Title: Mycorrhizal fungi stabilize bacterial growth and diversity in water-limited soils

Running Title: Mycorrhizae stabilize bacteria in water-limited soils

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Abstract

Drought disrupts soil microbial activity and many biogeochemical processes. Although mycorrhizal fungi are known to impact plant functions and nutrient cycling during drought, their effects on drought-exposed soil microbial communities are not well resolved. We used $H_2{}^{18}O$ quantitative stable isotope probing (qSIP) to investigate microbial response to water limitation in the hyphospheres of two distinct mycorrhizal lineages (*Rhizophagus irregularis* and *Serendipita bescii*) grown with the bioenergy model grass *Panicum hallii*. In the absence of mycorrhizal inocula, water limitation resulted in significantly lower bacterial growth rates and lower diversity in the actively growing bacterial community. Water limitation also decoupled growth from CO_2 efflux, resulting in a threefold lower growth efficiency. In contrast, both mycorrhizal lineages appeared to protect bacterial communities exposed to water limitation: bacterial growth rates, growth efficiency, and the diversity of the active bacterial community were similar in water-replete and water-limited soils inoculated with either fungus. Several of the bacterial taxa that responded positively to mycorrhizal inocula in water-limited soil belong to lineages that are considered drought-susceptible. Together, these results indicate that mycorrhizal-bacterial interactions can stabilize bacterial communities in water-limited environments. As drought frequency and severity increase, these synergistic relationships may support ecosystem resilience to moisture stress.

Keywords: *Panicum hallii*, mycorrhizal fungi, bacteria, drought, quantitative stable isotope probing, plant-microbe interactions, ^{18}O-water

Introduction

Drought is a destabilizing stressor that alters plant productivity[1], microbial community composition[2-4], microbial biomass[5,6], greenhouse gas emissions[7,8], and many critical biogeochemical processes[9]. Plant-microbial mutualisms mitigate plant drought response and aid in post-drought recovery through a variety of mechanisms[10,11]. In particular, mycorrhizal fungi—which associate with the majority of terrestrial root systems[12]—can support plants during drought by facilitating water transport[13], soil aggregation[14,15], root growth[16], plant nutrient uptake[17], photosynthesis[18,19], and stomatal conductance[18-20]. While mycorrhizal fungi may also influence soil microbial communities in water-limited environments, multipartite feedbacks during and after drought remain poorly quantified[11].

There is increasing evidence that the mycorrhizal hyphosphere—the region of soil that surrounds mycorrhizal fungi—is a hotspot for multipartite interactions. These interactions may be synergistic or antagonistic, with significant effects on resource exchange[21,22], stress tolerance[23], and organismal abundance and activity[24,25]. Recent work indicates that specific bacterial lineages are more abundant in the presence of arbuscular mycorrhizal (AM) fungi[26-28] and that fungal traits shape the composition of mycorrhizal-associated bacterial communities[28-30]. Together, these observations suggest that mycorrhizal-bacterial interactions have important ecological functions. Still, relatively little is known about how mycorrhizal fungi influence bacterial communities exposed to environmental stressors such as moisture limitation. A better understanding of these relationships would help us predict microbial response to global change,

harness beneficial plant-microbial relations for sustainable agriculture, and assess other applications of mycorrhizal-microbial mutualisms.

In situ investigation of mycorrhizal-bacterial interactions presents immense methodological challenges. The mycorrhizal hyphosphere is small, difficult to sample, and may not exert a detectable effect on microbial community composition or activity when assessed at a 'bulk' scale[28]. Furthermore, a substantial proportion of soil DNA may represent inactive organisms or extracellular 'relic' DNA[31-32]. Stable isotope probing (SIP) coupled with 16S rRNA gene profiling can distinguish active organisms from inactive ones by tracing isotope incorporation into newly-synthesized microbial DNA[33,34]. With quantitative SIP (qSIP), we can estimate growth rates of amplicon sequence variants (ASVs) based on shifts in DNA buoyant density caused by heavy isotope incorporation[35,36]. This enables sensitive detection of actively growing taxa, even in samples where a substantial quantity of DNA belongs to dead or dormant organisms. In challenging experimental systems like the mycorrhizal hyphosphere, qSIP has the potential to provide novel insight into taxon-specific interactions.

In this study, we sought to investigate how two mycorrhizal lineages—the AM fungus *Rhizophagus irregularis* and the Sebacinales fungus *Serendipita bescii*—mediate bacterial response to water limitation in a marginal soil planted with the switchgrass bioenergy model *Panicum hallii* (Hall's panicgrass). For the purpose of this study we define 'mycorrhiza' as a symbiotic relationship between a fungus and a plant root, where the fungus acquires C from the living plant in exchange for mineral nutrients or other resources[12]. Both AM and Sebacinales fungi associate with a wide range of plant species, including switchgrass[37-39], and support plant

function during drought[40-42]. However, *R. irregularis* has a reduced genome and is dependent upon host-provided C[43,44] and microbial transformation of nutrient sources into bioavailable forms[45,46]. In contrast, *S. bescii* and other Serendipita lineages are facultative symbionts with a broad enzymatic repertoire that enables direct resource acquisition from both living plants and detritus[47-49]. We hypothesized that both fungi would mitigate moisture stress, but that distinct bacterial communities would respond to each fungus. Our results demonstrate that mycorrhizal fungi have a stabilizing effect on bacterial communities in water-limited soil.

Materials and Methods

Soil collection and characterization

Soil used for this study was a Pond Creek fine sandy loam, classified as a superactive, thermic Pachic Argiustoll[50]. Soil was collected from a pasture in Caddo County, OK (35.072417/-98.303667) where switchgrass is endemic, on traditional land of the Anadarko (Nadaco) tribe and the Caddo Nation of Oklahoma. The soil is considered 'marginal' due to its high sand content (69%), low C, N, and P content (< 0.4%, < 0.04%, and < 6 ppm, respectively); its pH is ~5 (ref. 51). Surface soil (0-20 cm) was collected in May 2019, transported to Livermore, CA, sieved to 2 mm, and stored at 4 °C. A soil moisture retention curve was generated from air-dried soil using a pressure plate apparatus (WP4C, METER Environment, Pullman, WA) and the nonlinear fitting program SWRC-Fit to apply a Brooks and Corey model to the data[52].

Mycorrhizal inoculum

Spores of *R. irregularis* (formerly *Glomus intraradices*) isolate DAOM-197198 were purchased from Premier Tech (Rivière-du-Loup, Quebec, Canada). *S. bescii* (sourced from the Noble Research Institute) was grown in modified Melin-Norkran's (MMN) broth. Bentonite clay particles were mixed with MMN broth, inoculated with *S. bescii*, and incubated at 24 °C for 8 weeks according methods described by Ray et al. (ref. 53). Uninoculated bentonite clay particles were also mixed with MMN broth and incubated under the same conditions.

Greenhouse water limitation experiment

P. hallii seeds collected from the Edwards Plateau in central Texas were grown to seed, scarified, surface sterilized, stratified at 4 °C for one week, and germinated in petri plates. After 5 days, germinated seedlings were transferred into planting cones (Ray Leach Cone-tainers, Steuwe & Sons, Tangent, OR, USA) filled with double-autoclaved sand. Five days after transfer into cones, ~500 *R. irregularis* spores or 500 µL *S. bescii* inoculum were injected into the sand to inoculate the *P. hallii* seedlings. A subset of seedlings was left uninoculated. Eight weeks after inoculation, seedlings were transplanted into 960 cm^3 containers (Anderson Plant Bands, Steuwe & Sons) filled with a 50:50 (v:v) mixture of 'live' soil and double-autoclaved sand (CEMEX Lapis Lustre Specialty Sand, #2/12) packed to 1.7 g mL^{-1} bulk density at 15% moisture. Additional inoculum (consisting of 500 *R. irregularis* spores or 5 g bentonite clay particles coated with *S. bescii*) were placed near the roots of seedlings as they were transplanted. Five g of uncoated bentonite clay particles were placed near the roots of previously uninoculated plants and plants that had been inoculated with *R. irregularis*. Each microcosm contained a 25 µm mesh hyphal ingrowth core (2.3 cm diameter) filled with 75 g of the same 'live' soil (no sand) mixed with 0.5 g finely milled switchgrass biomass as mycorrhizal bait. The multi-phase mycorrhizal inoculation procedure

was intended to give *R. irregularis* and *S. bescii* a colonization advantage over other native mycorrhizal lineages or endophytes present in the soil.

After assembly, each microcosm was covered with 150 g double-autoclaved sand to prevent cross-contamination of mycorrhizal inocula. All microcosms were watered with a total of 20 mL ultrapure H_2O during the first week to facilitate plant and mycorrhizal establishment. Half of the microcosms were watered on a weekly or bi-weekly schedule to maintain soil moisture at approximately 15% (based on gravimetric measurements), a level that would not restrict plant growth. The other half of the microcosms were not watered for the remainder of the experiment and declined to 5% gravimetric soil moisture by 3 months. The moisture content of the soil surrounding the roots (i.e., not directly within the hyphal ingrowth cores) in 1 microcosm per treatment was monitored continuously with a volumetric water probe (ECH_2O EC-5, METER Group, Inc., Pullman, WA, USA). Another microcosm per treatment was weighed weekly to assess whole-microcosm gravimetric changes. Three replicate microcosms were maintained for each of the 6 treatment combinations (3 mycorrhizal inocula x 2 moisture regimes). Average daytime and nighttime temperatures were 27 °C and 24 °C, respectively, with a photoperiod of 16 h. After 3 months, the microcosms were destructively harvested. Soil from hyphal ingrowth cores was homogenized, flash frozen in liquid N_2 and stored at -80 °C for DNA extractions or at room temperature for qSIP assays (see Fig. 1a for experimental design).

Soil characteristics

Soil moisture content following the 3-month harvest was determined by mass difference after drying soil for 48 h at 105 °C. Total soil C and N contents were measured with a Costech ECS 4010 Elemental Analyzer (Costech Analytical Technologies Inc., Valencia, CA).

Soil $H_2{}^{18}O$ qSIP assay

Three days after the 3-month harvest, soil from 3 hyphal ingrowth cores per treatment was amended with either $H_2{}^{16}O$ or $H_2{}^{18}O$ (Fig. 1a). Each soil was divided into three subsamples: one 3.0 g dry weight equivalent sample for an initial $H_2{}^{16}O$ qSIP timepoint ("T0") and two 4.0 g dry weight equivalent samples for $H_2{}^{16}O$ and $H_2{}^{18}O$ 7-day qSIP timepoint ("T7"). This resulted in a total of 54 samples (18 T0 $H_2{}^{16}O$ samples, 18 T7 $H_2{}^{16}O$ samples, and 18 T7 $H_2{}^{18}O$ samples). Each subsample was air-dried to 0.047 gravimetric water content in a biosafety cabinet and then brought up to 22.13% soil moisture (60% field capacity) with either ultrapure water at natural isotopic abundance or water enriched with ^{18}O (98.38 atom % $H_2{}^{18}O$, Isoflex, San Francisco, CA, USA). The final enrichment of the samples containing $H_2{}^{18}O$ was 78.76 ^{18}O atom %. Standardization of soil moisture across treatments was necessary for qSIP calculations. The T0 subsample (amended with $H_2{}^{16}O$) was immediately flash frozen in liquid N_2 and stored at -80 °C. Each of the T7 soil samples was stored in the dark inside a separate 16 oz glass jar with a tight-fitting lid containing a septum for gas sampling. After 7 days, the soils were flash frozen in liquid N_2 and stored at -80 °C.

CO_2 efflux measurement

15 mL headspace gas samples were collected at the beginning and end of the qSIP assay. CO_2 concentrations were measured on a gas chromatograph equipped with a thermal conductivity detector (GC-14A, Shimadzu, Columbia, MD).

DNA extraction and qPCR

DNA was extracted from each soil sample in quadruplicate with the Qiagen PowerSoil Pro kit and then pooled per sample prior to downstream analysis. To assess the relative abundance of mycorrhizal inocula, we quantified *R. irregularis* and *S. bescii* gene copy number with qPCR primers designed specifically for each fungal lineage[53,54] (see Table S1 and SI text for additional information). All sample and standard curve qPCR reactions were performed in triplicate. We note that DNA can be unevenly distributed throughout fungal biomass and while not necessarily indicative of total biomass or activity is a relative indicator of inoculation success[55,56].

Density gradient fractionation of DNA

To separate isotopically enriched DNA from unenriched DNA, samples were subjected to a cesium chloride density gradient formed in an ultracentrifuge as previously described[57,58] with the following minor modifications. For each sample, 5 µg of DNA in 150 µL 1xTE was mixed with 1.00 mL gradient buffer, and 4.60 mL CsCl stock (1.885 g mL^{-1}) with a final average density of 1.730 g mL^{-1}. Samples were loaded into 5.2 mL ultracentrifuge tubes and spun at 20 ºC for 108 hours at 176,284 RCF_{avg} in a Beckman Coulter Optima XE-90 ultracentrifuge using a VTi65.2 rotor. Sample fractionation was automated using Lawrence Livermore National Laboratory's high-throughput SIP pipeline, which automates the fractionation and clean-up tasks for the density gradient SIP protocol. The content of each ultracentrifuge tube was fractionated

into 22 fractions (~236 µL each) using an Agilent Technologies 1260 isocratic pump to deliver water at 0.25 mL min^{-1} through a 25G needle inserted through the top of the ultracentrifuge tube. Each tube was mounted in a Beckman Coulter fraction recovery system with a side port needle inserted through the bottom. The side port needle was routed to an Agilent 1260 Infinity fraction collector, and fractions were collected in 96-well deep well plates. The density of each fraction was measured using a Reichart AR200 digital refractometer fitted with a prism covering to facilitate measurement from 5 µL, as previously described[59]. DNA in each fraction was purified and concentrated using a Hamilton Microlab Star liquid handling system programmed to automate glycogen/PEG precipitations[34]. Washed DNA pellets were suspended in 40 µL of 1xTE and the DNA concentration of each fraction was quantified using a PicoGreen fluorescence assay.

DNA sequencing

For each sample, we sequenced the 16S rRNA gene in unfractionated DNA as well as DNA fractions containing > 1.0 ng μL^{-1}, resulting in 6-11 sequenced fractions per sample. The V4 region of the 16S rRNA gene was amplified with the 515F/806R primer pair[60], processed and barcoded through the Illumina V2 PE150 sample preparation kit, and sequenced on an MiSeq v2 platform in 3 runs (Illumina, Inc., San Diego, CA; see SI text and Tables S2 & S3 for additional information about taxonomic assignment and ASV recovery).

Quantitative stable isotope probing (qSIP) analysis

We calculated taxon-specific growth using the ^{18}O qSIP approach[33,35]. Briefly, the qSIP mathematical model estimates taxon-specific ^{18}O incorporation into DNA based on the shift in

DNA density following exposure to natural abundance H_2O ($H_2{}^{16}O$) or 'heavy' $H_2{}^{18}O$. We determined baseline densities for each taxon in the absence of an isotopic tracer (i.e., $H_2{}^{16}O$), because even without tracer assimilation, DNA density varies by GC content[61]. For each ASV, we calculated median ^{18}O atom percent excess (APE) and gross growth rate. To increase confidence in these calculations, we first filtered out ASVs that were not recovered from all T0 and T7 replicates in a particular treatment.

Since ^{18}O incorporation could have occurred at any point during the qSIP assay, ^{18}O APE represents an integrated measure of activity. We note that a taxon's ^{18}O APE value may be high due to an increase in abundance (i.e., cells incorporate ^{18}O into DNA during replication; new cell production increases total population size and average DNA ^{18}O content) or population turnover (i.e., DNA from older unenriched cells degrades, while new cells grow and incorporate ^{18}O into DNA). During turnover, DNA ^{18}O APE can increase while population-level abundance either remains similar (if mortality matches growth rate) or declines (if mortality outpaces growth). Therefore, a high ^{18}O APE value is not necessarily indicative of a net increase in abundance.

Statistical analyses

We performed all statistical analyses in R[62]. We assessed differences in mycorrhizal gene copy number with a Wilcoxon rank sum test with a Benjamini-Hochberg correction for multiple comparisons. We assessed differences in soil moisture, soil C and N content, Inverse Simpson's diversity index, median ASV ^{18}O APE, and CO_2 efflux with a Tukey's HSD test comparing the means of all treatments. First, we created a linear model with a fixed effect for treatment. If the raw data did not meet the assumptions of normality, it was log-transformed prior to further

analysis. We calculated growth efficiency by dividing gross growth by CO_2 efflux per treatment[63]. We used 'vegan' to perform a principal coordinates analysis and permutational multivariate analysis of variance (PERMANOVA) on the weighted UniFrac distance between communities present under different treatments and qSIP timepoints[64,65]. We used 'metagenomeSeq'[66] with a wrench normalization for sparse data to identify significant differences in ASV relative abundance at T0 ($p < 0.05$ and $\log_2$ fold change > 2 or < -2). To compare taxon-specific growth between two treatments, we calculated ^{18}O-based 'growth ratios' by dividing an ASV's ^{18}O APE in one treatment by its ^{18}O APE in the second treatment. We used a Wilcoxon signed rank test with a Benjamini-Hochberg correction for multiple comparisons to assess whether mean phylum ratios were significantly greater than or less than 1.

Results

Mycorrhizal abundance and soil characteristics following 3-month water manipulation

After 3 months of *P. hallii* growth, *R. irregularis* and *S. bescii* abundances (measured with strain-specific qPCR primers) were significantly higher in the hyphal ingrowth cores of microcosms inoculated with each fungus, respectively (Fig. 1b; $p < 0.1$). Soil moisture was lower in the hyphal ingrowth cores of water-limited versus water-replete microcosms (4.9% ± 0.85 and 15.2% ± 0.57, respectively, Fig. 1c; $p < 0.001$), and was not significantly associated with mycorrhizal abundance ($p > 0.05$). Matric suction was also lower in water-limited hyphal ingrowth core soils (Fig. S1). Total soil C and N did not vary significantly between treatments (Fig. S2; $p > 0.05$).

Moisture and mycorrhizal effects on microbial community structure

We assessed the effects of moisture regime and mycorrhizal inoculum on microbial community structure with both traditional and qSIP-enabled 16S rRNA gene profiling (Tables S4-S6 & Fig. 2a,b). The differences we detected in mycorrhizal-inoculated samples represent the influence of *R. irregularis* or *S. bescii* in soils containing a background microbial community. A PERMANOVA of weighted UniFrac distances between the 'total' communities (i.e., 16S rRNA gene profiles sequenced from unfractionated DNA) suggests that moisture regime and mycorrhizal inoculum explained 29.5% of variation in community structure at the end of the qSIP assay (Table S4 & Fig. 2a; $p < 0.05$). Although there was a small shift in community structure during the qSIP assay (6.3% of variance explained; Table S5; $p < 0.001$), moisture and mycorrhizal effects persisted throughout the assay (Table S5; $p < 0.05$).

When we used qSIP to filter out inactive ASVs (i.e., those that did not incorporate significant quantities of ^{18}O) prior to conducting a PERMANOVA, we found that moisture regime, mycorrhizal inoculum, and their interactive effect explained over 75% of the variation in the structure of the actively growing community (20.4%, 27.4%, and 27.5%, respectively; Table S6 & Fig. 2b; $p < 0.001$). This is more than twice the effect size observed in the 'total' community identified through traditional 16S rRNA analysis. qSIP-based filtering also highlighted differences in alpha diversity. While there was no relationship between moisture regime or mycorrhizal inoculum and the alpha diversity of the 'total' communities sequenced (Fig. 2c; $p > 0.05$), water limitation was associated with lower alpha diversity of the actively growing community in uninoculated soils ($p < 0.01$), no difference in *R. irregularis*-inoculated soils ($p > 0.05$), and higher alpha diversity in soils inoculated with *S. bescii* ($p < 0.01$).

We conducted differential abundance analysis to evaluate how water limitation influenced ASV relative abundance in the presence or absence of mycorrhizal inocula. Within each mycorrhizal treatment, some ASVs were more abundant in water-limited soils while others were less abundant (Fig. S3). In uninoculated soils, 56 ASVs were less abundant following water limitation while only 24 were more abundant, contributing to a twofold difference in the magnitude of cumulative $\log_2$ fold change (Fig. S3a,d). In contrast, within *R. irregularis*- and *S. bescii*-inoculated soils, the number of ASVs that were more abundant following water limitation was similar to the number that were less abundant, contributing to a similar magnitude of cumulative $\log_2$ fold change (Fig. S3b,c,d).

Moisture and mycorrhizal effects on microbial growth and efficiency

We used $H_2{}^{18}O$ qSIP and CO_2 efflux measurements to quantify the effects of moisture regime and mycorrhizal inoculum on ASV ^{18}O incorporation, gross growth rates, and growth efficiency. Overall, DNA from soil amended with $H_2{}^{18}O$ was denser than DNA from soil amended with $H_2{}^{16}O$ (Fig. S4), indicating that many organisms incorporated ^{18}O into newly-synthesized DNA. Taxon-specific ^{18}O APE ranged from unenriched to 64.0 APE, with a median of 6.0. Between 35-59% of the total ASVs detected per treatment were significantly enriched (Table S3; lower 90% CI > 0); we refer to these as the 'actively growing' taxa.

In the absence of mycorrhizal inocula, microbial ASVs incorporated less ^{18}O in water-limited soils: median ASV ^{18}O APE was 2.14 compared to 9.06 in water-replete soils (Fig. 3a; $p < 0.05$). Gross growth was also lower in water-limited compared to water-replete soil (Fig. 3b). CO_2

efflux from uninoculated soils did not vary significantly between moisture regimes (Fig. 3c; $p > 0.05$). However, growth efficiency (gross growth divided by CO_2 efflux) was more than threefold lower in water-limited soil: only 8.4 (+/- 0.24) µg DNA mg^{-1} CO_2 compared to 28.3 (+/- 2.16) in water-replete soils (Table S7). In contrast, microbial communities in mycorrhizal-inoculated soils were less affected by water limitation. Microbial ^{18}O APE was 6.56 compared to 7.45 in water-limited versus water-replete *R. irregularis*-inoculated soils (Fig. 3a; $p > 0.05$), and 5.67 compared to 4.85 in water-limited versus water-replete *S. bescii*-inoculated soils (Fig. 3a; $p > 0.05$). Gross growth was also similar under both moisture regimes in mycorrhizal-inoculated soils (Fig. 3b). CO_2 efflux from *S. bescii*-inoculated soils was higher than from *R. irregularis*-inoculated soils, but the difference was only significant between water-replete soils (Fig. 3c; $p < 0.05$). Compared to uninoculated soils, moisture effects on growth efficiency in mycorrhizal-inoculated soils were relatively small. Growth efficiency was 14.4 (+/- 0.8) µg DNA mg^{-1} CO_2 compared to 20.2 (+/- 1.25) in water-limited versus water-replete soil inoculated with *R. irregularis*, and 12.5 (+/- 0.51) compared with 12.7 (+/- 0.75) in water-limited versus water-replete soils inoculated with *S. bescii* (Table S7).

Taxon-specific response to moisture regime and mycorrhizal inocula

Since compensatory dynamics may result in the increased activity of some organisms while others decline[67], we calculated ^{18}O-based 'growth ratios' for each actively growing ASV detected in both water-limited and water-replete soil. A ratio of greater than 1.0 suggests that the ASV sustained a higher growth rate in water-limited soil, a ratio of less than 1.0 suggests that the ASV sustained a lower growth rate in water-limited soil, and a ratio indistinguishable from 1.0 suggests that the ASV maintained a similar growth rate under both conditions. In the absence of

mycorrhizal inocula, most ASVs sustained lower growth rates in water-limited soil (Fig. 4a). When averaged at the phylum level, growth ratios were significantly less than 1 for 8 of the 10 most abundant phyla: Acidobacteria, Actinobacteria, Bacteroidetes, candidate division WPS-1, Gemmatimonadetes, Planctomycetes, Proteobacteria, and Verrucomicrobia (Fig. 4a; adjusted $p < 0.05$). In contrast, bacterial response to water limitation in mycorrhizal-inoculated soils was equivocal: many ASVs sustained lower growth rates, while many others sustained similar or greater growth rates. When averaged at the phylum level, this resulted in almost no significant effects of water limitation on microbial growth in mycorrhizal-inoculated soil (Fig 4b,c; adjusted $p > 0.05$ for most phyla). An exception to this pattern were the Acidobacteria, which sustained significantly higher growth rates in water-limited versus water-replete soils inoculated with *R. irregularis* (Fig. 4b; adjusted $p < 0.05$).

We also calculated ^{18}O-based growth ratios for ASVs detected in both uninoculated and mycorrhizal-inoculated water-limited soils. This allowed us to compare how mycorrhizal inocula influenced the growth of individual ASVs in water-limited soil. Most ASVs sustained higher growth rates in the presence of *R. irregularis* or *S. bescii* than in their absence (Fig. 5). Averaged at the phylum level, populations belonging to the phyla Acidobacteria, Actinobacteria, Bacteroidetes, candidate division WPS-1, Planctomycetes, Proteobacteria, and Verrucomicrobia were significantly more active in *R. irregularis*-inoculated soil (Fig. 5a,c; adjusted $p < 0.05$). In *S. bescii*-inoculated soils, populations from the phyla Actinobacteria and Proteobacteria were significantly more active than in uninoculated soil (Fig. 5b,d; adjusted $p < 0.05$). Some ASVs responded positively to both *R. irregularis* and *S. bescii*; others responded positively only to one

fungal lineage (Fig. 5c,d). Overall, the magnitude of positive growth response was greater in *R. irregularis*- than in *S. bescii*-inoculated soils.

Positive bacterial response to mycorrhizal inocula was also apparent in ASVs that did not grow under multiple conditions and therefore could not be compared via the ratios described above. For example, there were no Firmicutes ASVs with detectable growth rates in uninoculated water-limited soils (Fig. S5). However, several Firmicutes were active in mycorrhizal-inoculated soils regardless of moisture regime. More than double the number of Firmicutes ASVs were active in *R. irregularis*- compared to *S. bescii*-inoculated soils.

Discussion

Mycorrhizal fungi are well-known to improve plant drought tolerance[16-20,40-42], but their effects on bacterial communities in droughted soils are poorly quantified. With $H_2{}^{18}O$ qSIP, we show that mycorrhizal fungi protect bacterial communities from the suppressive and destabilizing effects of water limitation.

Water limitation suppresses bacterial growth and efficiency

Our findings suggest that water limitation suppresses taxon-specific bacterial growth rates and decouples growth from CO_2 efflux. Previous studies have shown that microbial carbon use efficiency is lower in water-limited soils[68,69]. This is consistent with a microbial stress response, wherein cells prioritize essential metabolic activities over replication[9,57]. Comparative transcriptomics analysis of drought-selected microbial communities has shown that there are trade-offs between stress tolerance and growth[70]. Slowed growth helps bacteria persist in the

presence of antibiotics[71,72], which can accumulate in dry soils as microorganisms compete for limited resources[73]. There is also evidence that lower microbial growth efficiency in dry soils may be due to reduced substrate diffusion[63,74]. Our observations indicate that water limitation reduces microbial resource use efficiency and capacity to produce new biomass. Because microbial biomass is an important precursor to soil organic matter[75], this may impact C storage in droughted soils.

Growth suppression was apparent across a broad range of bacterial lineages present following water limitation, including taxa that are considered to be drought tolerant. Several previous studies have reported increases in monoderm (e.g., Actinobacteria, most Firmicutes, and some Chloroflexi) compared to diderm (e.g., most Acidobacteria, Bacteroidetes, Proteobacteria, and Verrucomicrobia) abundance and activity in dry soils[2,9,76-78]. Monoderm physiology (i.e., thick cell walls and lack of an outer membrane) protects against oxidative damage under dry conditions[9,76,77,79]. Monoderms can also outcompete other organisms through their capacity to produce antibiotics[73,77,80] or utilize complex C substrates that remain available following water limitation[4,73]. However, we did not find evidence that monoderm growth rates were higher than those of diderms in water-limited soil. Taxa belonging to the monoderm phylum Actinobacteria may remain metabolically active but replicate more slowly than other organisms[81]. This lifestyle could explain the decoupling of growth and CO_2 efflux that we observed in water-limited soils.

Mycorrhizal fungi stabilize bacterial response to soil moisture

The AM fungus *R. irregularis* and the Sebacinales fungus *S. bescii* can substantially enhance plant performance in water-limited soils[40-42], thereby stabilizing plant communities during stress.

We found that both fungi also appeared to protect bacterial communities exposed to water limitation. Overall, bacterial growth and efficiency were similar in water-replete and water-limited soils inoculated with either fungus, and always higher in mycorrhizal-inoculated soils than in uninoculated soils. This 'protective' mycorrhizal effect extended broadly to the bacterial community, including many taxa that are considered drought-susceptible. While previous studies report a decrease in the relative abundances of Bacteroidetes, Planctomycetes, Verrucomicrobia, and many Proteobacteria and Acidobacteria in droughted soils[2-4,82], we found that in the presence of mycorrhizal inocula, many ASVs belonging to these phyla sustained similar growth rates in water-replete and water-limited soils. Mycorrhizal fungi could help bacteria tolerate water limitation through enhanced resource provisioning[21,83-85], soil structure[14,15], or biofilm formation[86]. By supporting microbial diversity and activity in water-limited soil, mycorrhizal-bacterial synergies may counteract the destabilizing effect of environmental stress[87] and improve capacity for recovery.

In contrast to their synergistic effect in water-limited soils, *R. irregularis* and *S. bescii* appeared to suppress bacterial growth and diversity in water-replete soils. Previous reports show that mycorrhizal fungi can compete with other biota for N[88] and P[24,25], that putative bacterial predators are more abundant on extraradical mycorrhizal hyphae than in surrounding soil[28], and that soil filtrates containing bacteria inhibit mycorrhizal proliferation[89]. Our results indicate that there is a trade-off between mycorrhizal and bacterial activity. While this trade-off may reduce maximum bacterial productivity under non-limiting conditions, it supports resilience under limiting conditions. Similar context-dependency is well-documented in other mutualisms[90]. We

interpret the context-dependency of mycorrhizal-bacterial relationships as further indication of their stabilizing ecological effect.

Magnitude of microbial response is mycorrhizal lineage-dependent

While both mycorrhizal lineages stabilized bacterial response to soil moisture, *R. irregularis* elicited a stronger positive response than *S. bescii*—both with respect to the number of ASVs and the magnitude of their response. Previous studies show that distinct microbial consortia associate with different mycorrhizal lineages[28,29]. Although empirical evidence remains sparse, different mycorrhizal exudate profiles, growth habits, and other functional traits may shape surrounding microbial community composition and activity[45,91], a phenomenon documented more extensively for root-microbial interactions[92-94]. Reduced bacterial growth rate and efficiency in *S. bescii*-compared to *R. irregularis*-inoculated soils may be due to *S. bescii*'s wider enzymatic repertoire, which could accelerate decomposition (as indicated by higher CO_2 efflux) or contribute to greater competition with other soil biota. It is also possible that greater abundance of *R. irregularis* compared to *S. bescii* contributed to the different magnitude of bacterial response. Together, these findings indicate that different mycorrhizal types elicit similar, but distinct bacterial response to water limitation, with significant effects on microbial community composition and growth rates.

$H_2{}^{18}O$ qSIP highlights microbial response

Previous studies report conflicting effects of drought on microbial biomass[5,6,95.96], activity[5,6,97,98], and community composition[2-4,82]. These variable responses may be due to differences in drought severity, biota examined, and experimental methods employed[10,99]. Previous drought exposure

can attenuate microbial response to additional water limitation[2]. With qSIP, we detected a strong negative microbial response to water limitation in soils that had repeatedly experienced below-average rainfall during the preceding decade[100]. This response was less apparent in our traditional microbial community analyses. For example, alpha diversity seemed unaffected by water limitation when assessed across 'total community' 16S rRNA gene profiles, but was significantly reduced in the actively growing community identified by qSIP. Similarly, compared to differential abundance analysis of 16S amplicons, qSIP identified tenfold more ASVs that were suppressed by water limitation (56 and 579, respectively). We attribute these differences to qSIP's capacity to distinguish actively growing organisms from the rest of the microbial community.

Conclusion

As global precipitation patterns change and water limitation becomes more prevalent, it is important to understand biotic response and conditions that might mitigate associated declines in function. Mycorrhizal fungi support plant performance and alter biogeochemistry in droughted soils, but their effect o`n soil bacterial response to water limitation is poorly explored. With $H_2{}^{18}O$ qSIP, we show that mycorrhizal fungi protect bacterial communities from the suppressive and destabilizing effects of water limitation. Both the AM fungus *R. irregularis* and the Sebacinales fungus *S. bescii* facilitated greater bacterial growth rates, growth efficiency, and the diversity of the active bacterial community in water-limited soil. While these divergent mycorrhizal lineages stimulated responses of differing magnitude, broad microbial patterns were similar, suggesting that the dominant underlying mechanisms are not limited to interactions between a small number of taxa. Remarkably, both *R. irregularis* and *S. bescii* prompted a

positive bacterial response despite the presence of a background microbial community in uninoculated soil. This finding is relevant for practical evaluation of mycorrhizal inoculants, whose ability to persist and elicit a positive effect in natural settings is not well-established. Together, our findings demonstrate that context-dependent mycorrhizal relationships support biotic resilience to moisture stress and may promote recovery once moisture is restored.

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Competing Interests

The authors declare no competing interests.

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Figures

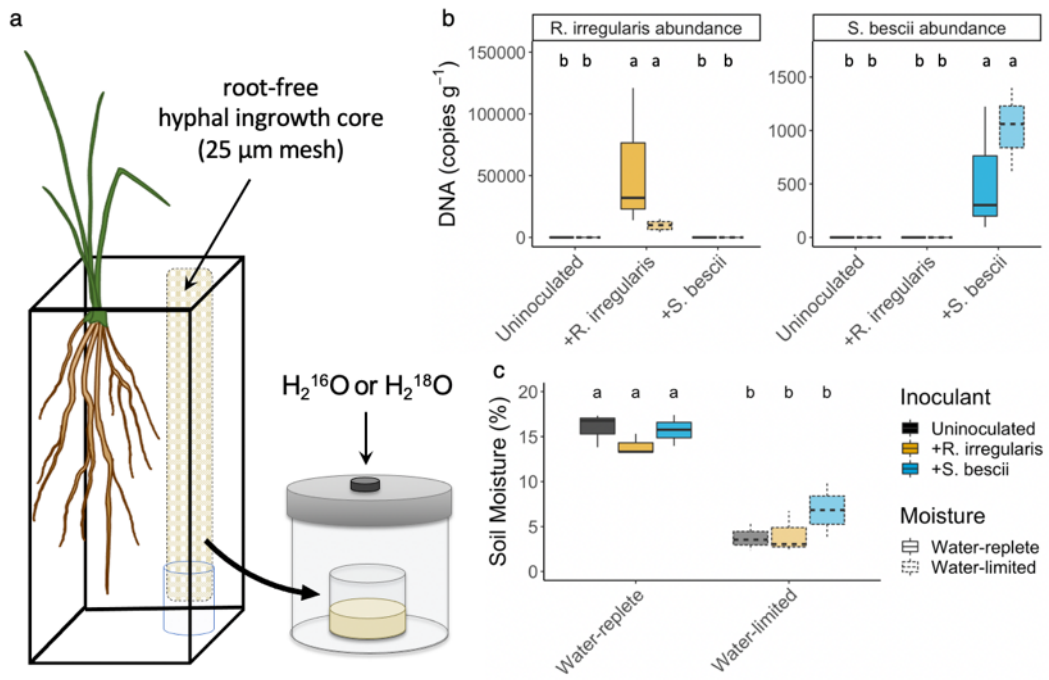

Figure 1. Experimental design and effect of mycorrhizal inoculation and moisture manipulation on mycorrhizal abundance and soil moisture after 3 months. Greenhouse experiment with *Panicum hallii* (Hall's panicgrass) and two mycorrhizal inocula grown in a marginal soil from central Oklahoma. **a** Illustration of microcosm experimental design and $H_2{}^{18}O$ stable isotope probing (SIP) incubation experiment conducted with soils collected from hyphal ingrowth cores that excluded plant roots. *P. hallii* was inoculated with either *R. irregularis*, *S. bescii* or left uninoculated (indicated in yellow, blue, or black, respectively) and grown for 3 months under water-replete or water-limited conditions (indicated in solid or dashed lines, respectively; n = 3 replicates per treatment). Soils harvested at 3 months were mixed with either enriched ($H_2{}^{18}O$) or natural abundance ($H_2{}^{16}O$) water and incubated for 7 days. **b** Abundance of *R. irregularis* and *S. bescii* (DNA copies g^{-1} soil) in hyphal ingrowth cores after 3 months of

plant growth and moisture manipulation, measured with strain-specific qPCR primers. **c** Soil moisture after 3-monts. Bold lines represent median value; whiskers represent upper and lower quartiles (n = 3 replicates per treatment). Letters denote the results of a Wilcoxon rank sum test performed separately for each qPCR plot ($p < 0.1$) and a Tukey's HSD test for soil moisture comparisons ($p < 0.001$).

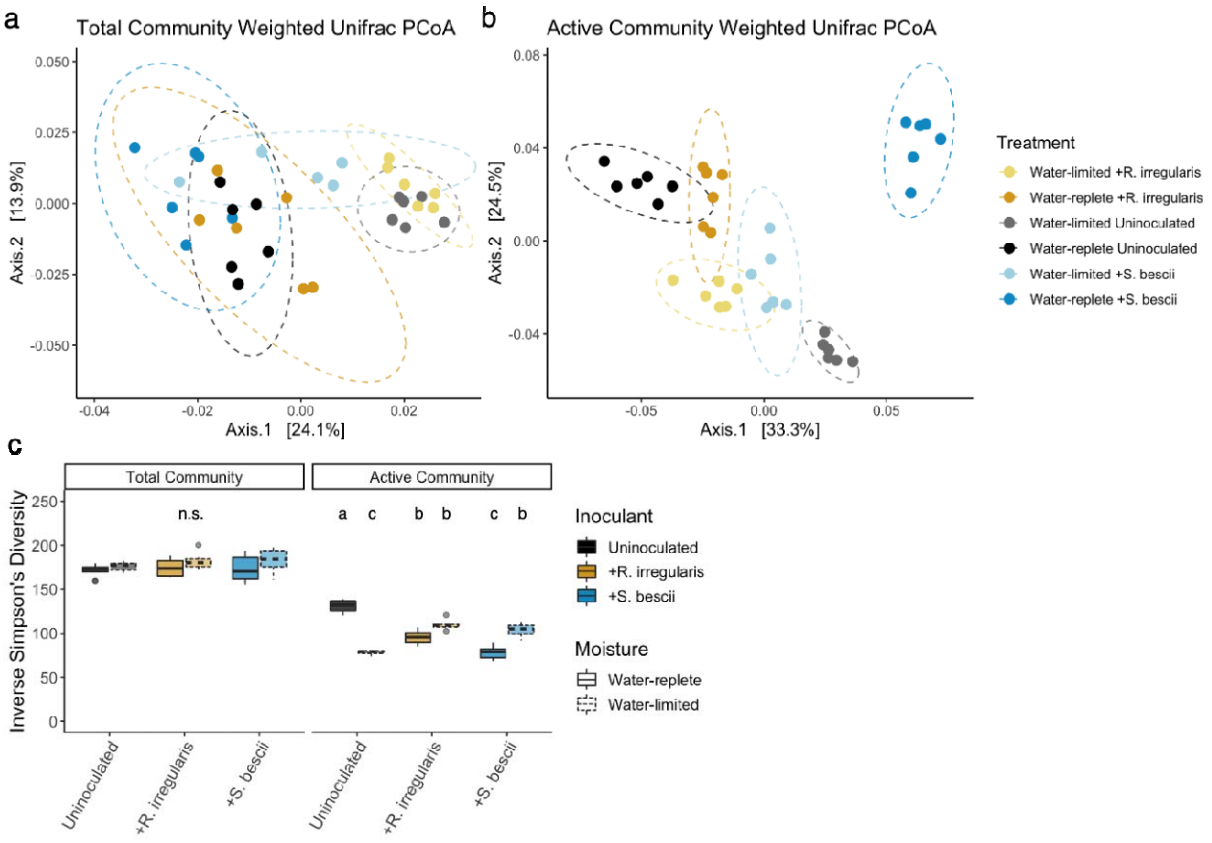

Figure 2. Structure and alpha diversity of total and active microbial communities based on traditional and $H_2{}^{18}O$ qSIP-filtered 16S rRNA gene profiling. Principal coordinates analysis (PCoA) of weighted UniFrac distances between 'total' microbial communities assessed with (**a)** traditional 16S rRNA gene analysis and **(b)** $H_2{}^{18}O$ qSIP-filtered 'actively growing' communities (i.e., ASVs that did not incorporate a significant quantity of ^{18}O were removed prior to analysis). Moisture regime and mycorrhizal inoculum explained a total of 29.5% of the variation in 'total' community structure ($p < 0.001$; n = 6 replicates) and 75.2% of variation in actively growing community structure ($p < 0.001$; n = 6 replicates). Ellipses show treatment groupings in **(a)** and **(b)**. **c** Inverse Simpson's diversity index in total and actively growing communities. Letters denote the results of a Tukey's HSD test (no significant differences between total communities; $p < 0.01$ for comparisons between active communities; n = 6 replicates). Bold lines represent median values; whiskers represent upper and lower quartiles. For all plots, uninoculated, *R. irregularis*-inoculated, and *S. bescii*-inoculated soils are represented in black, yellow, and blue, respectively. Water-replete and water-limited soils are represented in dark versus light color shades, respectively.

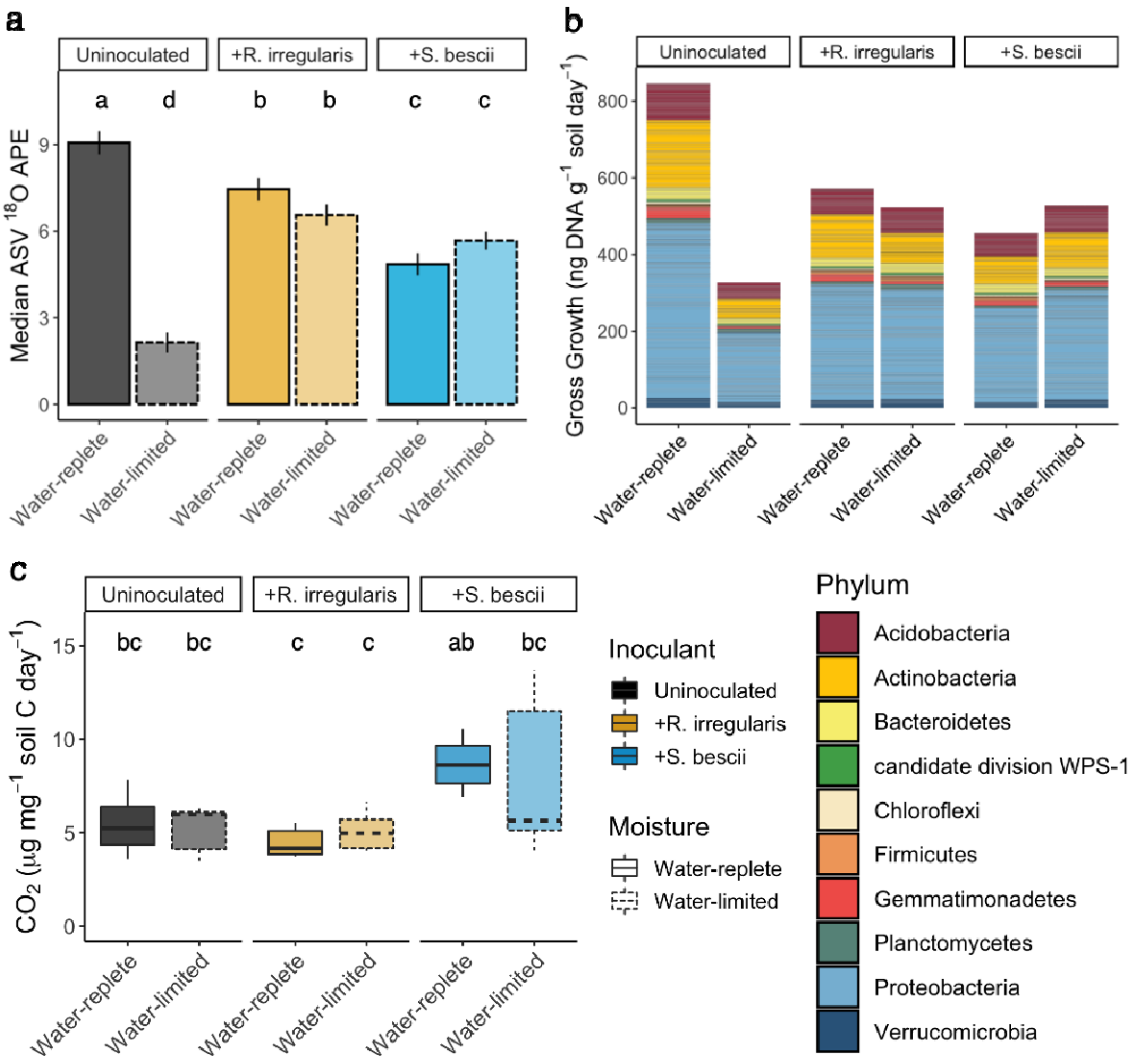

Figure 3. $H_2{}^{18}O$ qSIP-based microbial activity in hyphal ingrowth core soils exposed to different moisture regimes and mycorrhizal inocula. a Median ^{18}O APE of all bacterial and archaeal ASVs present under each treatment. Uninoculated, *R. irregularis*-inoculated, and *S. bescii*-inoculated soils are represented in black, yellow, and blue, respectively. Water-replete and water-limited soils are represented in solid and dashed lines, respectively. Letters denote the results of a Tukey's HSD test comparing the means of all treatments ($p < 0.01$; $n = 3$ replicates). **b** Taxon-specific gross growth rates (ng DNA g^{-1} soil day^{-1}) based on ASV ^{18}O incorporation,

summed by phylum for each treatment. **c** CO_2 efflux (µg CO_2 mg^{-1} soil C day^{-1}) from hyphal ingrowth core soils during $H_2{}^{18}O$ qSIP assay. Bold lines represent median values; whiskers represent upper and lower quartiles ($p < 0.05$; $n = 6$ replicates per treatment).

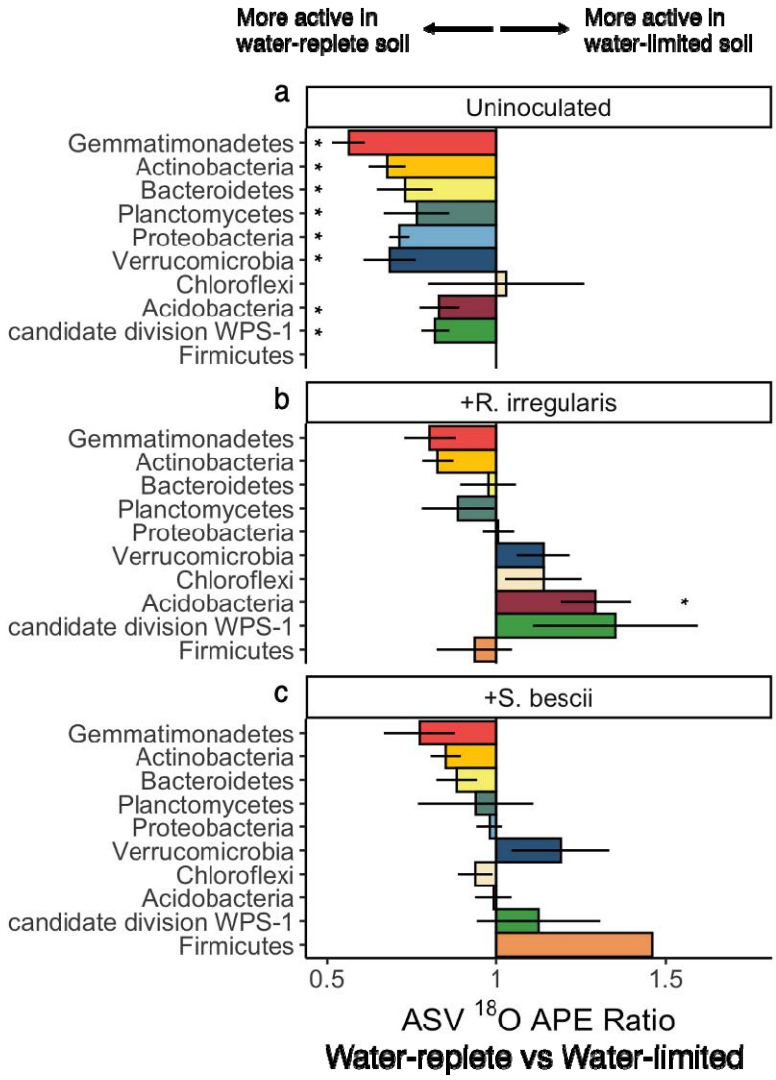

Figure 4. Comparison of taxon-specific activity in water-limited versus water-replete hyphal ingrowth core soils within each mycorrhizal treatment. ^{18}O atom percent excess (APE) 'active growth' ratios of ASVs present in both water-limited and water-replete soils that were either **(a)** uninoculated, **(b)** inoculated with *R. irregularis*, or **(c)** inoculated with *S. bescii*. Ratios were averaged at the phylum level. Ratios less than 1 (bars located to the left of the central line) indicate that water limitation suppressed growth. Ratios greater than 1 (bars located to the right of the central line) indicate that water limitation facilitated greater growth rates. Error bars represent the standard error. Asterisks denote phylum-level averages that are significantly

greater than or less than 1 (adjusted $p < 0.05$ based on Wilcoxon signed rank test and Benjamini-Hochberg correction for multiple comparisons). Only taxa that incorporated significant ^{18}O (lower 90% CI > 0) and belong to the 10 most abundant phyla are represented.

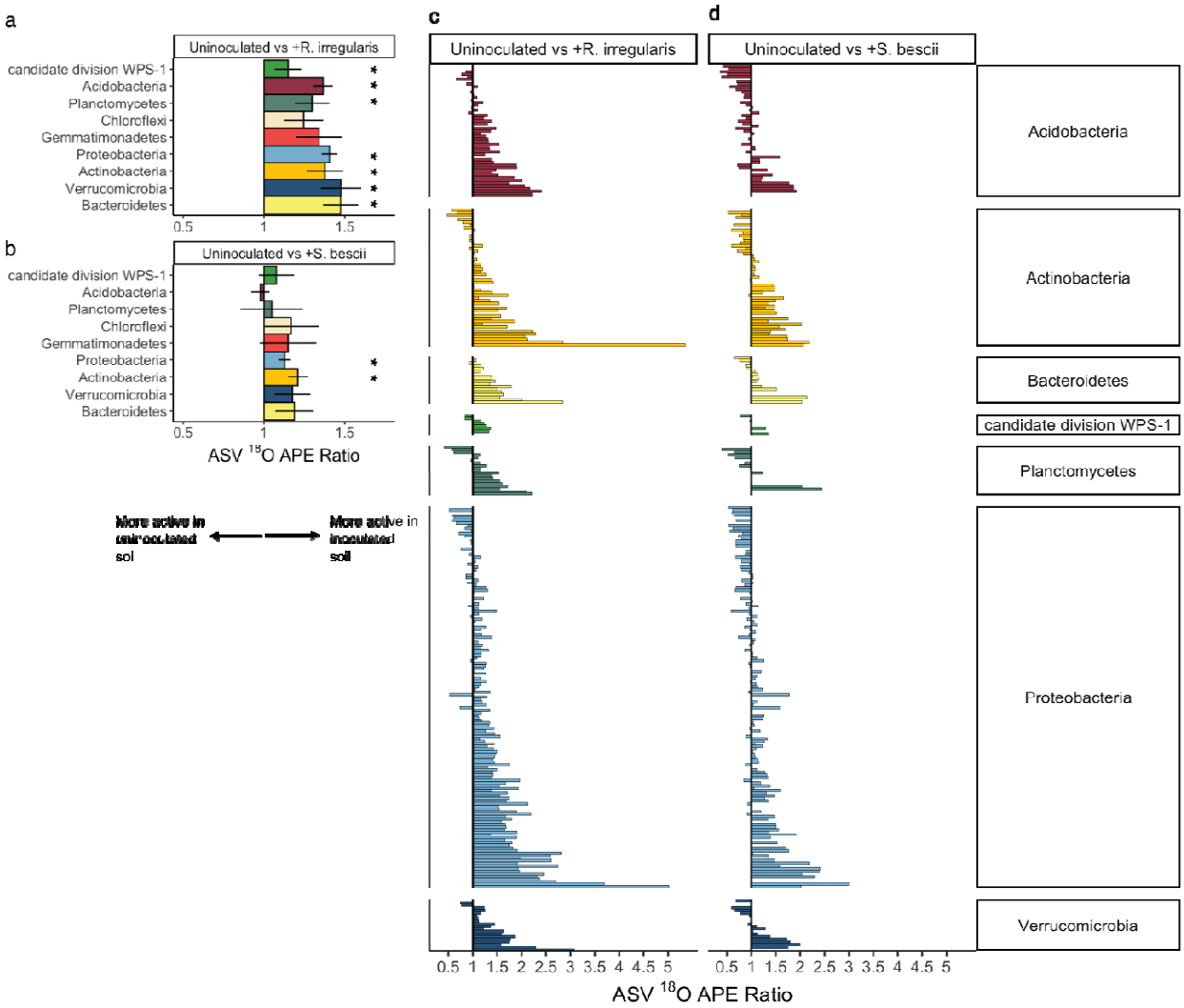

Figure 5. Comparison of taxon-specific activity in mycorrhizal-inoculated versus uninoculated hyphal ingrowth core soils following water limitation. ^{18}O atom percent excess (APE) 'active growth' ratios of bacterial ASVs present in water-limited soils that were either uninoculated or inoculated with *R. irregularis* or *S. bescii*, averaged at the phylum level **(a,b)** or

displayed by individual ASV **(c,d)**. Ratios greater than 1 (to the right of the central line) indicate that mycorrhizal fungi supported greater bacterial growth rates. For phylum level averages, asterisks represent ^{18}O APE ratios that are significantly greater than 1 (adjusted $p < 0.05$ based on Wilcoxon signed rank test and Benjamini-Hochberg correction for multiple comparisons). Error bars represent the standard error within each bacterial phylum. Only taxa that incorporated significant ^{18}O (lower 90% CI > 0) and belong to the 10 most abundant phyla are represented. ASVs belonging to phyla that incorporated significantly more ^{18}O in mycorrhizal-inoculated soils are represented in **c,d**.