



Water stress and disruption of mycorrhizas induce parallel shifts in phyllosphere microbiome composition

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Summary

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Received: 25 June 2021
Accepted: 6 October 2021

New Phytologist (2022) 234: 2018–2031
doi: 10.1111/nph.17817

Key words: aboveground–belowground interactions, drought, microbiome assembly, multi-kingdom interactions, mycorrhizas, plant health.

- Water and nutrient acquisition are key drivers of plant health and ecosystem function. These factors impact plant physiology directly as well as indirectly through soil- and root-associated microbial responses, but how they in turn affect aboveground plant–microbe interactions are not known.
- Through experimental manipulations in the field and growth chamber, we examine the interacting effects of water stress, soil fertility, and arbuscular mycorrhizal fungi on bacterial and fungal communities of the tomato (*Solanum lycopersicum*) phyllosphere.
- Both water stress and mycorrhizal disruption reduced leaf bacterial richness, homogenized bacterial community composition among plants, and reduced the relative abundance of dominant fungal taxa. We observed striking parallelism in the individual microbial taxa in the phyllosphere affected by irrigation and mycorrhizal associations.
- Our results show that soil conditions and belowground interactions can shape aboveground microbial communities, with important potential implications for plant health and sustainable agriculture.

Introduction

Soil water and nutrient content are among the most important predictors of plant health (Pautasso *et al.*, 2010; Gupta *et al.*, 2020). Disturbances that deplete water or alter nutrient composition in soil are expected to increase in frequency and severity with changing climates and altered management practices (St. Clair & Lynch, 2010; Pritchard, 2011; Seidl *et al.*, 2017; Bowles *et al.*, 2018). It is therefore crucial to understand how interacting ecological stressors will act in future years to shape plant function. A key piece of the puzzle is how these changes impact plant-associated microbial communities aboveground and belowground, and, in turn, how plant–microbiome interactions buffer or exacerbate these stressors. Plant microbiomes play key roles in plant growth, immune development, and disease resistance (Trivedi *et al.*, 2020), and a growing body of work indicates that these associations are vulnerable to disruption under changing global conditions, including climate and agricultural management. Belowground, for example, drought can induce complex changes in microbial dynamics (Naylor *et al.*, 2017; de Vries *et al.*, 2018; Xu *et al.*, 2018), including negative to positive impacts on mycorrhizal associations (Rillig *et al.*, 2002; Verbruggen & Toby Kiers, 2010), which are key drivers of plant performance (Smith & Read 1996; Latef *et al.*,

2016). Moreover, increased fertilization via agricultural intensification can cause plants to reduce resource allocations to microbial symbionts such as mycorrhizas and rhizobia, often selecting for less mutualistic strains (Johnson, 1993; Weese *et al.*, 2015). Together, shifts in water availability, soil nutrient content, and mycorrhizal communities in changing environments are likely to alter plant performance, including plant–microbiome interactions.

Although the majority of work in this space has focused on belowground plant–microbe interactions, the microbial communities associated with aboveground plant tissues, known as the phyllosphere, are gaining increased recognition in plant health. These microbiomes have been shown to play roles in nitrogen fixation, tolerance of extreme temperatures, and protection against pathogens (Lindow *et al.*, 1982; Fürnkranz *et al.*, 2008; Hubbard *et al.*, 2014; Berg & Koskella, 2018). Much like their belowground counterparts, phyllosphere microbiome functions can be disrupted by changes in climate and agricultural management. For example, experimental warming reduces phyllosphere microbial diversity and nitrogen fixation in peat moss (Carrell *et al.*, 2019). Soil fertilization reduces the resistance of phyllosphere communities to invasion by the foliar pathogen *Pseudomonas syringae* (Berg & Koskella, 2018). Finally, functional genes involved in carbon, nitrogen, phosphorus (P), and sulfur cycling are less abundant in the phyllosphere metagenomes of plants in farmlands than in forests (Xiang *et al.*, 2020). The building

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evidence makes it clear that plant microbiomes both shape and are shaped by their host plant's response to the local environment. What remains unclear are both how these responses might change under different or interacting stressors, and whether aboveground and belowground microbial communities impact one another's responses to such stress.

To begin addressing these questions and capture effects of soil conditions on the phyllosphere microbiome, we focused on soil properties that induce systemic changes in plant physiology. Water stress can impact nutrient and biomass allocation, phytohormone production, and toxin accumulation in leaves (Moles *et al.*, 2018; Zhou *et al.*, 2019; Gupta *et al.*, 2020), while soil fertility can affect nutrient content and acidity of aboveground plant tissues (Fageria & Barbosa Filho, 2007; Wang *et al.*, 2008). Arbuscular mycorrhizal fungi (AMF) colonize plant roots and modulate plant water and nutrient status, leaf gas exchange, and the expression of stress response genes (Liu *et al.*, 2007; Gerlach *et al.*, 2015). We therefore asked how water stress, fertilization, and mycorrhizal colonization affected bacterial and fungal communities in the tomato (*Solanum lycopersicum*) phyllosphere.

Through a combination of field and growth chamber experiments, we found that both plant water stress and disruption of mycorrhizas altered bacterial and fungal composition in the phyllosphere. Upon closer examination, we found that both stressors reduced bacterial richness within plants, homogenized bacterial communities across plants, and increased the evenness of fungal communities. Further, there was a high degree of overlap in the responses of individual indicator taxa to both treatments, suggesting that soil water availability and belowground mycorrhizal associations act in parallel on phyllosphere microbial communities.

Materials and Methods

Field site and treatments

The field trial was conducted at the University of California Davis Student Farm ($38^{\circ}32'29.49''N$, $121^{\circ}46'0.94''W$), a certified organic farm, during the 2019 growing season (see Supporting Information Methods S1 for details of field preparation). We conducted our experiment at a certified organic farm to minimize any chances of chemical inputs (e.g. fungicides) directly disrupting the microbial communities we aimed to study. Three treatments, water stress, P fertility, and mycorrhizal associations, were arranged in a split-plot design and assigned to levels in a fertilization gradient (Fig. S1). Water stress included two levels, fully watered (100% of crop evapotranspiration, ET_c) and deficit (50% ET_c) irrigation applied in alternating beds, with the deficit beginning 22 d after transplant, and water completely cut to the deficit beds 76 d after transplant. Phosphorus fertility was manipulated as a 12-level gradient of fertilizer established in each irrigation condition (24 plots total). We chose a fertilizer product approved for use in US organic systems. We applied an organic fertilizer, seabird guano (0-11-0, Down to Earth) on the surface of each plot, which was then incorporated with a cultivator a week before planting. The gradient ranged from 0 to 550 g of fertilizer $P\ m^{-2}$, including duplicate plots of the lowest ($0\ g\ P\ m^{-2}$)

and highest ($550\ g\ P\ m^{-2}$) treatments (Table S1). The 24 experimental plots were each split into two subplots, one with a tomato genotype deficient in mycorrhizal associations (*rmc*; Barker *et al.*, 1998) and another with its wild-type progenitor (76R), for a total of 48 experimental units. Seeds of the 76R and *rmc* genotypes were sourced from plants grown in the glasshouse. To avoid effects of adjacent irrigation treatments, three buffer plants were planted but not sampled between experimental units.

Soil samples were taken from three depths (0–15, 15–30, and 30–60 cm) at each plot. Gravimetric water content was measured as the difference between soil mass before and after the soils were dried at 105°C. Bioavailable P in soils was measured using the Olsen P method, based on phosphate extraction using sodium bicarbonate solution, at transplant, midseason, and harvest. Stem water potential was measured on one plant per plot, 53 d after transplant. Aboveground biomass was measured at harvest (116 d after transplant) from four plants per plot, of which a subsample was taken for further analysis. Total P in aboveground plant tissue was measured by Murphy–Riley colorimetry following microwave-assisted digestion in nitric acid and hydrogen peroxide.

Sample collection from field

Sixty days after transplant, three compound leaves per plant were sampled from both irrigation regimes and genotypes along the two lowest ($0\ g\ P\ m^{-2}$), two middle (220 and $330\ g\ P\ m^{-2}$), and two highest ($550\ g\ P\ m^{-2}$) positions in the fertilizer gradient. Four plants were sampled per replicate plot, for a total of eight plants per combination of irrigation regime, plant genotype, and fertilizer treatment. Samples were transported to the laboratory on ice. Samples used to generate inocula for growth chamber experiments were briefly stored at 4°C before processing, while samples used only for sequencing were frozen at –20°C.

To isolate microbial communities, leaves were pooled from the same plant into bags and suspended in sterile buffer. Bags were submerged in a Branson M5800 sonicating water bath for 10 min to gently dislodge microbial cells from the leaf surface. The resulting leaf wash was centrifuged at 3500 g for 10 min, resuspended in sterile buffer, and frozen at –80°C in a 50 : 50 mixture of King's B Medium and glycerol cryoprotectant. Up to 16 individual plants were processed each day until complete. Plant sonication batches were randomly assigned with respect to treatments, and were included as a covariate in all statistical models.

Mycorrhizal colonization was measured using roots collected during the midseason sampling period (51 d after transplant). Using a 6 cm diameter × 10 cm deep core, plant roots were collected 10 cm from the stem from one plant per subplot. Samples were rinsed in water, stored in ethanol, and stained using the ink and vinegar method (Vierheilig *et al.*, 1998). Colonization was then measured using the gridline intersect method (Giovannetti & Mosse, 1980).

Plant care in growth chamber experiments

We next assessed two of the field trial treatments in a more controlled setting. Surface-sterilized tomato seeds were germinated

on sterile agar, then transplanted into pots (see Methods S2 for growth chamber conditions). In the mycorrhiza experiment, an inoculum containing equal amounts of several arbuscular mycorrhizal fungi taxa (*Gigaspora rosea*, *Gigaspora albida*, *Acaulospora spinosa*, *Rhizophagus intradices*, and *Funneliformis mosseae*) was added to each pot at the transplant stage. Mycorrhizal inoculum was acquired from INVAM (West Virginia University, Morgantown, WV, USA). Half of the inoculum (14 g) was mixed into the potting mix, and the other half was added directly to the seedling transfer site at the top. In the fertilizer experiment, 105.6 mg of P (960 mg fertilizer) was added to half of the pots at the transplant stage, approximating the highest position of the P gradient in the field (550 g P m⁻³, assuming a fertilizer penetration depth of 20 cm). The other half of the pots were not fertilized (0 g P m⁻³). All pots were immediately randomized with respect to treatment and remained under controlled growth chamber conditions for the duration of the experiment.

Microbiome spray and pathogen challenge in growth chamber

Three-week-old plants were sprayed with microbial inocula from tomato plant leaves in the field. To track assembly of the same source communities under different conditions, each sample (which corresponded to a single plant in the field) was divided in half and used to generate inocula for two plants. In the mycorrhiza experiment, each field sample was used to inoculate one wild-type plant and one *rmc* plant in the growth chamber. In the P experiment, each field sample was used to inoculate one fertilized and one unfertilized plant in the growth chamber. Each plant was sprayed with 4 ml of microbial inocula at a standardized concentration of 10⁴ colony-forming unit (CFU) ml⁻¹.

The purpose of these experiments was two-fold. First, they served as an independent and more controlled test of the treatment effects in the field trial. Pots were completely randomized with respect to treatment, and the paired design allowed direct comparison of plants that received the same microbiota but were grown under different conditions. Second, after allowing communities to establish for 1 wk, we inoculated plants with the bacterial pathogen *P. syringae* pv tomato PT23. *Pseudomonas syringae* can grow to high densities in the tomato genotypes 76R and *rmc* but does not cause disease symptoms. This experiment allowed us to test whether soil conditions had altered microbiome resistance to invasion, a key property of ecological communities that has been shown to vary with nutrient availability (Berg & Koskella, 2018; Baumgartner *et al.*, 2021).

Three leaves per plant were inoculated with *P. syringae* at an optical density (OD₆₀₀) of 0.0002 (*c.* 10⁵ CFU ml⁻¹) by blunt-end syringe injection into the abaxial side of the leaf. Plants were destructively sampled 24 h after the *P. syringae* challenge. To measure *P. syringae* population density, three hole punches were taken from each of the inoculated leaves and homogenized in sterile buffer using a FastPrep-24 5G sample disruption instrument. *Pseudomonas* density on leaves was obtained through CFU plating and droplet digital polymerase chain reaction (ddPCR). To characterize epiphytic microbial communities, the remaining

aboveground plant material, which had been sprayed with microbial inocula but not directly challenged with *P. syringae*, was collected in a 15 ml tube. Microbial communities were isolated through sonication and centrifugation as described earlier. Total bacterial density was obtained through ddPCR with primers for conserved sequences in the 16S rRNA gene. Primer sequences and reaction conditions for ddPCR assays are available in Methods S3.

DNA extraction and sequencing

DNA was extracted from microbial communities isolated from the field and both growth chamber experiments. Extractions were performed using the DNeasy PowerSoil kit. Extraction batches of 24 samples were assigned randomly with respect to treatments and included in all statistical models. One extraction in each set of 24 was a negative control. Libraries were prepared by amplifying the V4 region of the 16S rRNA gene and the ITS2 gene (see Methods S3 for primer sequences). Libraries were amplified, cleaned, and sequenced alongside DNA extraction controls and PCR controls on the Illumina MiSeq platform at Microbiome Insights (Vancouver, BC, Canada).

Reads were analyzed using the recommended DADA2 workflow (Callahan *et al.*, 2016) to infer amplicon sequence variants (ASVs). Taxonomy was assigned using the SILVA and UNITE databases (Kõljalg *et al.*, 2005; Quast *et al.*, 2013). Because many internal transcribed spacer (ITS) sequences could not be classified at the kingdom level by UNITE, the hidden Markov model classifier ITSx was used to further delineate ITS sequences and remove nonfungal sequences (Bengtsson-Palme *et al.*, 2013). Thus, 16S variants were filtered to remove chloroplast and mitochondrial sequences. Potential contaminants were filtered based on prevalence in negative controls vs true samples using the package DECONTAM (Davis *et al.*, 2018). Additional details on quality control parameters are available in Methods S4.

Statistical analysis

Alpha diversity (species number) and beta diversity (species turnover) were analyzed using ANOVA and permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) with irrigation regime, plant genotype, soil fertilizer concentration, plant sonication batch, and DNA extraction batch as covariates. When treatments were allowed to interact, none of the interaction terms were significant, and model selection based on Akaike's information criterion favored the model without interactions. Indicator taxa were identified based on Dufrêne and Legendre's indicator value (R package MULTIPATT). To independently validate this analysis, we also modeled differential abundance across treatments (R package EDGER) (Robinson *et al.*, 2010).

The correlation between bacterial and fungal richness was assessed with Pearson's product correlation coefficient. Partial R² values were calculated to test whether this correlation could be attributed to shared responses of bacteria and fungi to other variables (R package RSQ). To test correlations between bacterial and fungal composition, a partial Mantel test was conducted on their respective Bray–Curtis dissimilarity matrices along with a

Euclidean distance matrix of field trial treatments and technical batches. To construct a co-occurrence network, the abundances of the top 500 bacterial taxa and the top 500 fungal taxa were first converted to presence–absence measures (as relative abundances would not be directly comparable across sequencing libraries). The two datasets were combined and the package RCORR was used to generate matrices of Pearson’s correlations and corresponding *P*-values. After Benjamini–Hochberg correction for multiple testing (false discovery rate (FDR) < 0.1), a network was constructed on significant correlations (R package iGRAPH).

While colonization of plants in the growth chamber by field communities was generally high, a subset of plants had low bacterial abundance and a high proportion of ASVs unique to the growth chamber, which appeared to confound estimates of bacterial diversity (Fig. S2). To address this, ASVs were classified as shared with the field trial or unique to the growth chamber. All analyses were conducted separately on the two categories. In support of this approach, ASVs shared with the field trial generally recapitulated treatment effects observed in the field, while the potential growth chamber contaminants appeared to be randomly distributed with respect to treatment (Fig. S3). A paired Wilcoxon signed-rank test was used to compare microbiome assembly on plants in the growth chamber that had received the same inoculum from the field.

Results

Effects of experimental treatments on soil conditions and field plants

In the field, wild-type plants had significantly higher AMF root colonization than the reduced mycorrhizal genotype (mean 76R = 17%, mean *rmc* = 11%; 95% confidence interval (CI) for difference in means = (0.60, 11), *P* = 0.032; Fig. S4a). Though there were no significant differences in soil gravimetric water content between fully watered and deficit irrigation plots at the mid-season sampling (51 d after transplant), there was a significant difference in soil water content at all three depths (0–15, 15–30, and 30–60 cm) at harvest (116 d after transplant), and at 53 d after transplant plants grown under deficit irrigation showed a trend toward an increase in stem water potential, i.e. experiencing more water stress (*P* = 0.07, Fig. S4b). Plant biomass from the reduced mycorrhizal genotype had significantly lower P concentrations than the wild-type as measured by percent dry biomass (absolute change of −0.2%, *P* < 0.001), as did deficit irrigation as compared to fully watered plants (−0.05%, *P* = 0.01), but the applied fertilizer gradient did not significantly impact P concentration in plant biomass or available soil P (Olsen P) at any of the sampling events (Fig. S4c–e). Finally, there were no significant differences in aboveground biomass at harvest among the treatments (data not shown).

Phyllosphere microbiome composition in field trial

We next measured the impact of the field trial treatments on microbial community composition in the phyllosphere. Water

regime and mycorrhizal associations significantly predicted variation in bacterial and fungal community composition, while the effect of fertilization was marginally significant in fungal communities only (Table 1).

Shifts in community composition often reflected changes in alpha diversity (species diversity per plant) and beta diversity (heterogeneity within treatments) (full model results in Tables S2, S3). Compared to fully watered plants, water-stressed plants harbored lower bacterial richness ($F = 10.468$, $df = 72$, $P = 0.0018$), lower Shannon diversity ($F = 4.990$, $df = 72$, $P = 0.029$), and more similar communities to one another ($F = 7.347$, $df = 92$, $P = 0.0080$). Simultaneous decreases in alpha and beta diversity often indicate loss of rare species (i.e. species that inhabit some sites, but not others, across a landscape) (Socolar *et al.*, 2016). Accordingly, rare ASVs showed the strongest discrepancies between the full water and deficit treatments (Fig. S5). In fungal communities, water stress increased the Shannon diversity index ($F = 7.966$, $df = 72$, $P = 0.0062$), a combined measure of richness and evenness. Further examination of the individual components revealed that this effect was driven by an increase in evenness under water-limited conditions ($F = 8.495$, $df = 72$, $P = 0.0047$), while richness was not affected (Fig. 1).

The reduced mycorrhizal (*rmc*) plants harbored lower phyllosphere bacterial richness ($F = 5.203$, $df = 72$, $P = 0.026$) and more similar communities to one another ($F = 7.548$, $df = 92$, $P = 0.0072$) than their wild-type counterparts. As with water stress, the strongest discrepancies between wild-type and *rmc* plants were among rare ASVs (Fig. S6). The *rmc* plants harbored lower fungal richness ($F = 11.417$, $df = 72$, $P = 0.0011$) and

Table 1 Full results of permutational multivariate analysis of variance models on Bray–Curtis dissimilarity matrices.

Treatment	df	Sum of squares	Mean square	F value	R ²	P-value
Bacterial community composition						
Irrigation regime	1	0.601	0.601	1.496	0.015	0.00079
Genotype	1	0.495	0.495	1.232	0.012	0.028
Fertilizer concentration	1	0.455	0.455	1.133	0.011	0.112
Plant sonication batch (randomized across treatments)	12	5.763	0.480	1.196	0.145	<0.0001
DNA extraction batch (randomized across treatments)	6	3.427	0.571	1.423	0.086	<0.0001
Fungal community composition						
Irrigation regime	1	0.470	0.470	1.436	0.014	0.011
Genotype	1	0.482	0.482	1.473	0.015	0.0089
Fertilizer concentration	1	0.404	0.404	1.234	0.013	0.061
Plant sonication batch (randomized across treatments)	12	4.633	0.386	1.180	0.141	0.0003
DNA extraction batch (randomized across treatments)	6	3.388	0.564	1.726	0.103	<0.0001

Terms that explain significant portions of variance (*P* < 0.05) are indicated in bold typeface.

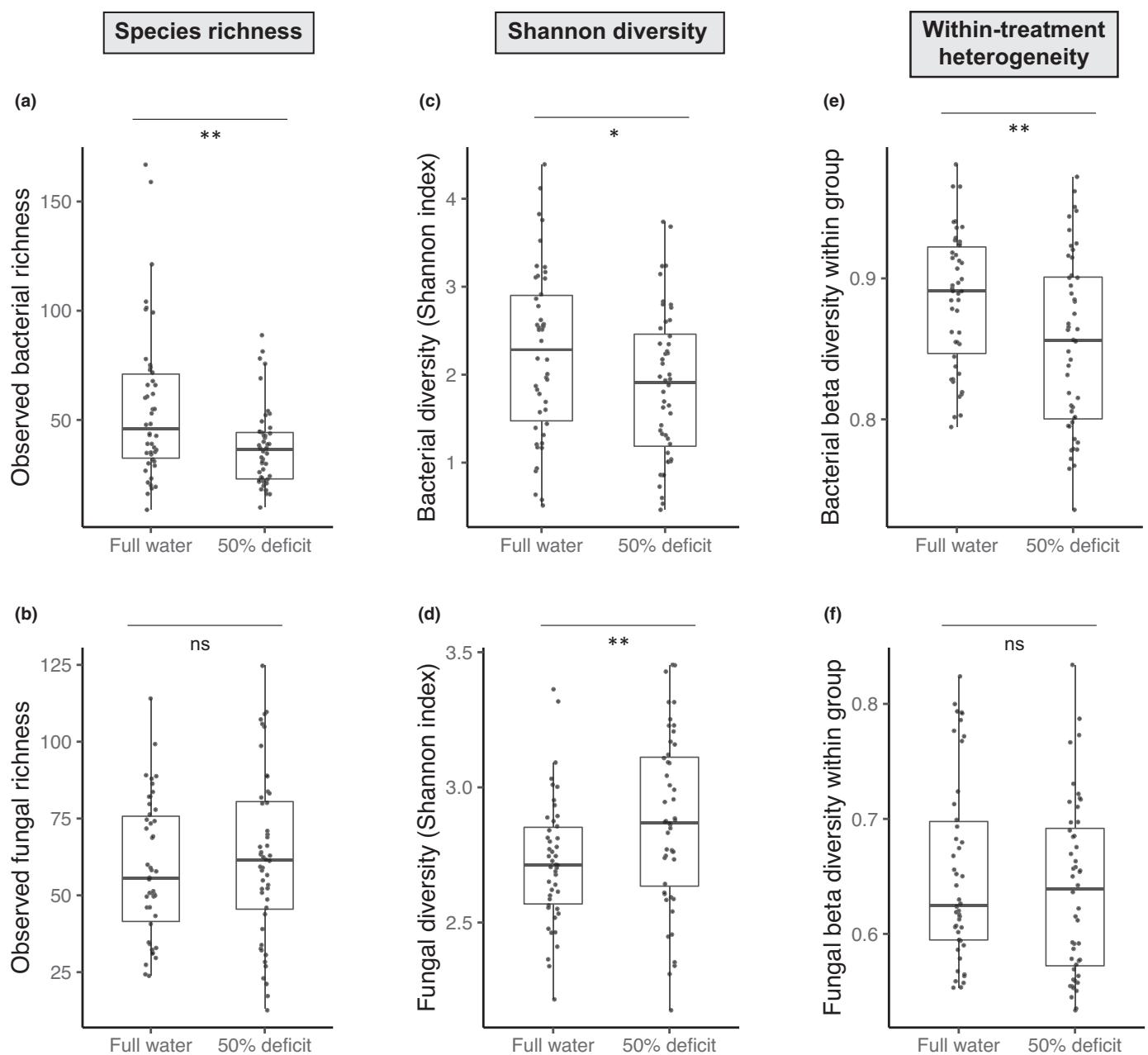


Fig. 1 Water stress reduces bacterial alpha and beta diversity and increases fungal alpha diversity in the tomato phyllosphere. (a, b) Number of amplicon sequence variants (ASVs) per sample. Bacterial richness values were log-transformed before analysis to meet normality and homoscedasticity assumptions. (c, d) Shannon–Weaver index of combined richness and evenness. (e, f) Within-group dispersion was quantified by generating a Bray–Curtis dissimilarity matrix. Points represent the mean Bray–Curtis dissimilarity between each sample and other samples within the same treatment group (full water or 50% deficit). The lower and upper hinges of each box plot represent the 25th and 75th percentiles of the data, respectively, while the upper and lower whiskers extend to values within 1.5 times the interquartile range. Levels of significance are indicated as follows: **, 0.001 ≤ P < 0.01; *, 0.01 ≤ P < 0.05; ns, P ≥ 0.05.

higher fungal evenness ($F = 13.182$, $df = 72$, $P < 0.001$) than wild-type plants (Fig. 2).

Having observed that water limitation and disruption of mycorrhizal associations induced similar changes in bacterial community metrics (i.e. reduction in alpha and beta diversity), we next asked whether similar taxa were affected in both treatments. A PERMANOVA on Bray–Curtis dissimilarity separated the fully watered wild-type plants from plants in deficit and/or reduced

mycorrhizal conditions ($P < 0.001$ accounting for effects of P fertilization and sample processing batches), while the remaining combinations of irrigation regime and mycorrhizal associations (wild-type/deficit, *rmd*/full water, *rmd*/deficit) were not statistically different from one another (Fig. 3a,b). To identify specific taxa affected by each treatment, we calculated Dufrêne and Legendre's indicator value for each bacterial ASV. There was a high degree of overlap in indicator taxa for the full water and wild-type

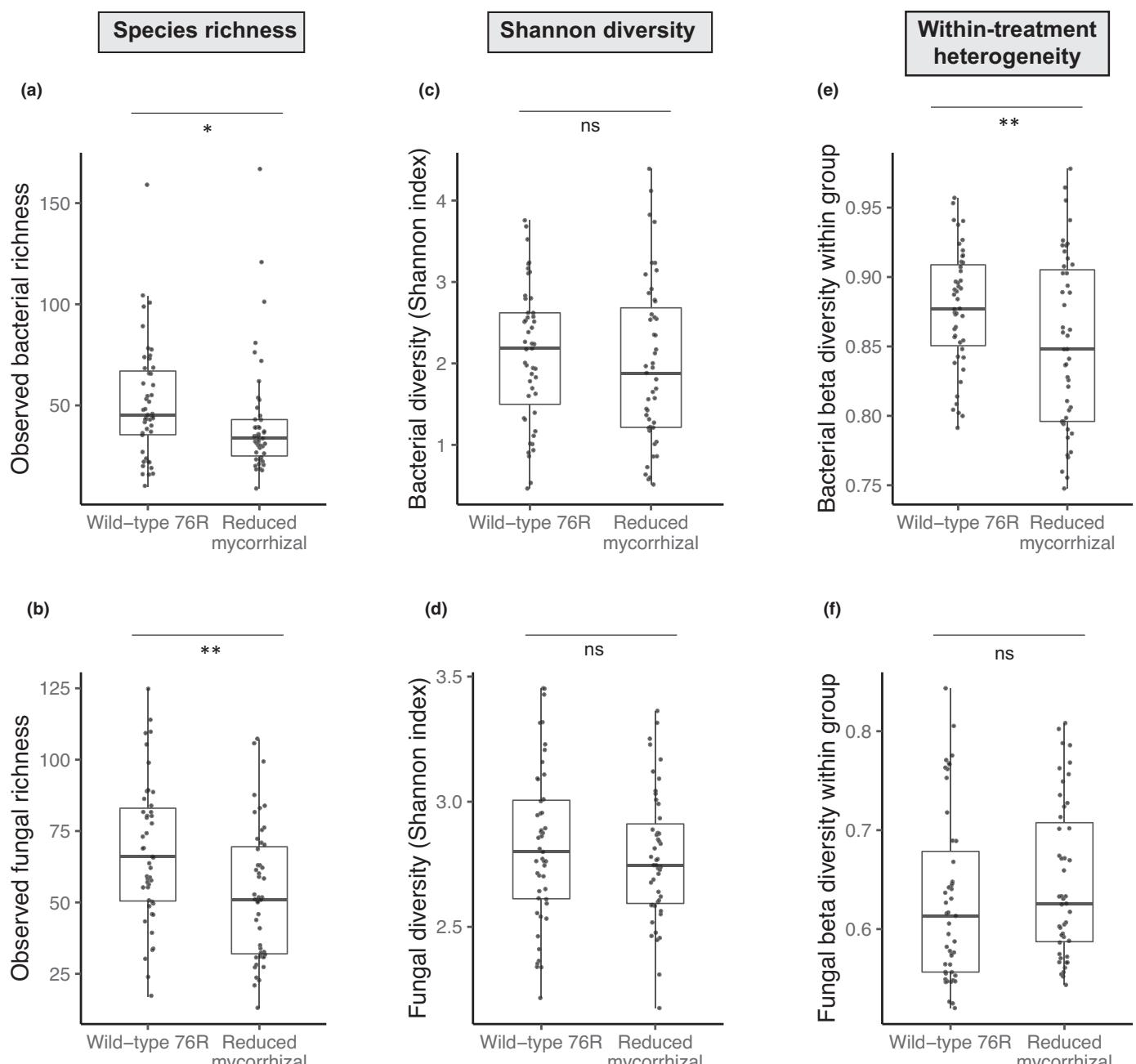


Fig. 2 The *rmc* genotype harbors lower bacterial alpha and beta diversity, and lower fungal alpha diversity, than its wild-type counterpart. (a, b) Number of amplicon sequence variants (ASVs) per sample. Bacterial richness values were log-transformed before analysis to meet normality and homoscedasticity assumptions. (c, d) Shannon–Weaver index of combined richness and evenness. (e, f) Within-group dispersion was quantified by generating a Bray–Curtis dissimilarity matrix. Points represent the mean Bray–Curtis dissimilarity between each sample and other samples within the same plant genotype (76R or *rmc*). The lower and upper hinges of each box plot represent the 25th and 75th percentiles of the data, respectively, while the upper and lower whiskers extend to values within 1.5 times the interquartile range. Levels of significance are indicated as follows: **, 0.001 ≤ P < 0.01; *, 0.01 ≤ P < 0.05; ns, P ≥ 0.05.

treatments, and in indicator taxa for the water-stressed and reduced mycorrhiza treatments (Fig. 3c). As indicator species analysis is based on occupancy patterns, we further validated this result using negative binomial regression, a method commonly used to identify taxa associated with treatments based on relative abundance patterns (Robinson *et al.*, 2010). We again found substantial parallels between the effects of irrigation regime and mycorrhizal associations on bacterial community composition (Fig. S7). We

next examined fungal communities and again observed substantial overlap in the distribution of individual indicator taxa (Fig. S8). Finally, we verified that our results could not be attributed to chance effects during random assignment to plant sonication or DNA extraction batches by comparing the effects of treatments on indicator taxa within, rather than across, technical batches. As expected, treatment effects within batches consistently mirrored those observed within the full dataset (Fig. S9).

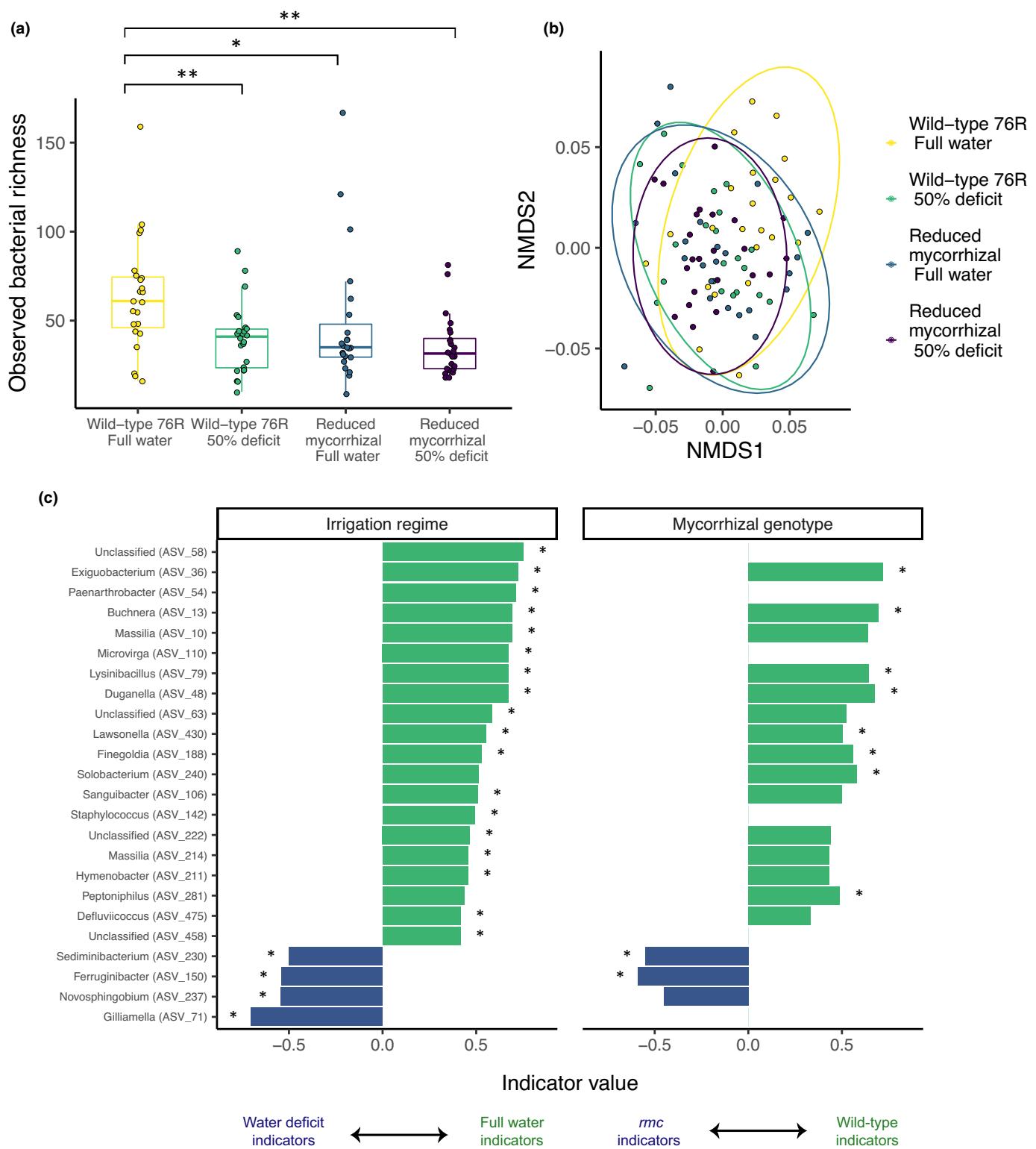


Fig. 3 Water stress and *rmc* genotype induce parallel shifts in bacterial composition. (a) Number of amplicon sequence variants (ASVs) per sample. Bacterial richness values were log-transformed before analysis to meet normality and homoscedasticity assumptions. The lower and upper hinges of each box plot represent the 25th and 75th percentiles of the data, respectively, while the upper and lower whiskers extend to values within 1.5 times the interquartile range. Levels of significance are indicated as follows: **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$; ns, $P \geq 0.05$. (b) Nonmetric multidimensional scaling (NMDS) ordination of plants in the field trial, colored by irrigation regime and mycorrhizal genotype. Ellipses indicate 95% confidence around groups. (c) Indicator ASVs identified for irrigation regime (on a wild-type background) or mycorrhizal genotype (on a full water background). Names indicate genus-level classification, with ASV name in parenthesis. Values represent the square root of Dufrêne and Legendre's indicator value. For ease of interpretation, the indicator values of opposing treatments (water deficit vs full water, reduced mycorrhizal vs wild-type) are displayed with opposing signs. Asterisks indicate nominal significance ($P < 0.05$). Missing bars indicate taxa that were too prevalent in both treatments to statistically test association.

By contrast, the fertilizer gradient did not impact bacterial alpha or beta diversity. However, increasing fertilizer concentrations were associated with higher fungal alpha diversity ($F=4.802$, $df=72$, $P=0.032$) and increased heterogeneity in fungal communities among plants ($F=6.293$, $df=72$, $P=0.0028$) (Fig. S10). Further examination revealed that none of the taxa identified as indicators of fertilization were present in the fertilizer product itself, suggesting that the effect of fertilizer was not directly attributable to the addition of microbes (Table S4).

Cross-kingdom associations

Next, we asked how bacterial and fungal communities co-associated in our study, and whether these associations changed under water or nutrient limitation. Bacterial richness and fungal richness were highly correlated across plants ($r=0.482$, $df=92$,

$P<0.0001$, Fig. 4a). The relationship remained significant in an ANOVA model that controlled for the field trial treatments (irrigation regime, mycorrhizal associations and soil fertilizer concentration) and technical effects (plant sonication batch, DNA extraction batch, and number of 16S and ITS reads per sample). Partial R^2 calculation indicated that bacterial richness accounted for 16.5% of the variation in fungal richness in this model. Bray–Curtis dissimilarity measures based on bacterial community composition and fungal community composition were correlated as well, including when controlling for field trial treatments and technical batches (partial Mantel statistic = 0.1563, $P=0.001$).

To assess the contributions of cross-kingdom associations to overall occupancy patterns, we constructed a co-occurrence network for each combination of irrigation regime and plant genotype (Fig. 4b). Across all treatment combinations, cross-kingdom

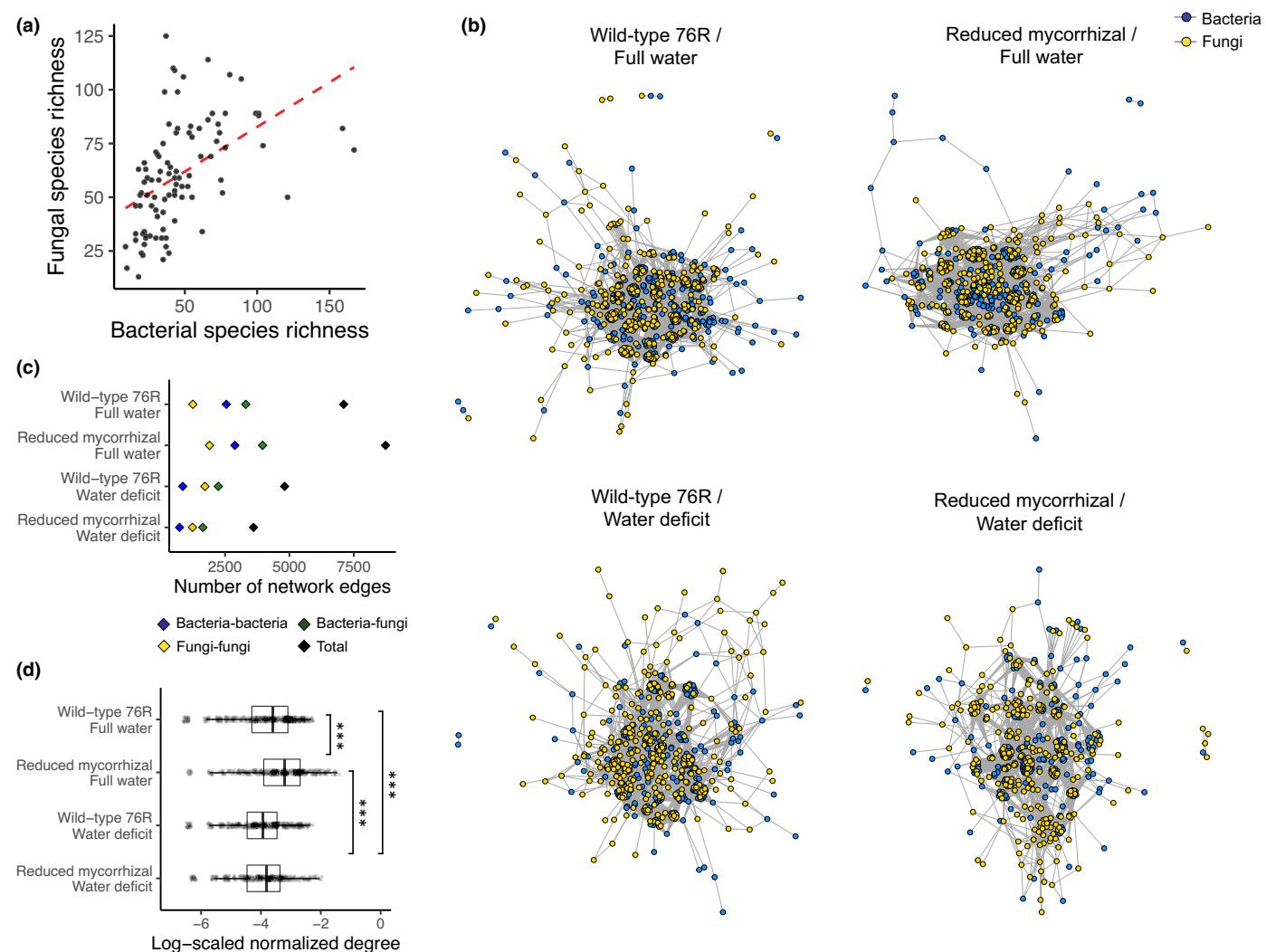


Fig. 4 Correlations between bacterial and fungal composition. (a) Number of amplicon sequence variants (ASVs) per sample. (b) Co-occurrence networks of top 500 bacterial taxa and top 500 fungal taxa, structured according to Fruchterman and Reingold's force-directed layout algorithm. (c) Number of network edges (positive and negative), displayed in total and according to the taxonomic identities of the nodes. (d) Distribution of degree per node (number of positive and negative connections), normalized to total network size and log-transformed. The lower and upper hinges of each box plot represent the 25th and 75th percentiles of the data, respectively, while the upper and lower whiskers extend to values within 1.5 times the interquartile range. Levels of significance are indicated as follows: ***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$; ns, $P \geq 0.05$.

pairs made up a larger proportion of the significant co-associations than either bacteria–bacteria or fungi–fungi pairs. Across mycorrhizal treatments, water stress decreased the average connectedness of nodes. These differences appeared to be driven by decreases in the number of bacteria–bacteria and bacteria–fungi associations in water-stressed plants (Fig. 4c,d).

Growth chamber experiments

We next sought to explore phyllosphere microbiome assembly and function in a more controlled setting. Microbial inocula from the field were sprayed onto plants in growth chambers. In the mycorrhiza experiment, wild-type and *rmc* plants in the growth chamber were sprayed with inocula from wild-type or *rmc* plants in the field, or a sterile buffer, for a fully reciprocal design. In the fertilizer experiment, plants treated with fertilizer matching the high and low extremes of the fertilizer gradient were sprayed with inocula from the corresponding positions in the field, or a sterile buffer. In both experiments, each individual sample (corresponding to one plant in the field) was divided in half and used to generate inocula for two plants, one in each growth chamber treatment.

One week after spraying, plants that had been sprayed with field inocula harbored higher absolute microbial abundances compared to plants sprayed only with sterile buffer ($t=2.181$, $df=19.075$, $P=0.041$). Microbial communities on these plants were primarily composed of ASVs from the field experiment (Fig. S2), further validating that the spray treatment was sufficient to alter microbiome composition in the growth chamber.

Compared to their inocula in the field, plants sprayed with field inocula in the growth chamber harbored less diverse bacterial communities (mycorrhiza experiment: $F=52.369$, $df=17$, $P<0.0001$; fertilizer experiment: $F=193.307$, $df=17$, $P<0.001$) and underwent a strong shift in bacterial community composition ($P<0.001$). In the mycorrhiza experiment, bacterial communities remained more similar to their inocula when sprayed onto a plant of the same genotype than a plant of the opposite genotype (Wilcoxon V-statistic = 10, $P=0.011$). Similarly, bacterial communities in the fertilizer experiment remained more similar when the fertilizer treatment of the field plant matched that of the growth chamber plant (Wilcoxon V-statistic = 8, $P=0.0061$) (Fig. 5). The loss of fungal diversity in the growth chamber was much greater, and many plants were dominated by fungal ASVs that were not present in the inoculum (Fig. S11), suggesting that the microbiome transplant methods we used were not optimized for fungi. As such, we focused on the bacterial communities in all analyses.

The results of the growth chamber experiments were generally similar to those observed in the field. In the mycorrhiza experiment, *rmc* plants harbored lower bacterial alpha and beta diversity. Surprisingly, the difference in alpha diversity was only significant among the plants sprayed with sterile buffer; plants sprayed with supplemental microbial communities from the field exhibited no effect of genotype on alpha diversity. In the fertilizer experiment, as in the field, bacterial alpha and beta diversity were similar across treatments (Fig. S12). We found that *P. syringae*

grew to higher densities on wild-type plants than on *rmc* plants, but only among the plants sprayed with sterile buffer; plants sprayed with microbial communities from the field showed no differences in resistance to invasion (Fig. S13).

Discussion

Changes in climate and land use threaten plant communities by depleting water and disrupting mycorrhizal communities in the soil (St. Clair & Lynch 2010; Pritchard, 2011; Seidl *et al.*, 2017). Many studies have asked how environmental change will affect plant physiology, yet microbiomes can change rapidly and may therefore improve plant resilience to changing environments (Suryanarayanan & Uma Shaanker, 2021). Systemic changes in nutrient and biomass allocation, defense regulation, leaf structure, and gas exchange in water- and nutrient-limited plants (Bowles *et al.*, 2016; Moles *et al.*, 2018; Zhou *et al.*, 2019) all suggest that soil conditions are likely to impact aboveground microbiota, with potential consequences for plant health. Here, we tested the effects of irrigation, mycorrhizas, and soil fertility on phyllosphere bacterial and fungal communities.

In the field and growth chamber, water stress and mycorrhizal disruption reduced bacterial richness and homogenized communities across host plants. Together, reduced species richness and increased homogenization reflect a disproportionate loss of bacterial species with low occupancy across plants (i.e. bacterial species that differentiate individual plants from one another). The phyllosphere environment has harsh temperature fluctuations and low relative humidity compared to the soil or rhizosphere (Knief *et al.*, 2012; Vorholt, 2012); low-occupancy species may be less well-adapted to these conditions, and therefore more strongly affected by stressors, than high-occupancy species. Alternatively, low metacommunity occupancy tends to correlate with small local population sizes (Fig. S14; Gaston, 1994), which are often more vulnerable to ecological stochasticity (Shoemaker *et al.*, 2020) or genetic drift (Lynch *et al.*, 1995).

By contrast, fungal community evenness increased under water stress and mycorrhizal disruption, reflecting a relative loss of dominant taxa. One explanation is that a small number of dominant taxa may comprise the majority of actively growing cells, which are more strongly affected by stressors, while rare sequences originate from dormant spores. In support of this explanation, RNA-based and stable isotope-based profiling show that only a minority of fungal sequences represent active populations (Cardoso *et al.*, 2017; Che *et al.*, 2018) and that this active fraction is more sensitive to disturbance (Che *et al.*, 2019).

In general, water stress affected bacterial communities more strongly than fungal communities, a pattern consistent with a large body of work comparing bacterial and fungal drought responses in soil (Yuste *et al.*, 2011; Barnard *et al.*, 2013; de Vries *et al.*, 2018; Ochoa-Hueso *et al.*, 2018; Preece *et al.*, 2019; Sun *et al.*, 2020). Interactions between bacteria and fungi may be an important part of the picture as well. We found strong positive correlations between bacterial and fungal composition that persisted when accounting for measured environmental variation. The connectedness of the multi-kingdom network decreased

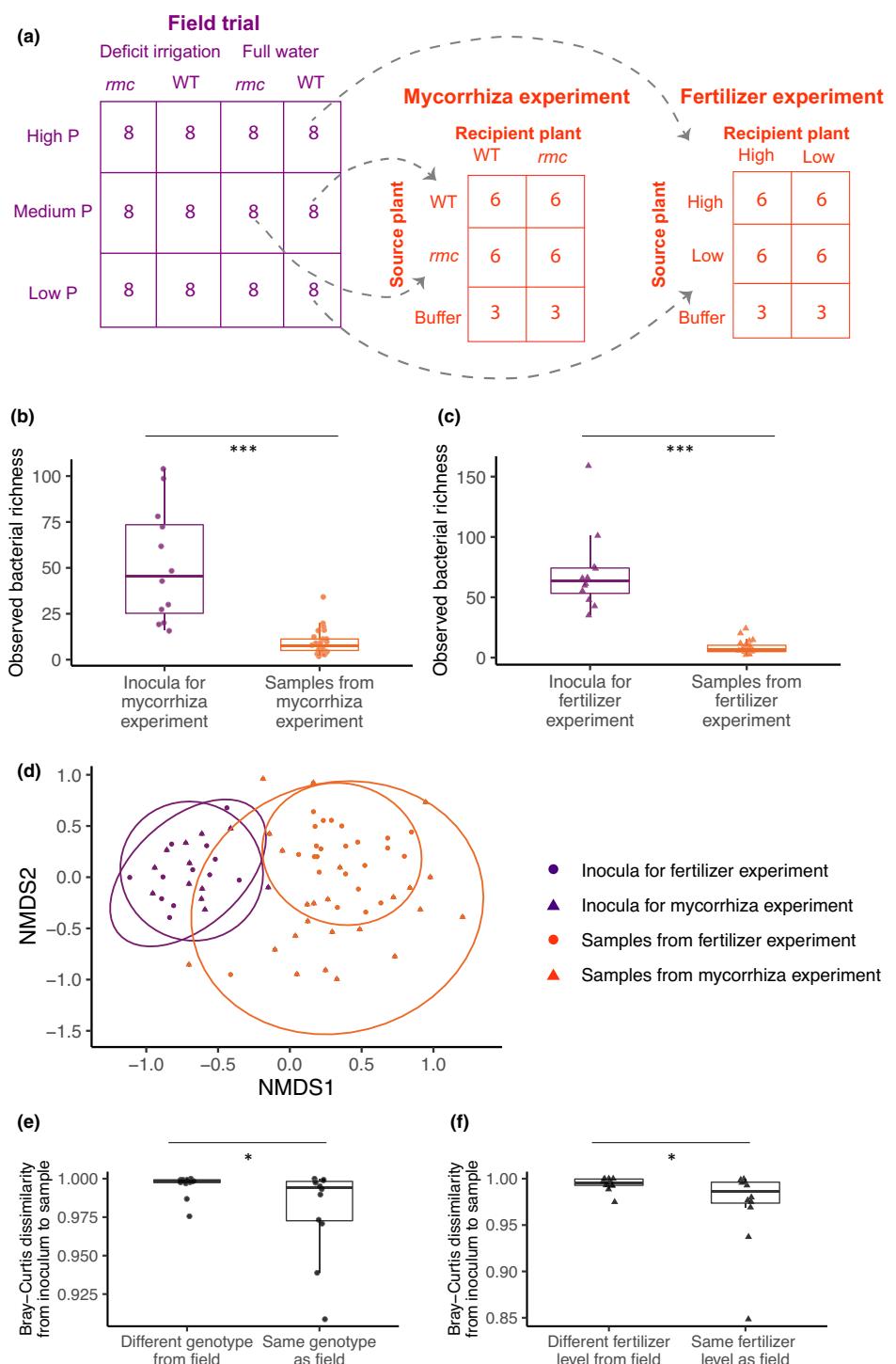


Fig. 5 Diversity and compositional changes from field to growth chamber. (a) Schematic indicating the subset of communities from the field that were transplanted onto plants in the growth chamber, with each cell number representing sample size. Each inoculum was used to inoculate two plants in the growth chamber (one in each treatment column). (b, c) Number of amplicon sequence variants (ASVs) per sample. Richness values were log-transformed before analysis to meet normality and homoscedasticity assumptions. (d) Nonmetric multidimensional scaling (NMDS) ordination shows that plants sampled in growth chamber experiments cluster together, away from their inocula. Ellipses indicate 95% confidence around clustering. (e, f) Bray–Curtis dissimilarity index between each field plant used to generate inocula and its corresponding inoculated plants in the growth chamber. Categories indicate whether the plant in the growth chamber received the same or different experiment treatment as its source plant in the field. Each individual field plant was used to generate inocula for both growth chamber treatments, thus allowing a paired test. The lower and upper hinges of each box plot represent the 25th and 75th percentiles of the data, respectively, while the upper and lower whiskers extend to values within 1.5 times the interquartile range. Levels of significance are indicated as follows: ***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$; ns, $P \geq 0.05$.

under water-limited conditions despite similar network sizes, largely due to decreases in the numbers of bacteria–fungi and bacteria–bacteria associations. Our ability to draw direct inferences about cross-kingdom interactions in this study is limited, as co-associations can be confounded by environmental heterogeneity or differences between measured and biologically relevant spatial scales (Blanchet *et al.*, 2020). However, other work has compiled fascinating examples of interactions between bacteria and fungi (Scherlach *et al.*, 2013), and it will be important to see whether and how environmental stressors disrupt such interactions.

We observed a striking parallelism in the responses of microbial communities to water stress and the *rmc* genotype in our study. Based on these observations, we propose that reduced soil moisture and disruption of mycorrhizas both induce systemic stress responses that alter aboveground plant physiology and microbiome composition. Both water limitation and mycorrhizal disruption can induce stomatal closure, reduce specific leaf area, and alter plant nutrient concentration (Easlon & Richards, 2009; Augé *et al.*, 2015; Bowles *et al.*, 2016), altering physical and chemical characteristics of the phyllosphere habitat. Interestingly, the effect of water stress in this study was strongest in wild-type plants, and the effect of mycorrhizal disruption was strongest in well-watered plants. In fact, the combined effects of water stress and mycorrhizal disruption were not associated with additional community turnover or loss of microbial diversity compared to either individually. This observation contrasts with previously observed interactions between irrigation and mycorrhizas, where mycorrhizal plants are typically more drought-tolerant (Al-Karaki *et al.*, 2004; Augé *et al.*, 2015). It is thus possible that mycorrhizas allow plants to access compensatory pathways that buffer their own fitness under water limitation, but do not extend to phyllosphere microbial communities. For example, mycorrhizal tomato plants closed their stomata more quickly than nonmycorrhizal plants under drought conditions in a prior study (Lazcano *et al.*, 2014). Rapid stomatal closure in mycorrhizal plants appeared to minimize plant biomass loss compared to nonmycorrhizal plants, but may have limited microbial access to stomata, an important source of water in the phyllosphere (Beattie & Lindow, 1999; Remus-Emsermann *et al.*, 2014). It is also possible that an interaction between water stress and mycorrhizas would have emerged if plants were sampled later in the growing season, when water stress was more severe.

Several alternative mechanisms may contribute to the effects of the belowground treatments in this study on phyllosphere microbiome composition. First, microbiome variation between wild-type and reduced mycorrhizal plants could simply reflect host genetic variation, independent of the presence of mycorrhizas. In addition to *CYCLOPS/IPD3*, which confers the reduced mycorrhizal phenotype, the *rmc* deletion spans parts of four other genes (Larkan *et al.*, 2013). At least one of the remaining genes regulates resistance to the root pathogen *Fusarium oxysporum* (Prihatna *et al.*, 2018), but we are not aware of any off-target effects on foliar phenotypes or phyllosphere microorganisms. Studies of 76R and *rmc* plants report similar biomass and nutrient uptake in the absence of mycorrhizal fungi (Cavagnaro *et al.*, 2006; Facelli *et al.*, 2010), and *CYCLOPS/IPD3* is not highly expressed in leaf tissue (Messinese *et al.*, 2007; Yano *et al.*, 2008).

Therefore, while our study design does not exclude the possibility of a direct effect of *rmc* on phyllosphere microorganisms, such an effect is at odds with other work on this system.

Another explanation for the effect of the reduced mycorrhizal genotype is that mycorrhizal colonization modifies host immune signaling both locally and systemically (Liu *et al.*, 2007), tending to increase resistance to necrotrophic pathogens and decrease resistance to biotrophic pathogens (Azcón-Aguilar *et al.*, 2009). This may explain why *P. syringae* colonized wild-type plants more successfully than *rmc* plants in the growth chamber, but it is unclear whether commensal microbiota on the leaf surface interact closely enough with plant cells to be regulated by immune signaling. Bacteria can often grow to high densities on the leaf surface with minimal changes to plant gene expression (Vogel *et al.*, 2016), and the results of studies that directly tested for immune control of phyllosphere microbiome composition have been mixed (Bodenhausen *et al.*, 2014; Chen *et al.*, 2020).

Soil is thought to be one of many sources of dispersal to the phyllosphere (Copeland *et al.*, 2015), so changes in the soil community could affect the phyllosphere community, independent of systemic plant modifications. However, the taxonomic patterns of enrichment and depletion that we observed in the phyllosphere were markedly different from those previously observed in soil and rhizosphere studies. In soil and rhizosphere habitats, drought is commonly associated with enrichment of many *Actinobacterial* taxa, and depletion of *Acidobacteria*, *Proteobacteria*, and *Bacteroidetes* (Barnard *et al.*, 2013; Naylor *et al.*, 2017; Xu *et al.*, 2018). By contrast, we did not observe significant enrichment in any families of *Actinobacteria* (though several were depleted under water-limited conditions), while several *Proteobacteria* and *Bacteroidetes* families were enriched under water stress (see Table S5 for annotated list of enriched and depleted taxa). Additionally, we would not expect to see parallel responses to water stress and the *rmc* genotype if the effect were driven by dispersal. While mycorrhizas can alter surrounding soil communities, their effects tend to be relatively minor and/or highly localized (Marschner & Baumann, 2003; Cavagnaro *et al.*, 2006).

Although irrigation regime and mycorrhizal associations were the strongest predictors of phyllosphere microbiome composition in our study, we observed some effects of fertilization. Fungal diversity and heterogeneity increased along the fertilization gradient, and bacterial communities remained more similar from the field to the growth chamber if they were transplanted onto a plant grown under the same nutrient conditions. Interestingly, these effects manifested aboveground even though fertilization did not appear to alter extractable phosphate availability in the soil. In light of this unexpected observation, we suspect that the added P was mainly in an organic form that was not sufficiently mineralized in the time period of the experiment to be detected by our measure of available P (Olsen P), but may have still altered plant physiology or belowground community composition in ways that subsequently impacted aboveground microbial communities. It is also possible that other components of this product, such as added calcium or resident microbiota, contributed to the observed changes in phyllosphere community composition. However, none of the fungal taxa that were enriched in the fertilized plots were detected

in the fertilizer product, making it unlikely that the effect of fertilizer was caused by the direct addition of microbes.

Our study demonstrates the effects of soil conditions on the diversity, composition, and distribution of aboveground microbiota. We observed strikingly parallel effects of water stress and mycorrhizal disruption despite differences in their nature (biotic or abiotic), yet these changes were distinct from enrichment patterns previously observed in the soil and rhizosphere. Taken together, our findings are unlikely to be explained by off-target effects of the *rmc* mutation, dispersal from soil, or stochastic colonization of plants in the field. Rather, they indicate that soil conditions induce systemic responses in plants which in turn alter selection on aboveground microbial communities. Our study highlights the importance of phyllosphere microbial communities both as indicators of plant stress and as potential mediators of plant responses to global environmental change.

Acknowledgements

The authors would like to thank the University of California, Davis Student Farm for access to the fields, members of the Koskella and Bowles laboratories for helpful discussion and comments, and Microbiome Insights, Inc. for their role in sequencing efforts. Support for this work was provided by the Army Research Office (W911NF-17-1-0231 to AG), the National Science Foundation (NSF Graduate Research Fellowships to RD, CAH, AG), the University of California, Berkeley (Berkeley Fellowships to YS, CAH), the Society for the Study of Evolution (grant no. 047408 to RD), the Hellman Fellows Fund (Hellman Fellows Award to BK) and the Marian E. Koshland Integrated Natural Sciences Center (KINSC Summer Scholarship to GK).

Author contributions

YS and TB designed the field trial. RD, YS, GK, RC and AD collected data and samples in the field. RD, GK, AG and BK designed the microbiome sampling design and growth chamber experiments. RD, GK and AG collected growth chamber data. RD, YS, GK and CAH analyzed the data. RD wrote the initial draft of the manuscript and all authors contributed substantially to revisions. TB and BK contributed equally to this work.

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Data availability

The data that support the findings of this study have been submitted to the DNA Data Bank of Japan (DDBJ) Sequence Read

Archive (accession no. PRJNA741547). Annotated scripts used to analyze the data are available at <https://github.com/reenadebray/davis-field-trial>.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Experimental design and layout of treatment plots in the field study.

Fig. S2 Colonization rates of plants in the growth chamber by field inocula.

Fig. S3 Analysis of the distribution of taxa that were detected in the growth chamber but not the field study.

Fig. S4 Effects of field trial treatments on root colonization by mycorrhizae, stem water potential, and phosphorus concentration in plant tissue.

Fig. S5 Effects of water stress are strongest on rare bacterial taxa and persist at broad taxonomic levels.

Fig. S6 Effects of the *rmc* genotype are strongest on rare bacterial taxa and persist at broad taxonomic levels.

Fig. S7 Negative binomial modeling identifies parallel shifts in bacterial community composition in water stress and *rmc* genotype.

Fig. S8 Overlap in fungal indicator species for water regime and mycorrhizal genotype.

Fig. S9 Treatment effects within batches mirror those observed within the full dataset.

Fig. S10 Phosphorus fertilization increases fungal alpha and beta diversity.

Fig. S11 Colonization of growth chamber plants by field fungal communities.

Fig. S12 Growth chamber experiments recapitulate effect of *rmc* genotype, and lack of effect of phosphorus fertilization, on bacterial alpha and beta diversity.

Fig. S13 *Pseudomonas syringae* density on growth chamber plants after experimental infection.

Fig. S14 Occupancy–abundance relationship for bacterial taxa in the field study.

Methods S1 Description of field trial preparation and sampling procedures.

Methods S2 Detailed methods for growth chamber experiments.

Methods S3 Droplet digital PCR reaction conditions and primers.

Methods S4 Amplicon sequencing workflow and analysis.

Table S1 Added quantities of organic fertilizer along the fertilization gradient in the field study.

Table S2 Full ANOVA model results for bacterial and fungal richness.

Table S3 Full ANOVA model results for bacterial and fungal Shannon diversity.

Table S4 Fungal taxa enriched in medium and high positions along the fertilizer gradient, and fungal taxa present in fertilizer product.

Table S5 Annotated list of bacterial families identified in enrichment analyses for irrigation regime and mycorrhizal genotype, including previous implication in drought response and/or disease.

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