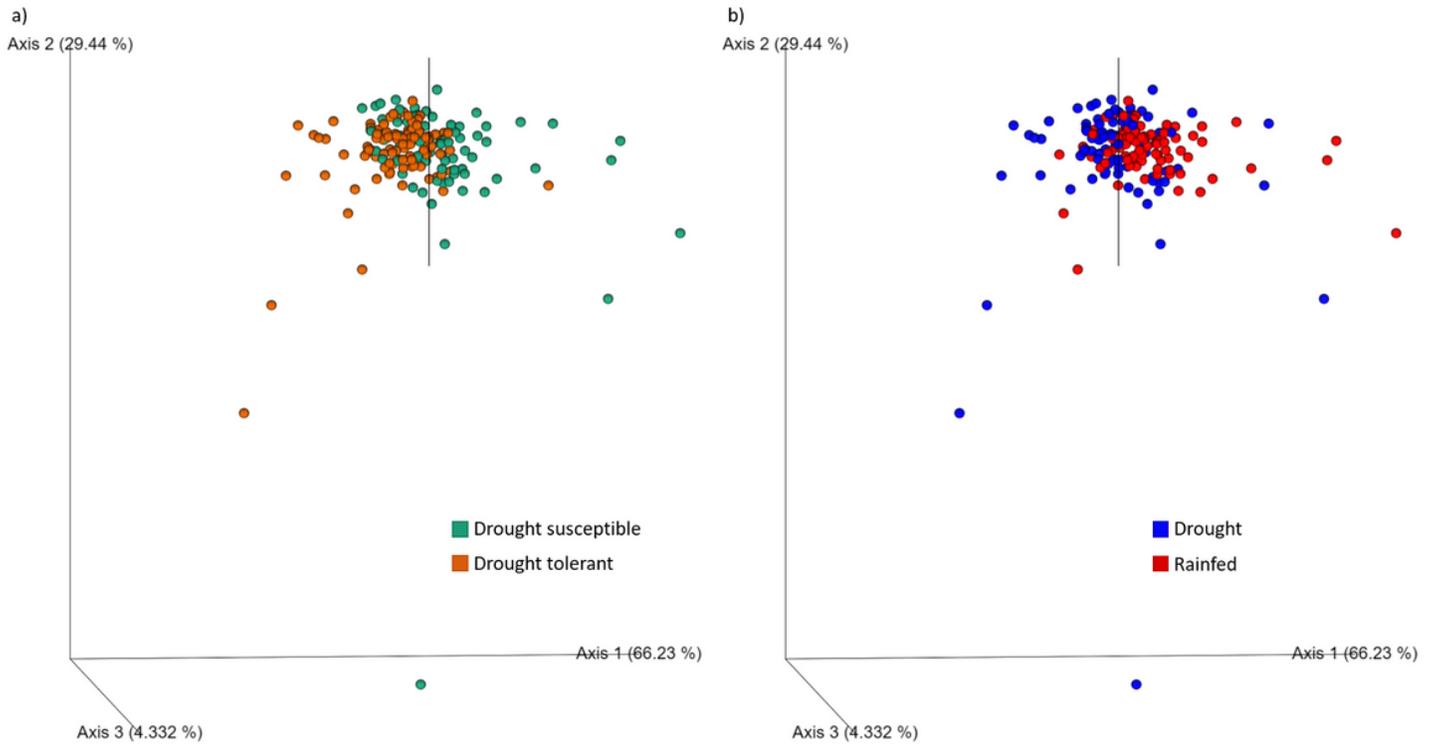


Figure 2

RPCA of the seed microbiomes of wheat lines a) subjected to drought (DC) or rainfed conditions (RC) b) from drought tolerant (DTL) or drought susceptible lines (DSL).

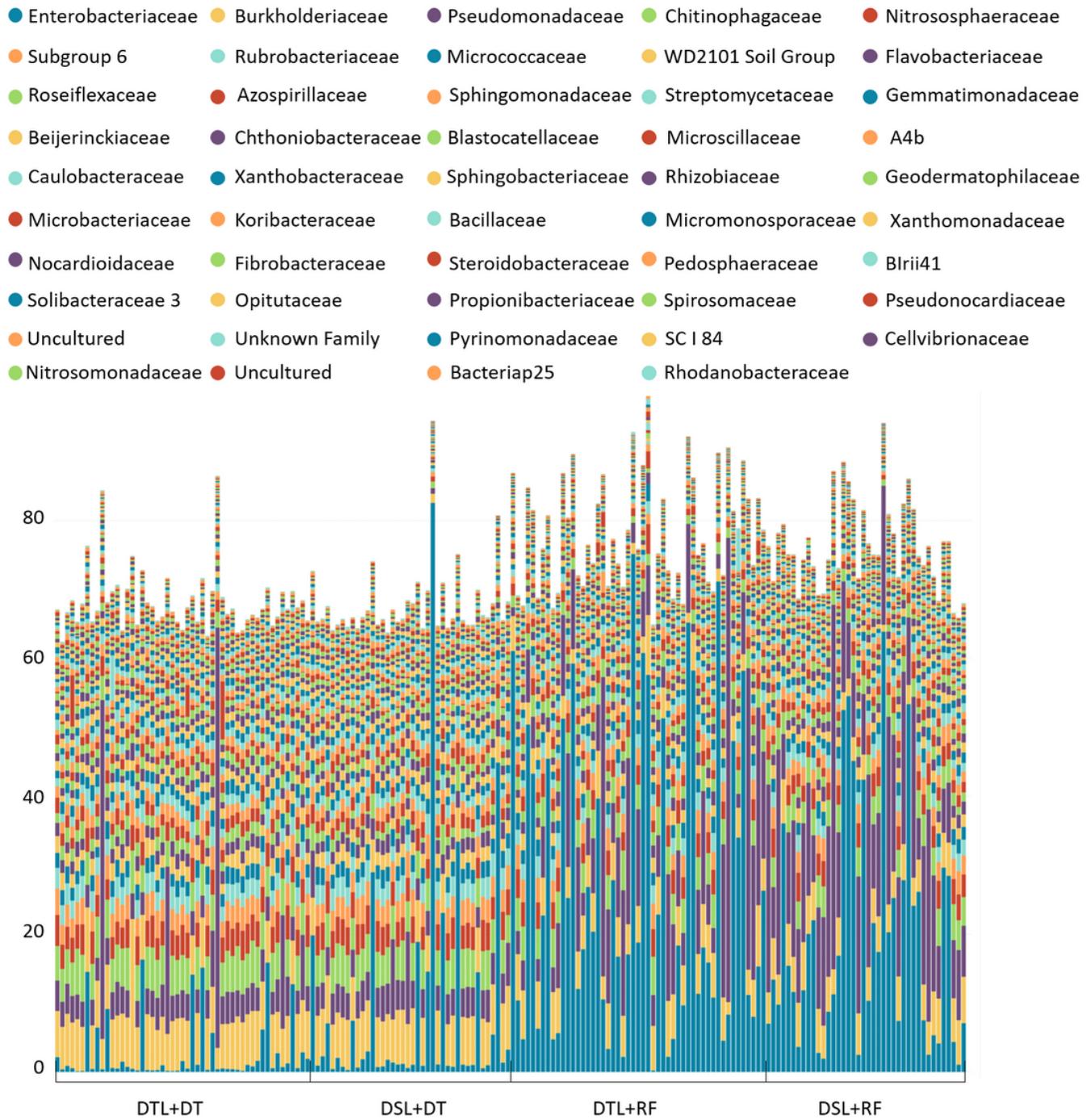


Figure 3

Taxonomy comparison the microbiomes of wheat plants under drought and rainfed conditions. Each column represents a microbial profile of a wheat line under specified conditions.

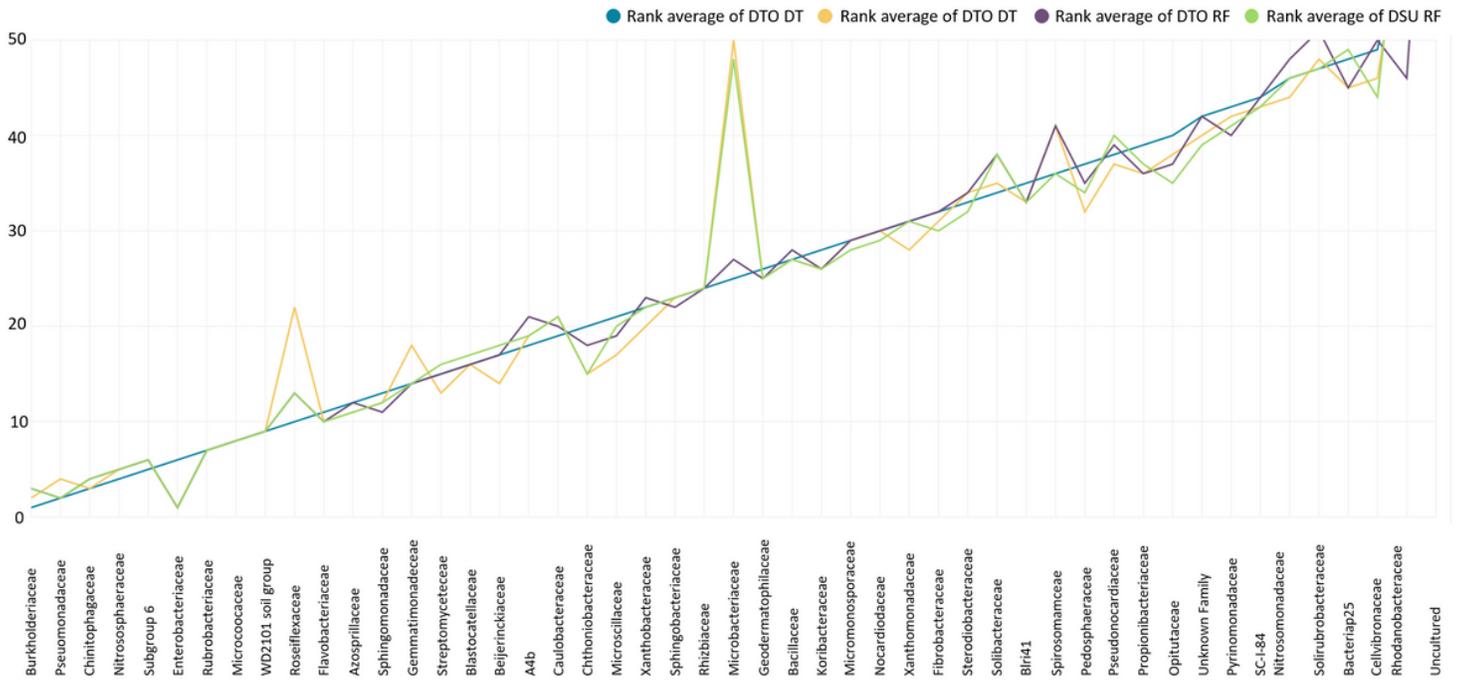


Figure 4

For each d4 level taxa an average percentage abundance across the four groups of plants was taken (DTL-DC; DSL-DC; DTL-RC; DSL-RC). Each of the four percentage columns were sorted from highest to lowest and ranked from highest value (Rank 1) to 50th value (Rank 50) for each group. The minimum rank value was taken across the four ranking columns, and the columns sorted from lowest rank to highest rank, and the rankings plotted as a line graph.

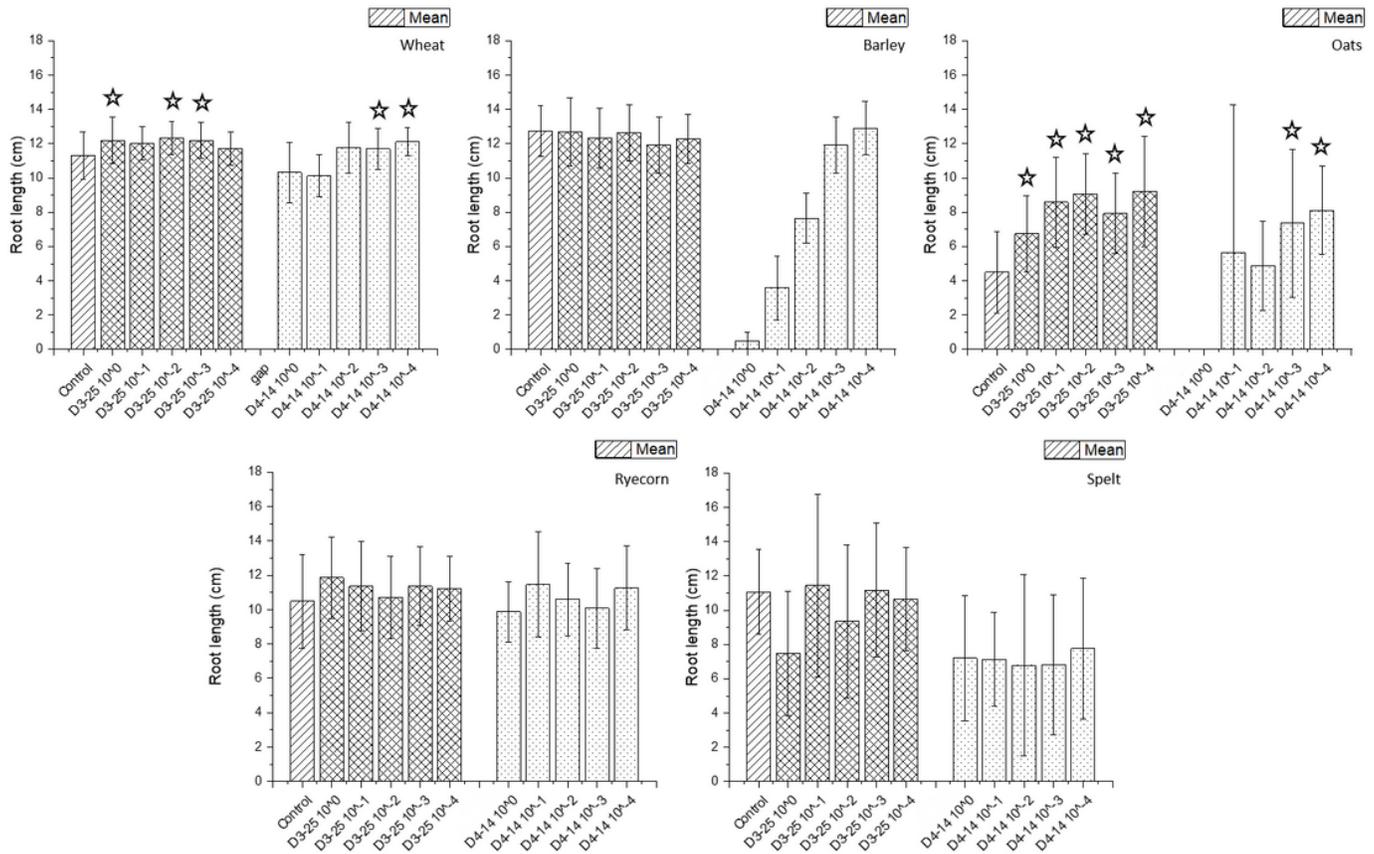


Figure 5

Root length (cm) of wheat, barley, oat, rye-corn and spelt seedlings inoculated with different concentrations (10⁰, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) of Cf D3-25 or Ar. sp D4-14. Stars indicate inoculum concentrations that had significantly longer roots.

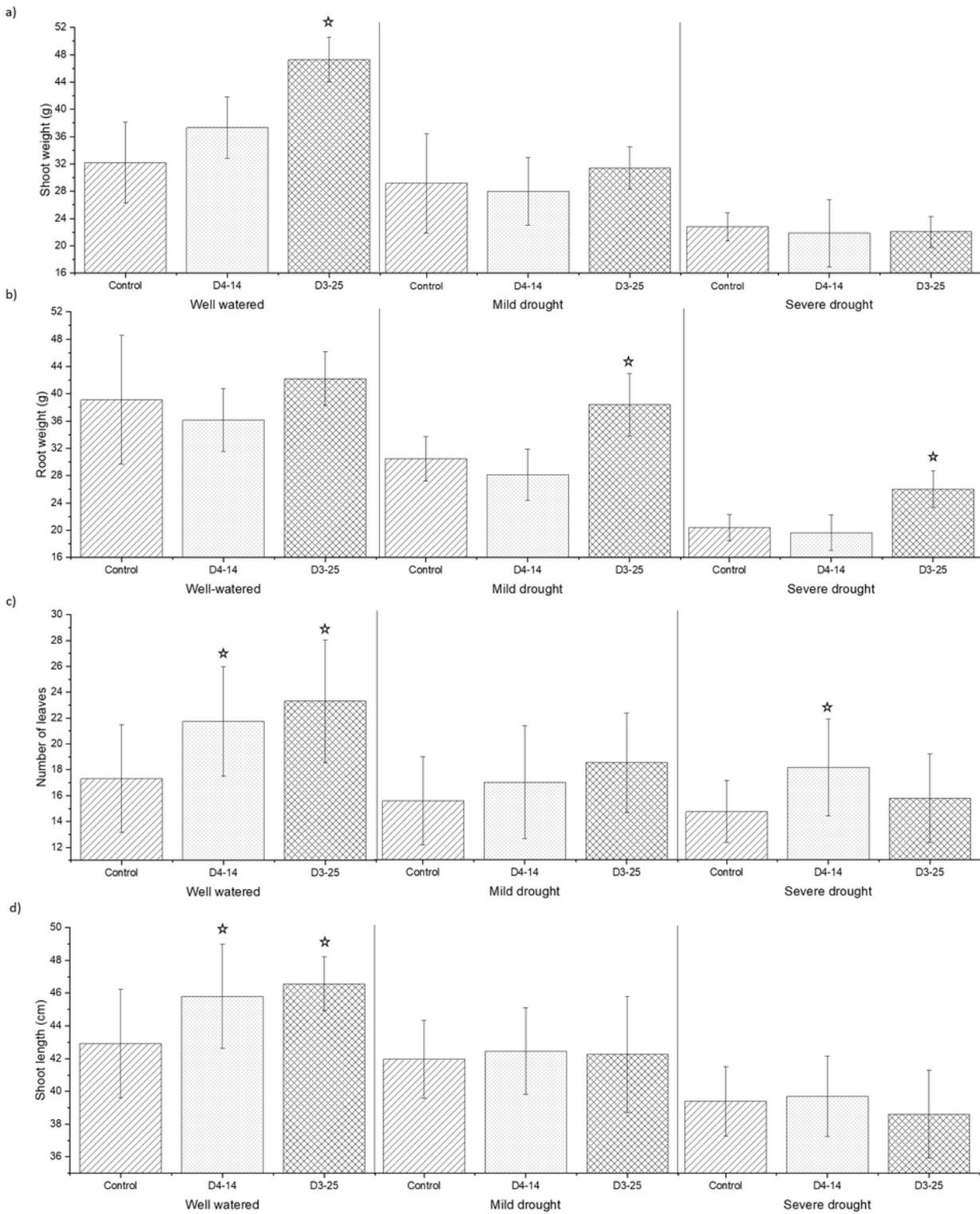


Figure 6

a) Wet shoot weight and b) wet root weight wheat plants in well-watered (300mL every 48 hs), moderate drought conditions (150mL every 48h) and severe drought condition (50mL every 48hs). c) Number of leaves and d) shoot lengths of wheat plants in well-watered, moderate drought conditions and severe drought condition. Stars mark statistically significant results.

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