The resistance of the wheat microbial community to water stress is more influenced by plant compartment than reduced water availability

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Abstract

Drought is a serious menace to agriculture across the world. However, it is still not clear how this will affect crop-associated microbial communities. Here, we experimentally manipulated precipitation in the field for two years and compared the bacterial communities associated with leaves, roots, and rhizosphere soils of two different wheat genotypes. The bacterial 16S rRNA gene was amplified and sequenced, while 542 microorganisms were isolated and screened for their tolerance to osmotic stress. The bacterial community was not significantly affected by the precipitation manipulation treatments but differed drastically from one plant compartment to the other. Forty-four isolates, mostly bacteria, showed high levels of resistance to osmotic stress by growing in liquid medium supplemented with 30% polyethylene glycol. The Actinobacteria were overrepresented among these isolates, and in contrast to our expectation, precipitation treatments did not influence the odds of isolating osmotic stress-resistant bacteria. However, the odds were significantly higher in the leaves as compared to the roots, the rhizosphere, or the seeds. Our results suggest that isolation efforts for wheat-compatible water stress resistant bacteria should be targeted at the leaf endosphere and that short-term experimental manipulation of precipitation does not result in a more resistant community.

Keywords: Drought stress, wheat, amplicon sequencing, microbial isolates.

Introduction

Global changes will result in altered precipitation patterns, which will lead to more frequent and longer drought periods in some regions of the world, including Canada (Stocker *et al.* 2013; Canada 2018). Drought is a factor that limits the growth of plants and can lead to losses of up to 50% in global agricultural productions, including wheat (Sangiorgio *et al.* 2020; Duggan, Domitruk and Fowler 2000; Bagci *et al.* 2007). In view of the rising world population and the scale and speed of global changes, we need innovative solutions to help crops withstand higher levels of water stress. One approach would be to manipulate or engineer the plant microbiota (Quiza, St-Arnaud and Yergeau 2015; Agoussar and Yergeau 2021) toward a community that would provide more beneficial services to the plant under drought. The first step down that road is to better understand how plant-associated microbial communities respond to water stress. Microorganisms can adapt to water stress through different mechanisms such as solutes accumulation, exopolysaccharides production, ribosome storage or sporulation (Ngumbi and Kloepper 2016). At the community level, wheat-associated microbial communities were shown to react strongly to changes in soil water availability (Azarbad 2018, 2020, 2021, Giard-Laliberté *et al.* 2019). Most of these changes were shown to be due to shifts in the relative abundance of microorganisms already present in the plant environment, with very little recruitment from outside sources (Giard-Laliberté *et al.* 2019), suggesting that plant-associated microbial communities have members that are already resistant to water stress. However, because it is a multigenic phenomenon, the resistance to water stress of bacteria is almost impossible to clearly predict from amplicon sequencing or metagenomic datasets. Most studies assume that the statistical association of OTUs with water-depleted treatments is indicative of a better resistance to water stress. For instance, the phylum *Actinobacteria* often becomes relatively more abundant when water availability decreases,

but it is not shown if individual microorganisms from this phylum are resistant to stress or simply less rapidly affected than other microbial groups. Combining microbial isolation with amplicon sequencing could help bridge this gap.

Not only does the tolerance to water stress vary among microorganisms (Evans and Wallenstein 2014), but they can also influence the plant resistance to such stress. Indeed, many specific bacteria of the phyla Actinobacteria and Proteobacteria were shown to improve plant tolerance to drought- or salinity-related stresses (Mayak, Tirosh and Glick 2004a, 2004b; Cheng, Park and Glick 2007; Saravanakumar and Samiyappan 2007). Fungal endophytes were also shown to improve plant performance under abiotic stress (Redman *et al.* 2011; Singh, Gill and Tuteja 2011; Hubbard 2012). Mycorrhizal fungi can improve water use efficiency and reduce drought stress in wheat (Al-Karaki, McMichael and Zak 2004), oat (Khan, Ahmad and Ayub 2003), and corn (Subramanian *et al.* 1995). Many mechanisms were shown to be involved in the enhancement of plant drought tolerance by microbes, such as modulation of plant drought stress genes (Timmusk and Wagner 1999), reduction of the stress hormone ethylene levels through degradation of its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) by the bacterial enzyme ACC deaminase (Mayak 2004a, b), increase in the production of phytohormones, proline and pigments induced by bacterial volatile organic compounds (Yasmin *et al.* 2021), stimulation of the expression of plant genes related to osmolytes (Barnawal, Singh and Singh 2019) or modulation of the plant epigenetics response to drought (Hubbard, Germida and Vujanovic 2014).

To help crops sustain increasing water stress levels, it could be useful to isolate microorganisms that are resistant to drought, but at the same time are compatible with crops and provide beneficial services under stressful conditions. Endophytic and rhizospheric microorganisms isolated from environments prone to drought tend to confer plants with better

resistance to drought (Redman *et al.* 2011; Timmusk *et al.* 2011). At the same time, compatibility with the host plant is highly important. For instance, *Arabidopsis* plants growing in their soil were more resistant to moderate drought than *Arabidopsis* plants growing in corn or pine soil (Zolla *et al.* 2013). This led to a significant reduction of the expression of drought stress-related genes in the plant, and it was suggested that the presence of specific soil bacteria could dampen the drought stress response of the plant. Recent work from our groups showed that the soil water stress history influences the soil microbial community composition, diversity and functions and plant root biomass when faced with a water stress event (Azarbad *et al.* 2018, 2020, 2021). Taken together, these results suggest that selecting an appropriate environment/sample type would be paramount to isolate microorganisms with the right properties to help crops resist to water stress.

Here, we hypothesize that experimentally decreasing precipitations would result in a microbial community that contains a higher frequency of osmotolerant isolates. To test this hypothesis, we designed and carried out a field experimental manipulation of the precipitation for two years and sampled leaves, roots, rhizosphere soils, and seeds of two wheat genotypes. On top of comparing bacterial communities using 16S rRNA gene sequencing, we went one step further and isolated 542 bacteria and fungi, screened them for osmotic stress resistance, and linked them back to the amplicon dataset.

Materials and methods

Experimental design

The experiment was conducted at the Institut national de la recherche scientifique experimental field (Laval, Qc, Canada) in 2016 and 2017. The field was divided into 6 blocks separated by 2 m, each containing eight 4 m^2 plots separated by 1 m. Each plot was subjected to one of the following four rainfall manipulation treatments from May to October each year: 100% precipitation, 75% precipitation, 50% precipitation, or 25 % precipitation by covering the plots with wooden shelters supporting various amounts of UV-transparent plastic sheets. The excess rainfall was collected in a gutter and directed into a bucket, which was manually emptied after significant rainfall events. Two wheat genotypes were used in this study: *Triticum aestivum* cv AC Nass (spring wheat) which was developed for the province of Quebec climate and known as a water stress-sensitive genotype, and *Triticum turgidum* subsp. *durum* cv. Strongfield (durum wheat) which was developed for the Canadian Prairies climate and is known as a water stress tolerant genotype. The first year, the seeds were obtained from Agriculture and Agri-Food Canada, and for the second year, the seeds harvested at the end of the first year were used. In May, each plot was seeded with approximately 2,000 seeds distributed in ten rows, for a density of 500 seeds/m^2. The experiment contained 6 fully randomized blocks, for a total of 48 plots (2 genotypes x 4 rainfall manipulation x 6 blocks). For the need of the current study, we sampled, in July 2017, roots, leaves and rhizosphere soil from the 25% and 100% precipitation treatments, resulting in 72 samples: 3 compartments x 2 rainfall manipulation treatments x 2 genotypes x 6 blocks). The soil water content (SWC) at the moment of sampling was on average 19.08 % ± 2.48 and 14.45% ± 3.14 for the 100% and 25% precipitation treatments, respectively. Seeds were collected for isolation work at the end of the growing season, in August 2017. Samples were stored at -20°C before DNA extraction and microbial isolation.

Isolation media

We used eight different solid culture mediums (tap-water yeast extract (TWYE), tryptic-soy agar (TSA), Reasoner's 2A agar (R2A), potato dextrose agar (PDA), Czapek-Dox agar, soil extract, plant extract and artificial root exudates (solution 2) (Baudoin, Benizri and Guckert 2003)) to maximize the diversity of isolates. For the soil and the plant extract media, 10 seeds of the two wheat genotypes were seeded separately in 500 g of commercial organic soil, maintaining the SWC at 50% of soil holding capacity for 20 days in triplicates pots. The plants were then harvested, and 50 g of plant leaves and root-adherent soil were crushed separately in 100 ml of autoclaved water then ground with an electric mixer. The extracted mixture was then filtered at 0.22 µm and the flow-through was mixed with the same volume of autoclaved 3% agar maintained at 45°C. The two media were then poured into sterile petri dishes.

Surface sterilization and microbial isolation

Bacteria and fungi were isolated from the rhizosphere, and surface-sterilized roots, seeds and leaves of the two wheat genotypes from the 25% and 100% treatments of blocks 1 and 2 of the precipitation manipulation field experiment. The surface sterilization of 0.1g of harvested plant material was done according to Tardif *et al.* (2016) with some modifications. Thoroughly rinsed samples were immersed for 5 min in 95% ethanol, for 10 min in 2.5% NaOCl with gentle shaking every minute, for 5 min in 95% ethanol, and rinsed 3 times in autoclaved distilled water. The final water wash was plated on three TSA plates (100 µl per petri) and incubated at 30°C for 24h to check the success of surface sterilization. Rhizosphere soils and surface sterile plant material (0.1g) were crushed with a sterile pestle and mortar in 1 ml of autoclaved saline water (0.9% NaCl). A

hundred microliters of this crushed plant and soil material were mixed with 0.9 ml of sterile saline water and then serially diluted in (10^{-1} to 10^{-6}) in sterile saline water. A hundred microliters of the four last dilutions were plated on the 8 different media and incubated at 28°C for 24h for the 10^{-3} and 10^{-4} dilutions on TWYE, TSA, R2A, for 48h for the 10^{-5} and 10^{-6} dilutions on TWYE, TSA, R2A, or for 5 to 7 days for all dilutions plated on the plant, soil and artificial root exudates media. The PDA and Czpaek-Dox agar plates were incubated at 25°C for 48h for the 10^{-3} and 10^{-4} dilutions and for 5 days for the 10^{-5} and 10^{-6} dilutions. The streaking method was used to isolate all colonies separately on TSA (bacteria) or PDA (fungi) plates. Each isolated bacterial colony was grown in TSB supplemented with 15% glycerol and then frozen at -20°C. For fungi, an agar plug with abundant mycelia was immersed in 70% glycerol before freezing at -20°C.

Screening of the isolates for growth in polyethylene-glycol

The isolated microorganisms were screened for their capacity to grow in liquid culture under reduced water availability in 96-well plates. Each isolate was inoculated in TSB (bacteria) or yeast-extract peptone dextrose broth (YPD; fungi) and incubated for 24h at 28°C with shaking at 240 rpm. A volume of 350 µl of each liquid culture was then inoculated in 0.2 ml of TSB (bacteria) or YPD (fungi) containing various concentrations of PEG-600. 0%, 10%, 20%, 30% of PEG (v/v) were used to simulate water stress (Marulanda, Barea and Azcón 2009) by generating hyperosmotic conditions (Michel and Kaufmann 1973). The plates were incubated for 24h at 28°C under aerobic conditions with shaking at 140 rpm. Growth was deemed positive when the opacity of the well changed.

DNA extraction

For bacterial isolates, DNA was extracted by phosphate-buffered saline (PBS) lysis using the thermal shock method. For that, 20 µl of the glycerol-preserved bacteria was diluted in 2 ml of TSB and incubated at 28°C for 24 h with shaking at 240 rpm. A volume of 1.5 ml of each culture was centrifuged at 1,400 rpm for 10 min. The pellet was washed in 500 µl of autoclaved PBS and centrifuged at 14,000 rpm for 10 min. Cell lysis was achieved by resuspending the pellet in 100 µl of PBS. Tubes were incubated at 100°C for 10 min followed by 10 min on ice. The supernatant containing the DNA was recovered in sterile Eppendorf tubes after 10 min of centrifugation at 14,000 rpm. For fungal isolates, glycerol-preserved fungal agar plugs were deposited on PDA and incubated at 25°C for 48h. A small quantity of mycelia was transferred to 100 µl of sterile water in a microcentrifuge tube and vortexed thoroughly and centrifuged at 10,000g for 2 min. The pellet was then resuspended in 100 µl of lysis solution (50 mmol/l sodium phosphate at pH 7·4, 1 mmol/l EDTA and 5% glycerol) and DNA was extracted as previously described (Zhang *et al.* 2010). For DNA extraction from plant tissue, leaves and roots were grounded in liquid nitrogen using a mortar and pestle. Five hundred milligram of rhizosphere soil or ground plant tissues (pooled from 5-6 individual plants) was then used for DNA extraction using a phenol-chloroform extraction method (Dellaporta, Wood and Hicks 1983) as modified by Azarbad et al. (2018).

PCR amplification and library preparation

To identify isolates, the bacterial 16S rRNA gene was amplified by PCR using the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Suzuki and Giovannoni 1996) and the fungal ITS1 region was amplified using ITS1-F (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’). PCR products were purified and sent for forward and

reverse Sanger sequencing at the Centre d'expertise et de services Génome Québec (CESGQ) (Montréal, Canada). For Illumina 16S rRNA gene amplicon sequencing, the amplicon library construction was done as described previously in Yergeau et al. (2015) based on a dual-indexing strategy that followed the '16S Metagenomic Sequencing Library preparation' Illumina guide (Part #15 044 223 Rev. B). The V4 hypervariable region was amplified using the universal primers 520F (5' - AGCAGCCGCGGTAAT- 3') and 799R2 (5' - CAGGGTATC TAATCCTGTT- 3') (Edwards *et al.* 2007), that exclude plant plastids 16S rRNA genes. PCR products were purified, pooled and submitted for 2 × 250 bp Illumina MiSeq sequencing at the CESGQ.

Bioinformatic analyses

16S rRNA gene amplicon sequencing data was analyzed using AmpliconTagger (Tremblay and Yergeau 2019). Raw reads were controlled for quality. Remaining high quality reads and free of sequencing adapters artefacts were dereplicated at 100% identity and clustered/denoised at 99% (DNAclust v3). Clusters of less than three reads were discarded and remaining clusters were scanned for chimeras using UCHIME, first in de novo mode then in reference mode (Edgar *et al.* 2011). Remaining clusters were clustered at 97% identity (DNAclust v3) to produce OTUs. OTUs were assigned a taxonomic lineage with the RDP classifier (Wang *et al.* 2007) using the Silva release 128 database (Quast *et al.* 2013) supplemented with eukaryotic sequences from the Silva database and a customized set of mitochondria, plasmid and bacterial 16S sequences (see the AmpliconTagger databases DOI:10.5281/zenodo.3560150). The RDP classifier gave a score (0 to 1) to each taxonomic depth of each OTU. For each OTU, the taxonomic lineage was reconstructed by keeping only the taxa that had a score ≥ 0.5. To normalize the OTU table, thousand-reads

rarefactions were then performed 500 times and the average number of reads of each OTU of each sample was then computed to obtain a consensus normalized OTU table.

For Sanger sequencing and the identification of isolates, sequence data were treated using the Genious software (Genious Prime 2021.0.3, Biomatters, *Inc*., San Diego, USA). We first proceeded by inspecting the electropherogram and replacing unresolved sequencing peaks by Ns for both reverse and forward sequence reads. The two sequences were then merged by the *de novo* assembly method and the merged sequences were identified through Blast searches in the NCBI database, keeping only hits with more than 97% similarity. The bacterial isolates that were highly resistant to osmotic stress (i.e. that grew in PEG 30%) were matched to their representative OTUs (from the normalized OTU table) by locally Blasting the 16S rRNA gene sequences of the isolates against the sequence representative of each OTU and keeping only hits that had >97% identity over at least 200 bp. We thereby created a truncated normalized OTU table that matched the osmotic stress resistant isolates.

Statistical analysis

Statistical analyses were performed in R (version 4.0.3, The R Foundation for Statistical Computing). Shapiro-Wilks and Levene tests revealed that, even after log or square root transformation, the alpha diversity and relative abundance data did not meet the assumptions for parametric ANOVA. Therefore, four independent one-way Kruskal-Wallis tests by rank were performed for the effects of Precipitation, Compartment, Genotype and Block. In the case of compartments, when the Kruskal-Wallis tests were significant, we performed pairwise Mann-Whitney U-tests to determine which compartment significantly differed from the others. The effect of Precipitation, Compartment and Genotype on the bacterial community structure was visualized

using principal coordinate analyses (PCoA) and tested using Permanova with 1,000 permutations (including Blocks as a controlling factor), both based on Bray-Curtis dissimilarity calculated from the normalized OTU table. For significant Permanova results, we further tested if this effect was due to differences in the dispersion of the samples within each group (beta-dispersion). Indicator species analysis was performed on the OTU table to single out OTUs that were strongly linked to either the 25% or 100% precipitation treatments. Chi-square test for contingency tables was used to test, for the different treatments (compartment, wheat genotypes and precipitation treatments), if the observed frequency of isolates that could grow in PEG 30% differed significantly from expected values under the null hypothesis.

Data availability

The raw amplicon datasets and associated metadata are available through NCBI BioProject accession PRJNA767855.

Results

Bacterial community structure, composition and diversity

The principal coordinate analyses (PCoA) showed that the plant compartment (leaf, root, rhizosphere) was the main factor influencing the bacterial community structure, with a visible effect of wheat genotype and precipitation levels for some compartments (Fig. 1). Accordingly, in Permanova tests, plant compartment and genotype were the factors having the strongest influence followed by precipitation (Table 1). In the case of compartment, there was also significantly different ($P<0.001$) dispersion among groups in beta-dispersion analyses. Compartment and genotype also significantly interacted to shape the microbial community structure. This can be visualized by the separation of the DS genotype from the DT genotype for the leaf samples, a pattern that was not clear for the other compartments (Fig. 1). Beta-dispersion analysis was also significant for the Compartment:Cultivar interaction ($P=0.033$). In Permanova, the effect of the precipitation treatments had a marginally significant main effect ($P=0.072$) and a similarly marginally significant interactive effect with compartment ($P=0.070$, Table 1). In the PCoA, the precipitation treatments are well separated for the root samples, but not for the other compartments (Fig. 1). The interactive effects were confirmed by running Permanova for each compartment separately (Table 2). We found a significant genotype effect only in the leaf samples, and a significant effect of the precipitation manipulations only in the root samples (Table 2).

Bacterial alpha diversity (Shannon index) had a mean of 4.45 and did not vary significantly across the treatments ($P>0.05$ in Kruskal-Wallis tests). In contrast, the community composition was significantly affected by the compartments, with compartment having a highly significant effect on all dominant phyla/classes (Table 3). Post-hoc pairwise tests showed that for Actinobacteria, Betaproteobacteria, and Gammaproteobacteria, the three compartments were

significantly different from each other, with Gammaproteobacteria being more abundant in the leaves, Actinobacteria in the rhizosphere and Betaproteobacteria in the roots (Fig. 2, Table 3). Bacteroidetes were significantly more abundant in the roots, as compared to the two other compartments, whereas Gemmatimonadetes and Deltaproteobacteria were significantly more abundant in the rhizosphere as compared to the leaves and roots (Fig. 2, Table 3). The Alphaproteobacteia were significantly less abundant in the leaves as compared to the two other compartments (Fig. 2, Table 3). Finally, the Firmicutes were significantly less abundant in the roots as compared to the rhizosphere, but their relative abundance in these two compartments did not differ significantly from their relative abundance in the leaves (Fig. 2, Table 3). The Firmicutes were significantly more abundant on the DT cultivar as compared to the DS cultivar (Fig. 2, Table 3). Indicator species analyses highlighted 63 OTUs that were indicators of the 25% precipitation treatment and 26 that were indicators of the 100% precipitation treatment (Table S1). Among the 25% treatment indicators, 26 belonged to the Proteobacteria, 17 to the Bacteroidetes, 9 to the Actinobacteria and 3 to the Firmicutes (Table S1). Among the 100% treatment indicators, 14 belonged to the Proteobacteria, 2 to the Bacteroidetes, 2 to the Actinobacteria and 2 to the Firmicutes (Table S1).

Microbial isolation and screening

We isolated 542 microorganisms from the different wheat compartments (147 (27.1%) from roots, 152 (28.0%) from leaves, 204 (37.6%) from rhizosphere soil and 39 (7.2%) from seeds) of the two genotypes (304 (56.1%) for DT and 238 (43.9%) for DS) growing under different precipitation treatments (297 (54.8%) for 100% precipitation and 245 (45.2%) for 25% precipitation). Among these, 223 isolates (41.1% of all isolates) could grow in liquid media supplemented with 20% PEG.

In liquid media supplemented with 30% PEG, only 44 isolates (8.1% of all isolates) were able to grow, comprising 32 bacteria, 9 fungi and 3 unidentified isolates (Table 4). Among the 32 bacterial isolates that grew in PEG 30%, 52% belonged to the Firmicutes, 39% to the Actinobacteria and 9.7% to the Proteobacteria (Table 4), as compared to relative frequencies of 67.6% for Firmicutes, 26.6% for Actinobacteria and 5.8% for Proteobacteria for all bacterial isolates that could be identified by Sanger sequencing. Eight of the nine fungal isolates growing in liquid media supplemented with PEG 30% were *Penicillium* spp. and the other was *Cladosporium* sp. (Table 4). Isolates from leaves were more often able to grow in liquid media supplemented with 30% PEG (15/152, 9.9% of leaf isolates) than isolates from other compartments (3/39, 7.7% for seeds, 16/204, 7.8% for rhizosphere and 10/147, 6.8% for roots) (Fig. 3). To test if the frequency of isolates being able to grow in liquid media supplemented with 30% PEG significantly differed from expected values if the treatments had no effect, we performed a chi-square test for each of the experimental factors. Precipitation and genotype did not significantly change the frequency of isolates that could grow in PEG 30% (χ^2=0.00, df=1, P=1 and χ^2=0.09, df=1, P=0.763, respectively). However, there was a significant effect of the compartment on the frequency of isolates that could grow in PEG 30% (χ^2=9.64, df=3, P=0.0219).

OTUs corresponding to the PEG 30% isolates

We Blasted the 16S rRNA gene sequences from the 32 bacterial isolates that grew in liquid media supplemented with 30% PEG against the consensus sequences of the OTUs derived from the amplicon sequencing dataset, to identify which OTUs corresponded to our isolates. These OTUs represented 0.51% of all OTUs (24 OTUs out of 4,672 OTUs) and 3.2% of all reads (37,348 reads out of 1,173,132 reads). We performed statistical analyses on this subset of OTUs, to determine if

they responded to the treatments as the general community. Compartment, Genotype, Precipitation, and the Cultivar:Genotype and the three-way interactions were all significant (Table 5). Three OTUs that matched our PEG 30% bacterial isolates were also found as indicators for the 25% precipitation treatment and were identified as *Rhodococcus* (Actinobacteria), *Pseudomonas* (Gammaproteobacteria) and *Microbacterium* (Actinobacteria) (Table S1).

Discussion

Here, we experimentally manipulated precipitation levels in the field and assessed its effect on the microbial community, not only through an amplicon sequencing approach but also by retrieving microbial isolates and testing their resistance to osmotic stress in link with the experimental treatments. In contrast to our hypothesis, the precipitation reduction only modified the community structure in the roots and, consequently, did not increase the frequency of water stress-resistant microorganisms in the wheat environment. Osmotic stress-resistant microorganisms were more frequently isolated from the leaf endosphere, and the bacterial OTU community matching these isolates was significantly influenced by the plant compartment. This suggests that the frequency of water-stress resistance naturally varies across plant-associated habitats. Even though they were relatively depleted in Actinobacteria (the phylum to which belonged most of our resistant isolates), the leaf endosphere yielded more frequently isolates that could grow at high osmotic pressure (PEG 30%). The assembly of the leaf endophytic bacteria results either from the colonization of the rhizosphere followed by migration or through colonization from the leaf surface community (Compant, Clément and Sessitsch 2010). The model put forward by Xiong et al. (2021) suggests that plant-associated bacterial communities are gradually filtered from the bulk soil to the different plant compartments. This would result in leaf microbiomes with lower diversity, less complex networks (Xiong *et al.* 2021) and lower biomass (Azarbad *et al.* 2018). This also suggests that leaf endophytic bacteria must possess several very specific physiological adaptations to migrate through the xylem or nutrient-rich intracellular spaces and finally colonize the leaf endosphere (Compant, Clément and Sessitsch 2010). One of these adaptations could be growing at high osmotic pressure. Alternatively, leaf surfaces are believed to be one of the harshest microbial plant environments, with very little nutrient and water available and high solar irradiation (Kowalchuk

et al. 2010; Bulgarelli *et al.* 2013). It would therefore not be surprising if microbes that migrated from this environment into the leaf endosphere would be more frequently able to grow under high osmotic pressure. However, the frequency of resistant microorganisms did not exceed 10% of the leaf isolates retrieved, which suggests that the ability to grow at high osmotic pressure is not a major trait needed for adaptation to the leaf endosphere environment. Because microorganisms that were not isolated here (either because they are not culturable or because our isolation effort was not enough) could grow at high osmotic pressure, it is difficult to conclude if this trait is important for life in the leaf endosphere. Other mechanisms of adaptation to low water availability, such as dormancy, could also be more prevalent among the leaf endospheric community.

Even though the bacterial communities were generally not affected by the precipitation manipulations, some results suggested an effect on the osmotic stress resistant microorganisms. Many bacterial OTUs were indicators for the 25% precipitation treatment, including three that matched with the PEG 30% bacterial isolates. Precipitation treatment was also significant when focusing the analyses on the OTUs that matched the PEG 30% bacterial isolates. This suggests that experimentally reducing precipitations favored bacteria with a high resistance to osmotic stress, even though this reduction in precipitation had minor effects on soil water content (reduction of soil water content by 23%, from 19% to 14.5%) and did not visibly stress the plants. Since these osmotic-stress resistant bacteria were a relatively minor part of the community (the OTUs matching PEG 30% isolates accounted for 3.2% of the total reads), the effects of the precipitation treatments were not clearly visible in global tests and on the isolation frequency. Rare microorganisms are thought to play central roles under changing environmental conditions, and the relatively infrequent osmotic stress resistant bacteria detected here could play a key role under more severe water stress.

As compared to leaf isolates, root and rhizosphere isolates were less frequently able to grow at high osmotic pressures, and when taken separately, the root bacterial community was significantly affected by the precipitation treatments. This heightened sensitivity of the root microbial communities to changes in water availability was previously shown for field-grown sorghum, where root microbial community composition was more sensitive to drought which also resulted in a decreased total bacterial abundance (Xu *et al.* 2018). In addition, drought stress can reduce root biomass and development (Azarbad *et al.* 2018; Xu *et al.* 2018), induce changes in exudates (Rizaludin *et al.* 2021) and have a significant effect on root transcriptional (Xu *et al.* 2018) and metabolomics activity (Liu *et al.* 2020), suggesting an indirect plant-mediated route by which root-associated microbes can be affected by changing water availability. This might explain why, the later the drought event occurs in the development of the plant, the lower the impact on the composition of the root microbial community (Xu *et al.* 2018). Taken together, this implies that a lower frequency of osmotic stress resistant microorganisms and the potential for indirect plant-mediated effects make the root microbial communities particularly sensitive to change in soil water availability.

Significant differences between the two wheat genotypes were also observed. Differences between the microbial communities of different plant genotypes were often reported (Berg *et al.* 2006; Garbeva, van Elsas and van Veen 2008; Azarbad *et al.* 2020; Wagner, Busby and Balint-Kurti 2020) and linked to differences in protein-level responses to drought for different genotypes (Budak *et al.* 2013), or specific root exudation patterns across genotypes (Garbeva, van Elsas and van Veen 2008; Sasse, Martinoia and Northen 2018). However, recent reports showed that the strength of the wheat genotype effect on microbial communities is often subtle and can be dwarfed by other factors (such as plant compartments in the present study), and can even sometimes

completely disappear (Yergeau, Quiza and Tremblay 2020). We also isolated osmotic stress resistant bacteria from the two cultivars at the same frequency, suggesting that the differences between the plant genotype in their resistance to water stress are not necessarily mirrored in their associated microbial communities.

Our result showed that the Actinobacteria were overrepresented among the isolates having a high resistance to osmotic stress, being represented by five genera (*Streptomyces, Micrococcus, Artrobacter, Microbacterium* and *Rhodococcus*). Together with the Firmicutes, they represented over 90% of the osmotic stress resistant isolates, which is consistent with recent research that demonstrated that Actinobacteria and Firmicutes are more resistant to drought conditions as compared to the Proteobacteria (Xu *et al.* 2018). In amplicon sequencing studies, the Actinobacteria generally increase their abundance under drier conditions (Barnard, Osborne and Firestone 2013; Xu *et al.* 2018; Azarbad *et al.* 2020, 2021; Simmons *et al.* 2020; Wipf, Bùi and Coleman-Derr 2020), which can be modulated by the duration of the dry period (Xu *et al.* 2018) and the plant development stage (Xu *et al.* 2018; Simmons *et al.* 2020). In contrast to other phyla containing water stress-resistant bacteria, such as the Proteobacteria, Chloroflexi, Firmicutes, Gemmatimonadetes and Planctomycetes, the Actinobacteria seem to be particularly adapted to life in dry environments as they synthesize and accumulates ribosomes throughout dry periods, which makes them able to get a head start as soon as conditions become more favorable for nutrient acquisition (Barnard, Osborne and Firestone 2013). Our culture-based approach allows us to indicate that this pattern of increased abundance could also be due to a better ability of the Actinobacteria to grow under high osmotic stress. In contrast, although the relative abundance of the Firmicutes is known to increase under dry conditions (Xu *et al.* 2018; Azarbad *et al.* 2021), they were underrepresented among the isolates that were able to grow in liquid media

supplemented with 30% PEG, suggesting that they might use different mechanisms to survive drier conditions, such as spore formation.

In conclusion, our study shows the value of combining culture-based approaches to more commonly used amplicon sequencing approaches. Indeed, using this combined methodology we were able to confirm the osmotic stress resistance of the bacterial communities associated with wheat plants subjected to reduced precipitation. Although the reduction in precipitation did not increase the frequency of isolation of osmotic stress resistant microorganisms, we were able to show that wheat leaves are a potential source of such isolates and that reduction in precipitations does affect the osmotic stress resistant bacterial community. With these isolates in hand, the next steps would be to test for their capacity to improve wheat resistance to drought, toward the development of microbe-based solutions to mitigate the impact of global changes on crops.

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Conflicts of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Principal coordinates analysis of Bray–Curtis dissimilarities calculated from the bacterial 16S rRNA gene OTUs relative abundance for leaf, root and rhizosphere samples from two wheat cultivars (drought-tolerant and drought-sensitive) growing in the field under two precipitation levels (25% and 100%).

Figure 2. Relative abundance of the phyla/classes that accounted for, on average, more than 1% of the bacterial 16S rRNA gene reads retrieved from leaf, root and rhizosphere samples from two wheat cultivars (drought-tolerant and drought-sensitive) growing in the field under two precipitation levels (25% and 100%).

Figure 3. Number of microbial isolates from leaves, rhizosphere, roots and seeds that could grow in liquid media supplemented with 0%, 10%, 20% or 30% of polyethylene glycol (PEG-600).

Table 1. Permanova tests for the effects of precipitation manipulation, genotype, compartments and their interactions on the bacterial community structure based on Bray-Curtis dissimilarities.

Factor	Df	R^2	F	P
Compartment (C)	2	0.439	39.02	0.001 ***
Genotype (G)	1	0.047	8.389	0.001 ***
Precipitation (P)	1	0.012	2.193	0.072 .
Block	5	0.047	1.681	0.029 *
C:G	2	0.094	8.426	0.001 ***
C:P	2	0.021	1.792	0.070 .
G:P	1	0.010	1.832	0.131
C:G:P	2	0.018	1.630	0.101
Residual	55	0.309		
Total	71	1.000		

***: P<0.001, **: 0.001<P<0.01, *0.01<P<0.05, .: 0.05<P<0.10

Table 2. Permanova tests for the effects of precipitation and genotype on rhizosphere, root and leaf-associated bacterial community structure based on Bray-Curtis dissimilarities.

	Rhizosphere				Leaf				Root			
	Df	F	R^2	P	Df	F	R^2	P	Df	F	R^2	P
Genotype	1	1.21	0.05	0.21	**1**	**12.02**	**0.35**	**0.001*****	1	1.20	0.05	0.24
Precipitation	1	0.71	0.31	0.86	1	1.17	0.05	0.259	1	**2.27**	**0.09**	**0.01****

***: P<0.001, **: 0.001<P<0.01, *0.01<P<0.05, .: 0.05<P<0.10

Table 3. Kruskal-Wallis and post-hoc Mann-Whitney U tests for the effects of precipitation, genotype, and compartments on the relative abundance of the bacterial phyla/classes that represented, on average, over 1 % of the reads in the 16S rRNA gene amplicon dataset.

Factor		Actino.	Bacteroid.	Firmicutes	Gemma.	Alpha.	Beta.	Delta.	Gamma.
Compartment	Df	2	2	2	2	2	2	2	2
	X^2	55.58	34.85	9.76	37.99	31.54	45.21	31.93	58.76
	P	**8.5×10^{-13}**	**2.7×10^{-8}**	**0.007**	**5.6×10^{-9}**	**1.4×10^{-7}**	**1.5×10^{-10}**	**1.2×10^{-7}**	**1.7×10^{-13}**
	Post-hoc	L Rh R a b c	L Rh R a a b	L Rh R ab b a	L Rh R a b a	L Rh R a b b	L Rh R a b c	L Rh R a b a	L Rh R a b c
Genotype	Df	1	1	1	1	1	1	1	1
	X^2	0.07	3.67	8.73	3.60	1.97	1.63	1.16	0.51
	P	0.79	0.05	**0.003**	0.057	0.16	0.20	0.28	0.47
Precipitation	Df	1	1	1	1	1	1	1	1
	X^2	0.49	0.68	0.00	0.02	0.02	0.86	0.54	0.22
	P	0.48	0.41	0.99	0.87	0.87	0.35	0.46	0.74
Block	Df	5	5	5	5	5	5	5	5
	X^2	0.81	2.23	2.56	1.01	4.61	1.68	1.71	1.23
	P	0.97	0.82	0.77	0.96	0.46	0.89	0.88	0.94

Values in boldtype face are significant at $P<0.05$.

Table 4. Taxonomical assignment and source of isolation of bacterial and fungal isolates that grew in media supplemented with 30% PEG based on Blast searched in NCBI.

Genotype	Compartment	Precipitation	Identity (%)	Assignment
Bacteria				
DT	Leaves	25%	99.5	*Bacillus sp.*
DS	Leaves	25%	99.5	*Bacillus pumillus*
DS	Leaves	25%	97.3	*Paenibacillus amylolyticus*
DT	Leaves	100%	99.9	*Bacillus sp.*
DT	Leaves	100%	100	*Psychrobacillus sp.*
DS	Leaves	25%	100	*Bacillus pumilus*
DT	Leaves	25%	99.1	*Micrococcus sp.*
DS	Rhizosphere	100%	99.6	*Streptomyces sp.*
DS	Rhizosphere	100%	99.4	*Rhodococcus fascians*
DT	Rhizosphere	100%	100	*Microbacterium maritypicum*
DT	Rhizosphere	100%	100	*Microbacterium oxydans*
DT	Rhizosphere	100%	97.6	*Microbacterium arborescens*
DT	Rhizosphere	100%	99.9	*Sporosarcina ureae*
DT	Rhizosphere	25%	99.9	*Pseudomonas sp.*
DT	Rhizosphere	25%	99.9	*Rhodococcus erythropolis*
DT	Rhizosphere	25%	100	*Microbacterium oleivorans*
DS	Rhizosphere	25%	100	*Microbacterium sp.*
DS	Rhizosphere	25%	99.9	*Alcaligenes sp.*
DS	Rhizosphere	25%	99.9	*Alcaligenes sp.*
DS	Rhizosphere	100%	99.7	*Bacillus aryabhattai*
DS	Rhizosphere	100%	98.8	*Pseudarthrobacter sp.*
DT	Roots	25%	97.7	*Sphingobacterium sp.*
DT	Roots	25%	99.9	*Microbacterium phyllosphaerae*
DT	Roots	100%	98.1	*Bacillus sp.*
DT	Roots	100%	99.8	*Solibacillus silvestris*
DT	Roots	25%	97.0	*Bacillus megaterium*
DS	Roots	25%	99.6	*Bacillus megaterium*
DS	Roots	100%	100	*Bacillus aryabhattai*
DS	Roots	100%	99.9	*Arthrobacter sp.*
DS	Roots	100%	98.7	*Bacillus aryabhattai*
DS	Seeds	100%	99.5	*Bacillus pumilus*
DS	Seeds	100%	99.7	*Bacillus aerius*
Fungi				
DT	Seeds	25%	97.5	*Penicillium sp.*
DT	Rhizosphere	100%	98.8	*Penicillium sp.*
DS	Rhizosphere	25%	99.2	*Cladosporium sp.*
DT	Shoot	25%	100	*Penicillium sp.*
DT	Shoot	25%	98.9	*Penicillium sp.*
DT	Shoot	25%	99.1	*Penicillium sp.*
DS	Root	25%	98.9	*Penicillium sp.*

DS	Shoot	100%	99.3	*Penicillium sp.*
DS	Shoot	100%	99.2	*Penicillium sp.*

DS : Drought sensitive.
DT : Drought tolerant.

Table 5. Permanova test for the effects of precipitation manipulation, genotype, compartments, and their interactions on the community structure of the OTUs that matched the 16S rRNA gene of the bacterial isolates growing in TSB supplemented with 30% PEG.

Factor	Df	R^2	F	P
Compartment (C)	2	0.26	15.52	0.001 ***
Genotype (G)	1	0.033	3.881	0.006 **
Precipitation (P)	1	0.216	2.529	0.032 *
Block	5	0.073	1.724	0.024 *
C:G	2	0.063	3.699	0.001 ***
C:P	2	0.027	1.618	0.098 .
G:P	1	0.013	1.551	0.165
C:G:P	2	0.035	2.031	0.035 *

***: P<0.001, **: 0.001<P<0.01, *0.01<P<0.05, .: 0.05<P<0.10

Figure 1

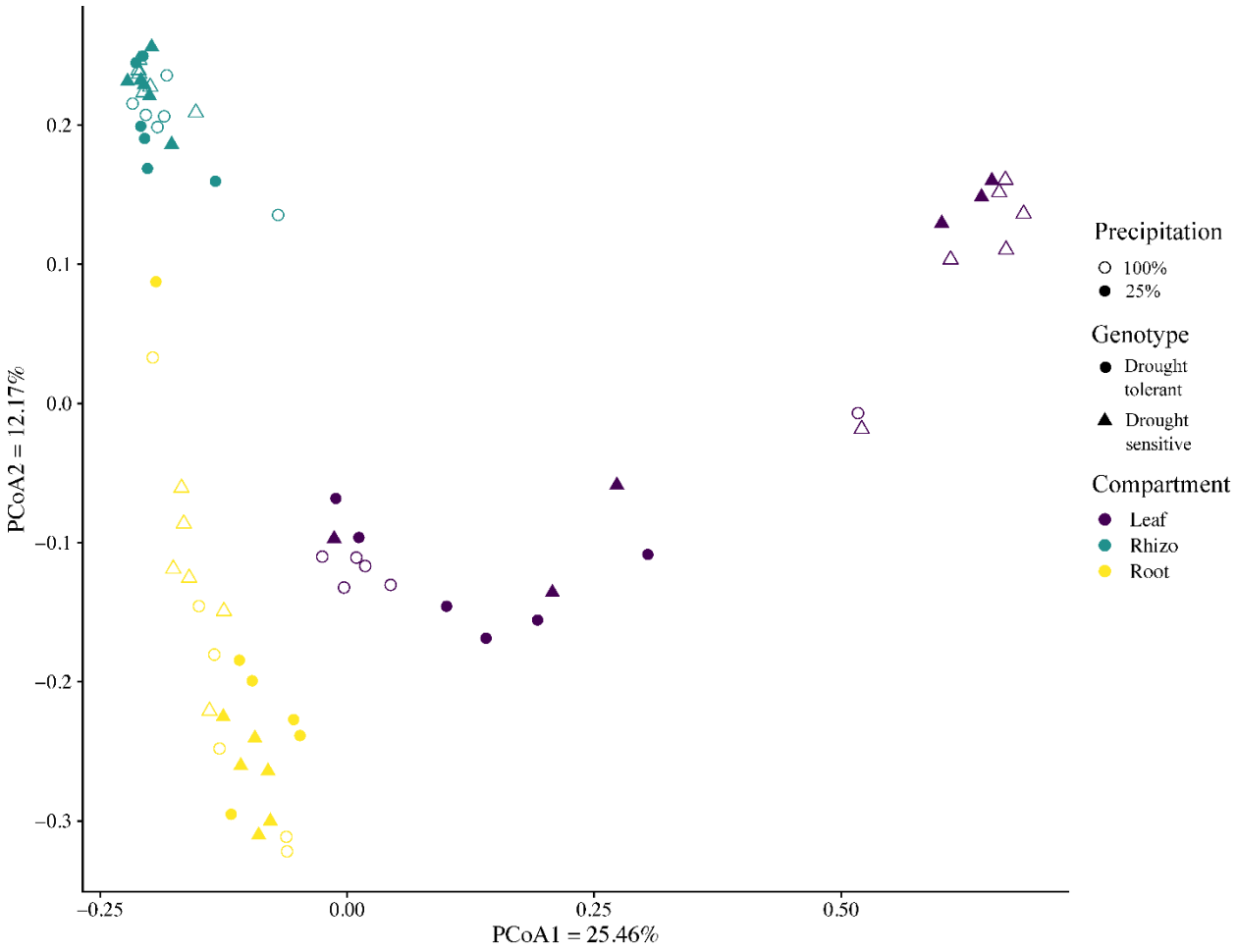

0.2
0.1
0.0
−0.1
−0.2
−0.3
PCoA2 = 12.17%
−0.25
0.00
0.25
0.50
PCoA1 = 25.46%
Precipitation
100%
25%
Genotype
Drought tolerant
Drought sensitive
Compartment
Leaf
Rhizo
Root

Figure 2.

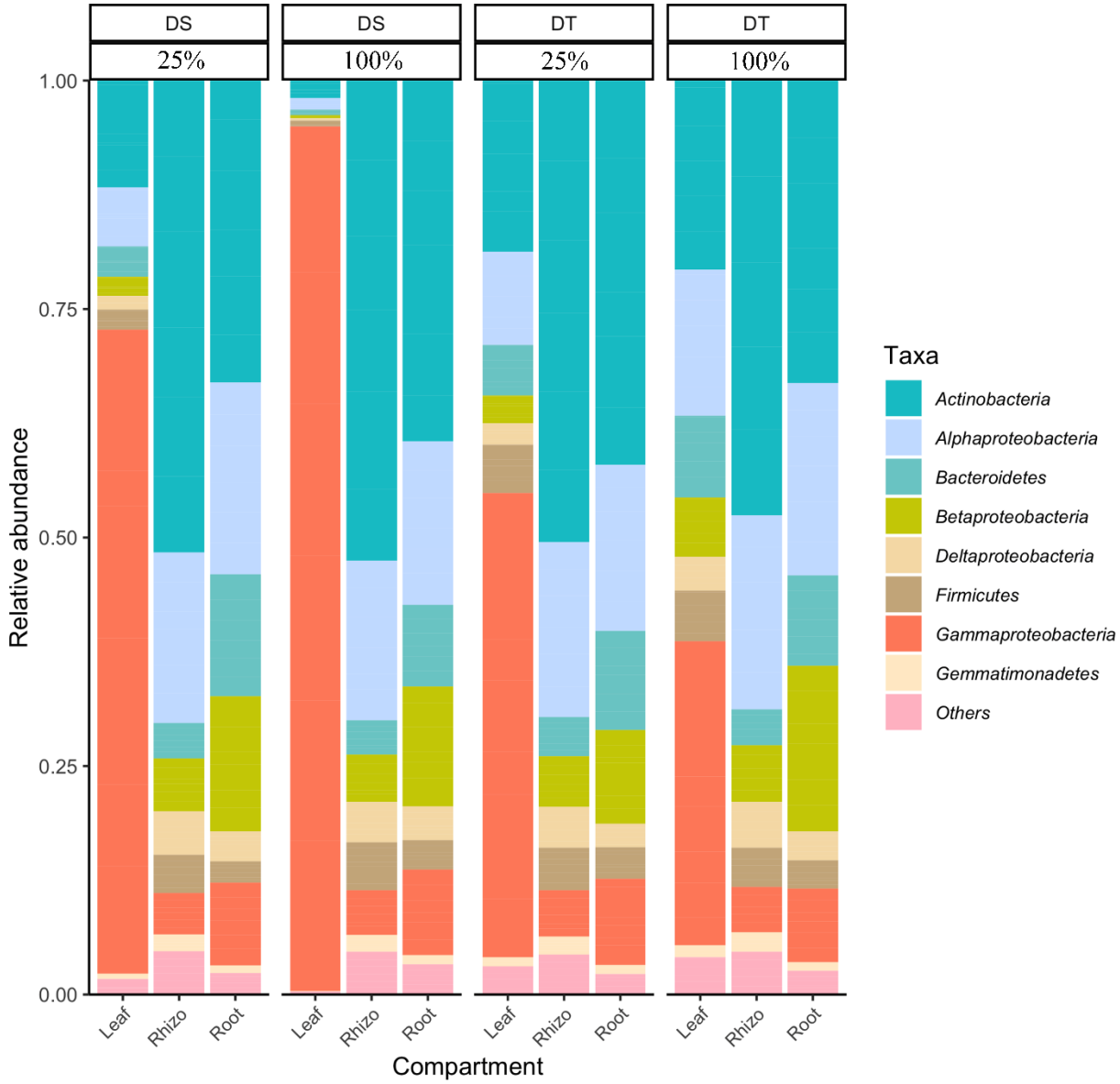
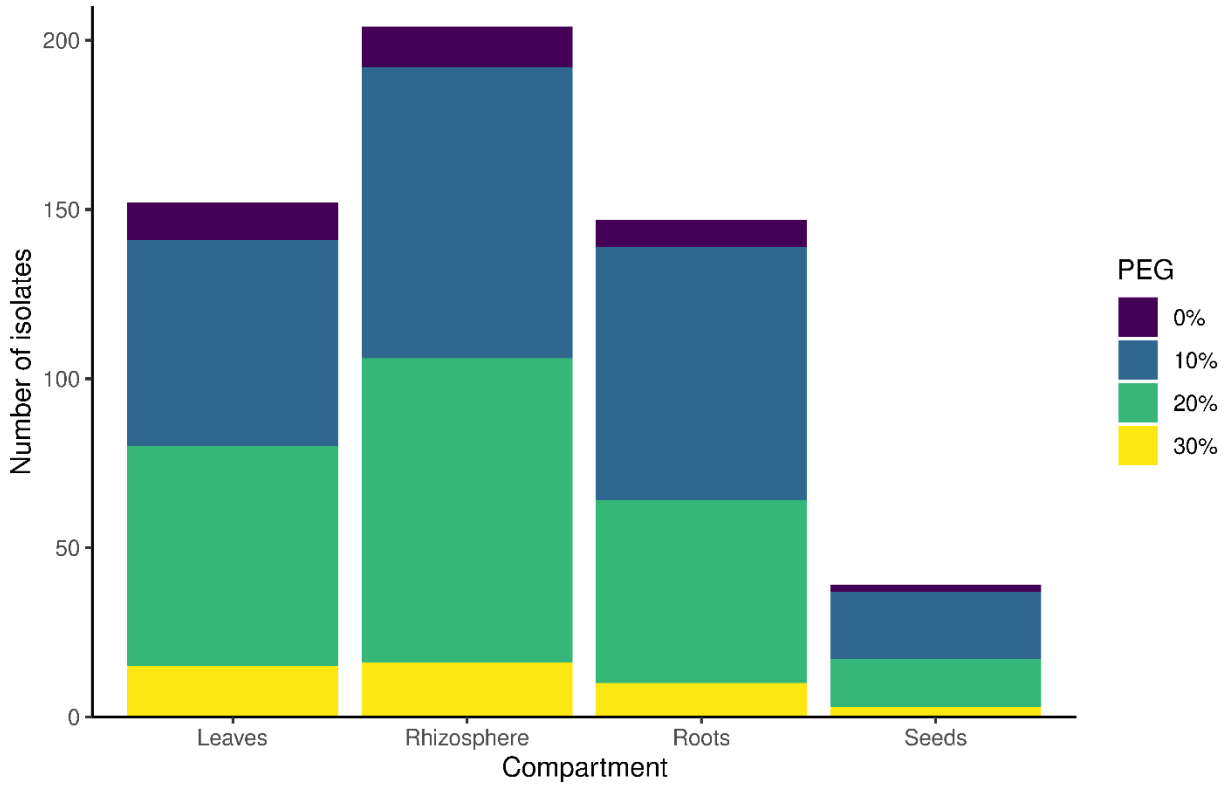

DS
25%
DS
100%
DT
25%
DT
100%
1.00
0.75
0.50
0.25
0.00
Relative abundance
Leaf
Rhizo
Root
Compartment
Taxa
Actinobacteria
Alphaproteobacteria
Bacteroidetes
Betaproteobacteria
Deltaproteobacteria
Firmicutes
Gammaproteobacteria
Gemmatimonadetes
Others

Figure 3.


200
150
100
50
0
Number of isolates
Leaves
Rhizosphere
Roots
Seeds
Compartment
PEG
0%
10%
20%
30%