Science of the Total Environment

The effects of mixed-species root zones on the resistance of soil bacteria and fungi to long-term experimental and natural reductions in soil moisture

--Manuscript Draft--

Manuscript Number:	STOTEN-D-22-26442R2
Article Type:	Research Paper
Keywords:	plant-soil interactions, forest soil microbiome, drought resistance, precipitation gradient, beech-spruce forest, and root complementarity
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Response to Reviewers:	

Cornell CALS College of Agriculture and Life Sciences

February 11th, 2023

Dear Editors of the *Science of the Total Environment*,

We have made the requested minor revisions to our article of original research entitled: "T**he effects of mixed-species root zones on the resistance of soil bacteria and fungi to long-term experimental and natural reductions in soil moisture**." We thank the reviewers for their thoughtful feedback and hope the manuscript now meets the standards of STOTEN.

Sincerely,

Dr. Taryn L. Bauerle

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Reviewers/Editor comments:

Reviewer #1: Thank you for the good job in revising the article. Another question came to my mind while reading the discussion section.

You attribute the greater community tolerance in spruce stands than in beech stands to factors such as the litter quality, pH, etc (mainly 4.1 part, but also 4.3). These are certainly the most important factors that make the difference between the communities of the two species. However, the physiological nature of these tree species and their rooting depth contribute to the fact that beech stands are much wetter in the upper soil layer during drought periods (which has been confirmed by studies with lysimeters), while spruce stands generally tolerate drought periods less well and the water almost disappears from the upper soil layers. Could not spruce communities be trained to such conditions over time and thus select more drought-resistant species? I would welcome a discussion on this point as well.

This is an important point and was a clear oversight on our part. We are grateful for your suggestion. In section 4.1, we now write: “Physiological differences in rooting depth result in consistently drier conditions in shallow soils in spruce stands relative to beach (Allen et al., 2019; Zwetsloot and Bauerle, 2021). Our findings indicate that this effect is large enough to select for higher proportion of drought stress-tolerant bacteria in spruce soils.” (L384) and, later on, we write: “These adverse conditions, along with consistently lower shallow soil moisture levels in spruce stands, may select for stress-tolerant populations, which may better endure water stress.” (L399).

The effects of mixed-species root zones on the resistance of soil bacteria and fungi to long-term experimental and natural reductions in soil moisture

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Running title: Drought-microbiome effects in mixed rooting zones

Abstract

Mixed forest stands tend to be more resistant to drought than species-specific stands partially due to complementarity in root ecology and physiology. We asked whether complementary differences in the drought resistance of soil microbiomes might contribute to this phenomenon. We experimented on the effects of reduced soil moisture on bacterial and fungal community composition in species-specific (single species) and mixed-species root zones of Norway spruce and European beech forests in a 5-year-old throughfall-exclusion experiment and across seasonal (spring-summer-fall) and latitudinal moisture gradients. Bacteria were most responsive to changes in soil moisture, especially members of Rhizobiales, while fungi were largely unaffected, including ectomycorrhizal fungi (EMF). Community resistance was higher in spruce relative to beech root zones, corresponding with the proportions of drought-favored (more in spruce) and drought-sensitive bacterial taxa (more in beech). The spruce soil microbiome also exhibited greater resistance to seasonal changes between spring (wettest) and fall (driest). Mixed-species root zones contained a hybrid of beech- and spruce-associated microbiomes. Several bacterial populations exhibited either enhanced resistance or greater susceptibility to drought in mixed root zones. Overall, patterns in the relative abundances of soil bacteria closely tracked moisture in seasonal and latitudinal precipitation gradients and were more predictive of soil water content than other environmental variables. We conclude that complementary differences in the drought resistance of soil microbiomes can occur and the likeliest form of complementarity in mixed-root zones coincides with the enrichment of drought-tolerant bacteria associated with spruce and the sustenance of EMF by beech.

Key words: plant–soil interactions**,** forest soil microbiome, drought resistance, precipitation gradient, beech-spruce forest, and root complementarity.

1. Introduction

Plant species diversity positively correlates with ecosystem productivity (Hooper and Vitousek, 1997; Liang et al., 2016; Tilman, 2001) and with increased resistance to extremes in water availability, at least in grasslands (Craven et al., 2016; Isbell et al., 2015). Forest ecosystems are vulnerable to the increasing frequency, intensity, and duration of drought caused by changing precipitation patterns (Dai, 2013; IPCC, 2018). However, it remains to be proven whether forests with higher plant diversity or functional richness are more resistant to drought than single species ('species-specific') plantations (García-Valdés et al., 2021). The general relationship between plant species diversity and productivity is, at least, partially due to the effects of biotic feedbacks between plants and soil microorganisms (Hendriks et al., 2013; Schnitzer et al., 2011). Yet, to date, research into diversity-productivity relationships in forests has been primarily focused on aboveground parameters, e.g. annual growth (Paquette and Messier, 2011; Pretzsch et al., 2020, 2010). More recently, belowground parameters were found to differ between species-specific and mixed-species forest stands, including tree root lifespan dynamics (Zwetsloot et al., 2019) and root niche partitioning during drought (Altinalmazis-Kondylis et al., 2021; Zwetsloot and Bauerle, 2021). These observations raise questions about the potential influence of other belowground biotic interactions on the drought resistance of mixed forest stands.

Belowground interactions between root systems of European beech (henceforth 'beech') and Norway spruce (henceforth 'spruce'), a common forest type in Central Europe, can differ between monoculture and mixed-species forests (Schmid and Kazda, 2002). Spruce and beech exhibit anatomical (tracheid vs. xylem vessel elements) and physiological (isohydric vs. anisohydric) differences that effect their hydrological processes and drought response (Lyr et al., 1992; Pretzsch et al., 2013) and their root systems can interact in complementary ways that reduce competition and facilitate access to soil moisture. These effects are evident in the differences

between species-specific and mixed root zones, where beech roots exhibit lower mortality than in monospecific root zones, due to decreased intraspecific competition (Zwetsloot et al., 2019), and where spruce roots maintain higher fine root mass during chronic drought (Zwetsloot and Bauerle, 2021). This complementarity is due to several aspects of root ecophysiology, which include: (i) differences in rooting depth, with beech colonizing lower soil layers in mixed root zones (Bolte and Villanueva, 2006), and (ii) diverging responses in fine root growth during drought, where spruce become more dormant (cease growth and enhance suberization), while beech maintain a degree of root activity, producing new, albeit shorter lived, fine root mass (Nikolova et al., 2020; Rötzer et al., 2017). Beech and spruce also select for broadly different soil microbiomes (Bárta et al., 2017; Uroz et al., 2016). Complementarity in the function and drought-tolerance between their associated microbiomes might contribute to the altered plant drought responses where roots mix.

Root-associated microbes support the nutritional needs of trees (Brzostek et al., 2013; Calvaruso et al., 2014; Gan et al., 2021; Nicolitch et al., 2016), and provide protection from phytopathogens (Lehr et al., 2008; Terhonen et al., 2016) and the effects of mild drought (Pena et al., 2013; Shi et al., 2002). Prior research has shown that the soil microbiome in mixed-species forests can have greater functional and metabolic diversity than species-specific forests (Beugnon et al., 2021; Prada-Salcedo et al., 2021). Thus, complementarity in the function and drought-tolerance of soil microbiomes associated with beech and spruce might confer benefits during drought. Several observations indicate the potential unevenness in the susceptibility or tolerance of members of the beech- and spruce-associated microbiomes. The spruce soil microbiome is characterized by a higher relative abundance of Actinobacteria, Armatimonadetes, and Acidobacteria (and a higher denitrification potential) and greater proportion of ectomycorrhizal fungi (EMF) relative to saprotrophic fungi than the beech soil microbiome, which has higher

proportions of Alphaproteobacteria, Planctomycetes, and Verrucomicrobia and different dominant EMF taxa (*Scleroderma*, *Russula* and *Laccaria*) than spruce (*Boletus* and *Thelephora*) (Bárta et al., 2017; Felsmann et al., 2015; Nacke et al., 2016; Uroz et al., 2016). Populations of Alphaproteobacteria and Planctomycetes (beech-associated) are generally more sensitive to drought, while Armatimonadetes and Actinobacteria (spruce-associated) are more tolerant (Bastida et al., 2017; Bouskill et al., 2013; Chodak et al., 2015; Curiel Yuste et al., 2014; Evans et al., 2014; Felsmann et al., 2015; Hartmann et al., 2017; Preece et al., 2019; Zeng et al., 2016; Zhou et al., 2018). EMF also differ in their drought tolerance with more sensitive (ex. within *Laccaria* and *Lactarius*) and more tolerant (ex. within *Russula*, *Cenococcum*, and *Scleroderma*) species among those that associate with beech and spruce (Coleman et al., 1989; Di Pietro et al., 2007; Ortega et al., 2004). Disparities in the drought tolerance or susceptibility of beech- and spruce-associated microbiomes have yet to be experimentally tested.

With our study, we investigated whether the effects of experimental and natural reductions in soil moisture on the structure of beech and spruce microbiomes differed in species-specific (single species) versus mixed-species root zones. Drought-affected bacteria and fungi were identified in a 5-year-old throughfall exclusion experiment and changes in these populations were profiled in seasonal and latitudinal gradients in soil moisture and precipitation, respectively (Figure 1). The composition of bacterial and fungal communities and root-associated 'rhizobacteria' (rhizoplane and root endophytes) were determined with 16S rRNA gene and ITS region amplicon sequencing. We expected EMF and rhizobacterial populations to be most sensitive to reductions in soil moisture given the associated reduction in fine root mass (Zwetsloot and Bauerle, 2021). We hypothesized that the impact of reduced soil moisture would affect beech and spruce microbiomes differently, and that these responses would be further altered in soils where roots

mixed. Specifically, our experiment tested the null hypothesis that the responses of beech- and spruce-associated bacteria and fungi would not differ in mixed-species root zones relative to species-specific zones. We then tested whether similar differences occurred in response to seasonal variation and latitudinal differences in soil moisture. Our experiment advances an understanding of the effects of drought and natural gradients in soil moisture on beech and spruce soil microbiomes and establishes the potential for complementation to occur where root systems mix.

2. Methods

2.1 Study overview and experimental design

In 2013, a latitudinal precipitation gradient was established in Bavaria, Germany (Pretzsch et al., 2014). The gradient is composed of five forest sites containing 60 to 90-year-old Norway spruce (*Picea abies* [L.] Karst) and European beech (*Fagus sylvatica* L.) which are (from driest to wettest): Arnstein, Kelheim, Kranzberg, Wasserburg, and Traunstein (Figure 1A). All five sites are similar in mean annual temperature (MAT) and soil type (Cambisol and Luvisol), but differ in their mean annual precipitation (MAP; Table S1). Relative differences in total annual precipitation were consistent among sites during the duration of the experiment (Table S2). However, periods of drought occurred throughout Germany in summer in 2015 and 2018 (Schuldt et al., 2020). Individual sites consist of a species-specific spruce and beech root zone, as well as a mixed spruce and beech root zone with varying degrees of mixing (Table S3). A throughfall-exclusion experiment was established at the middle site of the precipitation gradient, (Kranzberg Forest Roof Experiment or 'KROOF'; Figure 1B) and is composed of six drought plots, each with a species-specific beech and spruce and mixed-species stand covered by a throughfall exclusion roof, and six corresponding uncovered reference plots, with corresponding stand compositions. At the time of the last sampling in 2018, seasonal throughfall had been excluded for five years, resulting in an overall reduction of ~70% soil moisture across time and significantly greater pre-dawn water stress

for beech ($\bar{x}$ = -0.66 ± 0.2 MPa water potential) and spruce ($\bar{x}$ = -0.98 ± 0.3 MPa) compared to reference plots (Grams et al., 2021).

2.2 Soil and root sampling

Soils from each root zone were sampled during the fall of 2017 (November), and the spring (April), summer (July), and fall of 2018 (October) at all five sites and from the throughfall exclusion experiment. During each sampling, ten soil cores (1.6 cm in diameter and 30 cm long) were harvested from five plots (approximately 10 m^2) randomly located within in each tree root zone and/or throughfall exclusion treatment plot. The O_l (litter layer) was manually removed, and each soil core was divided into upper (0-8 cm deep) and lower soil layers (9-30 cm) comprised of the $O_f + {}_hA_h$ and A_lB_v, respectively (KA5 classifications; Nickel et al., 2018). The upper and lower layers of ten cores were separately composited to yield 5 replicates per tree root zone per site and/or per throughfall exclusion treatment. Five grams of the upper and lower composite sample (excluding roots and soil particles > 2 mm) was weighed into Whirl-pak® bags in the field (Nasco Sampling, Madison, WI, USA), and transported on dry ice to the Technische Universität München (TUM) campus in Freising, where they were dried at 60 ºC to a constant weight. Oven drying was used to minimize the period in which cells are active and has negligible impact on bacterial and fungal community composition (Castaño et al., 2016; Tzeneva et al., 2009). Dried samples were shipped to Cornell University (Ithaca, NY, USA) and stored at room temperature until DNA was extracted within two months. Over short periods, changes in microbiome composition due to air drying and storage are minor (Clark and Hirsch, 2008; Lauber et al., 2010; Tzeneva et al., 2009). The remaining soil samples were stored at TUM at 4 ºC for additional analyses and to determine fine root biomass (mg) (≤ 1 mm diameter), also dried to 60 ºC to a constant weight. Prior to the onset of KROOF, in 2013, fine root samples were taken from upper and lower layers in species-specific spruce and beech root zones for the purpose of identifying root-associated bacteria

('rhizobacteria') associated with each tree (n = 20), as opposed to 'soil bacteria,' which we use to refer to the amplicon data generated from whole soil cores. Roots were thoroughly cleaned in tap water, identified under a stereomicroscope and frozen until DNA was extracted. The bacterial populations identified via this method correspond to a combination of 'rhizoplane' and root endophyte populations.

2.3 Analyses of soil properties

The remaining soil from each sample was used to measure soil water content (SWC), pH, and soil organic matter (SOM). SWC was averaged from triplicate measurements using 3 g of fresh soil, which was calculated as the percentage mass lost after drying for 24 hours at 105°C. Soil pH was measured using a VWR Scientific Products model 2000 pH meter, in a 1:10 (w/v) dilution with distilled deionized water, after the sample was shaken for one minute and allowed to settle for 10 minutes. SOM was estimated using loss on ignition method, according to (Howard and Howard, 1990), by weighing 2 g of soil onto clay trays that were put in a muffle furnace where temperature was slowly increased by 10 ºC increments until a final temperature of 550 ºC was reached and maintained for 3 h.

2.4 Analyses of soil bacterial and fungal communities and rhizobacteria

DNA was extracted from 0.25 g of soil sample using the Qiagen PowerSoil kits (Germantown, Maryland, USA) according to the manufacturer's protocol with a bead beating treatment of 3 min at 5.5 $m \cdot s^{-1}$ (Bio-spec, Bartellsville, OK, USA). DNA quantification and PCR amplification of bacterial (V4 region of 16S rRNA gene; 515f/806r) and fungal phylogenetic gene markers (ITS1; nBITS2f/58A2r) was performed as previously described (Sridhar et al., 2022). Duplicate PCR reactions per sample were pooled prior to Illumina MiSeq (2 x 250 bp; v2) sequencing, which was performed at Cornell Biotechnology Resource Center (Ithaca, NY) using dual-indexed bar-coded primers (Koechli et al., 2019; Kozich et al., 2013). Seventy-five ITS

sequencing libraries from the Kranzberg throughfall-exclusion experiment were discarded due to poor sequencing quality. These were discarded at random without impacting the balance of our experimental design (Table S4). To identify rhizobacteria, DNA was extracted from 0.35-0.45 g of root using the PowerSoil kit as previously described (Nickel et al., 2018) and 16S rRNA amplicon libraries were prepared targeting rhizobacteria using the same methods (details in SI) except that the V3-V4 region was targeted using primers optimized for plant DNA-rich samples (335f/769r), as previously described (Dorn-In et al., 2015). All sample metadata is provided in Table S5, and raw sequencing data was archived with the European Nucleotide Archive under the BioProject accession: PRJEB36981 (data reference, see Wilhelm et al., 2022).

Sequencing data was processed using QIIME2 (v. 2020.2; Bolyen et al., 2019) with a dependency on DADA2 (v. 1.10; Callahan et al., 2016) to assign sequences to operational taxonomic units (i.e., amplicon sequence variants). Taxonomic classification was performed using the QIIME2 'q2-feature-classifier' trained on the Silva database (v. 132; Quast et al., 2013) and UNITE database (v. 7.2; Nilsson et al., 2019) for bacteria and fungi, respectively. OTUs found in the non-template controls and in low abundance were removed, namely those present in fewer than three samples, or at a total relative abundance < 0.01%. All counts were normalized by proportion of total reads and presented as counts per thousand reads. R package *phyloseq* (v. 1.34; McMurdie and Holmes, 2013) was used to characterize the communities and estimate diversity parameters on rarified libraries (n_{bact} = 15,300 and n_{fungi} = 2,544 reads per sample). Fungal taxonomic classifications were used to identify EMF, endophytic, and saprotrophic fungi using the FUNGuild database (v. 1; Nguyen et al., 2016). Samples from throughfall-exclusion plots were removed during all analyses of seasonal or latitudinal effects.

183 *2.5 Bioinformatic and statistical analyses*

184 The differential abundance of OTUs between reference and throughfall exclusion plots
185 (i.e., 'drought-affected') were identified using the *R* software package *indicspecies* (v. 1.7.9;
186 Cáceres and Legendre, 2009). Indicator species analysis was performed independently for soil
187 layers, season, and forest type, then combined and de-duplicated. Non-significant indicator OTUs
188 ($p_{adj} < 0.05$) and those with low indicator values (< 0.35) were excluded. Alpha-diversity was
189 measured as species richness, Shannon diversity, and Pielou's evenness. Beta-diversity was
190 assessed using Bray-Curtis dissimilarity and differences in community composition were
191 visualized using principal coordinates analysis and tested with PERMANOVA ($n_{perm} = 999$) using
192 'adonis' from the *R* package *vegan* (v. 2.5.7; Oksanen et al., 2015). Resistance (R) to change in
193 microbiome composition due to throughfall exclusion was measured as the Bray-Curtis
194 dissimilarity between reference and exclusion plots (D), such that R = 1 – D. A lower R value
195 indicates a greater dissimilarity between reference and throughfall exclusion plots, indicating a
196 greater drought effect (De Vries and Shade, 2013). R was calculated for the average of all
197 permutations of replicates between reference and exclusion plots. The environmental and
198 microbiome features that were most predictive of SWC were identified using random forest-based
199 feature selection implemented in the *R* package *Boruta* (v. 7.0; Kursa and Rudnicki, 2010).
200 Features included in model selection were environmental (soil layer, root zone, site, season, year)
201 and microbiome (aggregated counts at the taxonomic rank of Order scaled with the 'scale' function
202 in *R*). Subsequently, Pearson's correlations between selected bacterial and fungal features and
203 SWC were performed using 'rcorr' from the *R* package *Hmisc* (v. 4.5; Harrell and Dupont, 2015).
204 Differences in the relative abundance of OTUs among tree root zones were tested with ANOVA
205 ('aov' function in *R*). The main effects and interactions between throughfall exclusion, season, or
206 tree root zone on the relative abundances of OTUs, were determined by fitting to fixed effects

linear models ('lm' function). P-values were adjusted for multiple test correction according to the Benjamini and Hochberg false discovery rate. Only the most abundant OTUs (> 0.05% of sample reads) were included in statistical testing. Significant effects are denoted by asterisk: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All analyses can be reproduced with scripts included in the Supplementary Data package available through the Open Science Foundation (doi: 10.17605/OSF.IO/DN9CH).

3. Results

Our initial analyses were performed to establish the drought susceptible and tolerant (section 3.1) and spruce- and beech-associated soil microbiome and rhizobacteria (section 3.2) prior to testing whether the response of these groups differed in species-specific versus mixed root zones (section 3.3). Additional analyses were performed to characterize the natural variation (seasonal and precipitation gradient) in drought-affected populations among tree root zones and soil layers (section 3.4).

3.1.1 General effects of throughfall exclusion on soil properties and the soil microbiome

Across the full 5-year experimental period, throughfall exclusion resulted in a 70% reduction in SWC, on average, during the growing season (Grams et al., 2021) and, at the time of our sampling, the throughfall exclusion plots had significantly lower SWC in upper ($\bar{x}$ = -27.8%) and lower soil layers ($\bar{x}$ = -22.4%) across all seasons (Figure S1). Upper layers had higher average SWC though the extent of moisture reduction was comparable in both layers (t = -3.9 vs. -3.2, respectively). Throughfall exclusion significantly decreased fine root mass, with a greater effect on upper layers ($\bar{x}$ = -51.1%; t = -4.2; $p < 0.001$) compared to lower layer soils ($\bar{x}$ = -29.8%; t = -3.2; $p < 0.001$). Exclusion had minor effects on soil pH (+1% in upper layer; $p = 0.03$); and had mostly insignificant effects on SOM and DNA yield (Figure S1). Throughfall exclusion accounted for a relatively low proportion of variation in the beta-diversity of soil bacterial and fungal

communities (Figure 2AB), which was primarily attributable to differences among soil layer and tree root zone. Communities from mixed root zones exhibited an intermediate similarity between species-specific root zones (Figure 2A).

3.1.2 Drought-affected soil bacteria and fungi

The differences in the relative abundance of OTUs between reference and throughfall excluded plots were used to identify 'drought-favored' or 'drought-sensitive' bacterial and fungal populations. The soil microbiome at Kranzberg was comprised of 10,357 bacterial and 3,282 fungal OTUs. Of these, a total of 369 bacterial and 23 fungal OTUs were indicative of throughfall exclusion (Table S6). More OTUs were drought-sensitive (n_{bact} = 194 and n_{fungi} = 15) than drought-favored (n_{bact} = 175 and n_{fungi} = 8). More drought-affected OTUs were observed in upper (n = 215) versus lower soil layers (n = 130), but OTUs did not differ in their likelihood of being drought-affected based on their soil layer association (Fisher's test; p = 0.8).

Drought-favored fungi were primarily classified as Ascomycota (80%) and belonged to genera designated as saprotrophic (*Pseudogymnoascus*, *Niesslia*, and *Ciliolarina*). Two of the eight drought sensitive fungal OTUs were classified to genera of EMF (*Inocybe* and *Lactarius*). However, the overall relative abundance of EMF was largely unaffected by throughfall exclusion (Figure S2A). Fungal endophytes (genus *Phialocephala*) were significantly more abundant in throughfall-excluded upper soils (Figure S3A) and were relatively more abundant in spruce root zones (Figure S3B).

Throughfall exclusion affected a phylogenetically diverse group of bacteria, but most of the affected OTUs were classified to Planctomycetes, Alphaproteobacteria and Actinobacteria (Figure S4; complete list in Table S6). Actinobacteria were near uniformly drought-favored (primarily classified as Pseudonocardiales, Solirubrobacterales, and Microccocales), while Planctomycetes were primarily drought-sensitive (Pirellulales, Gemmatales, and

Planctomycetales), though certain clades of Planctomycetes also contained a high proportion of drought-favored taxa (Tepidisphaerales and Isosphaerales). Within Alphaproteobacteria, several orders were favored by throughfall exclusion, namely populations of Caulobacterales (*Phenylobacterium*), Elsterales, and Sphingomonadales (*Sphingomonas*) while others were sensitive, namely populations of Rhizobiales (*Roseiarcus*, *Rhodoplanes*, and *Bradyrhizobium*) and Reyranellales (*Reyranella*). Other major bacterial groups that were drought sensitive included: Deltaproteobacteria (Myxococcales), Verrucomicrobia (Pedosphaeraceae and Xiphinematobacteriaceae), and Dependentiae (Vermiphilaceae).

3.2.1 Beech- and spruce-associated soil bacteria and fungi and rhizobacteria

Soil microbiome composition significantly differed between beech and spruce root zones, though the most abundant bacterial and fungal OTUs (≥ 0.2% of total reads) were present in all root zones (97% of bacteria and 52% of fungi; Figure S5). The relative abundances of beech- and spruce-associated OTUs in mixed zones were characteristically intermediate between the relative abundance in the species-specific zones (Figure S6). The spruce-associated microbiome was dominated by several orders of Actinobacteria (Frankiales and Solirubrobacterales) and Acidobacteria (Acidobacteriales and Solibacterales; Figure 3A) and had a higher Ascomycota:Basidiomycota ratio compared to beech (Figure S7). The beech microbiome had a higher proportion of Proteobacteria (Alpha-, Gamma- and Delta-), Verrucomicrobia and Planctomyces and a higher proportion of EMF (Figure S2B). Beech-associated taxa were significantly more likely to be drought-sensitive than spruce-associated taxa (Fisher's Exact, O.R. = 23.8; $p < 0.001$), with spruce-associated taxa tending to increase in relative abundance in throughfall excluded plots.

Rhizobacterial populations associated with beech and spruce roots were identified using indicator analysis. Beech-associated rhizobacterial populations were enriched in

Alphaproteobacteria from the family Xanthobacteraceae relative to spruce, which were enriched in Acidobacteriales (*Acidipila*, *Granulicella* and *Occallatibacter*) and Frankiales (*Acidothermus*; Figure 3B, complete list in Table S7). Bacterial species richness and evenness were significantly higher in beech than spruce root zones, while no differences were evident for fungi (Figure S8).

3.2.2 Corresponding differences in plant and soil properties among root zones

Trends in the soil microbiome corresponded primarily with differences in fine root mass and soil organic matter content in root zones. The upper soil layer in spruce root zones had a significantly higher percentages of soil organic matter (2-fold) and SWC than beech, except for at Arnstein, which received the lowest precipitation (Figure S9A). In contrast, the beech root zone had significantly higher fine root mass in both the upper (66%) and lower layers (60%) at all sites, while mixed zones had intermediate root mass. In all sites, fine root mass followed seasonal trends in SWC between spring and fall, increasing in wetter sites (Wasserburg and Traunstein) and declining in drier sites (Arnstein and Kranzberg), with the trend most pronounced in beech stands (Figure S9B). Soil pH was significantly more acidic in spruce ($\bar{x}$ = 4.04) than beech ($\bar{x}$ = 4.30), with intermediates values in mixed root zones ($\bar{x}$ = 4.21).

3.3.1 Contrasting the effects of soil moisture on microbiomes among root zones

We tested for the generalized effects of soil moisture reduction on soil bacteria and fungi among root zones using the community resistance (R) metric. In our study, lower R values indicate a greater change in community composition resulting from reduced soil moisture (i.e., a lower resistance). On average, R values for bacterial communities differed by tree root zone, but not for fungal communities. Bacterial communities in the spruce root zones had higher R values than in the beech root zones, though this difference was only significant in the lower soil layer (Figure 4A). Differences in R among root zones corresponded with the proportion of drought-favored (higher in spruce) and drought-sensitive bacterial OTUs (higher in beech; Figure 4B). The spruce

soil microbiome also exhibited greater R values in relation to seasonal changes between spring (wettest) and fall (driest) at Kranzberg and, in this case, the mixed root zone also exhibited significantly higher R than species-specific beech (Figure S10A). These differences also corresponded to significant seasonal increases in drought-favored and drought-sensitive taxa (Figure S10B). R values for fungal communities were variable and did not significantly differ among tree root zones (Figure S11A). Yet, the relative abundance of drought-favored fungal OTUs was also significantly higher in spruce root zones (Figure S11B).

3.3.2 Contrasting responses in the soil microbiome of species-specific and mixed root zones

The effects of throughfall exclusion on several abundant beech- and spruce-associated bacteria were significantly altered in the mixed-species root zone. Several beech- and spruce-associated OTUs that were affected by throughfall exclusion in species-specific root zones exhibited enhanced tolerance in mixed root zones (Figure 5AB; Table S8). Conversely, several OTUs that were unaffected by throughfall exclusion in species-specific root zones exhibited different responses in mixed-species root zones. These could be divided into beach-associated OTUs, that exhibited a greater susceptibility (Figure 5C), and spruce-associated OTUs, that exhibited a greater resistance, in mixed-species root zones (Figure 5D; Table S9). These trends were evident when profiling the same populations across seasonal differences in SWC at Kranzberg (lower panels in Figure 5C and 5D), and at other sites, though not uniformly (Figure S12). There were no significant interactions between root mixing and throughfall exclusion for soil fungi.

3.4.1 Seasonal and latitudinal trends in drought-affected populations

Complementary to the throughfall exclusion experiment, we profiled changes in the soil microbiome of species-specific and mixed-species root zones across seasonal and latitudinal gradients in soil moisture and precipitation, respectively (Figure 1A). A random forest, decision-

tree-based feature selection was used to identify the environmental variables and microbial taxa that were most predictive of soil water content in these gradient (Table 1). Several bacterial orders were selected as predictors of SWC, and the abundance patterns of Rhizobiales outranked all other features in importance, including environmental parameters (Table 1). No fungal taxa were selected as predictors of SWC. The abundance patterns of the top microbiome-based predictors of SWC closely tracked seasonal and latitudinal differences in precipitation in upper (Figure 6) and lower soil layers (Figure S13) and were among the same groups impacted by throughfall exclusion. Among the OTUs identified as drought-affected in data from the throughfall exclusion experiment, the number that significantly differed among high and low precipitation sites in the latitudinal gradient was lowest in spring (n = 92), when SWC was highest, and progressively increased in summer (n = 133) and fall (n = 220).

EMF did not exhibit a clear relationship to SWC as their relative abundance did not vary significantly by season or site position in the precipitation gradient. The exception was at Arnstein during the summer, when increased relative abundance of EMF coincided with some of the lowest SWC values measured (Figure S2B). The EMF taxa that increased in Arnstein during the summer included taxa from the genera: *Amphinema*, *Clavulina*, *Otidea*, *Sebacina*, *Tricholoma*, *Inocybe*, and *Lactarius*.

3.4.2 Moisture related shifts in rhizobacterial abundance between soil layers

Reductions in soil moisture can cause stratification of roots in spruce, to upper soil layers, and beech, to lower soil layers, according to observed root behavior (Bolte and Villanueva, 2006). We found that the relative abundance of spruce- and beech-associated rhizobacteria tended to differ between upper and lower soil layers correspondingly, particularly at Kranzberg (Figure 7). The enrichment of spruce-associated rhizobacteria in upper layer soils was even more pronounced during fall, when SWC was lowest, though this interaction was only significant at Kranzberg and

Wasserburg (Figure 7). In contrast, beech-associated rhizobacteria were more prevalent in the lower soil layer, and no shift was seen due to throughfall exclusion or season. Additionally, at Kranzberg, the seasonal shift to the upper soil layer was most pronounced in mixed root zones ($t_{interact.}$ = 8.1; $p < 0.001$) compared to species-specific spruce (t = 3.9) or beech root zones (t = 3.6). No shifts in spruce- and beech-associated fungi were evident, though we did not obtain fungal sequence data from rhizoplane / endophyte samples.

4. Discussion

Our study reveals major differences in the response of Norway spruce and European beech soil microbiomes to changes in soil moisture and their interplay in mixed root zones. A significantly higher proportion of beech-associated taxa were sensitive to reductions in soil moisture compared to the more drought-tolerant populations associated with spruce. Furthermore, several populations of rhizobacteria and soil bacteria had increased resistance or susceptibility to drought in mixed-species root zones relative to species-specific. These findings lead us to reject the hypothesis that the response of microbial populations to changes in moisture would not differ in mixed-species root zones, allowing for the possibility that complementation can occur. Here, we discuss the possible reasons why drought may impact spruce- and beech-associated soil microbiomes differently, and the potential consequences of the phenomena observed in mixed-species root zones.

4.1 Explaining the contrasting effects of reduced soil moisture on beech and spruce microbiomes

Taxa that were consistently impacted by experimental or natural reductions in soil moisture were broadly characteristic of the spruce and beech microbiomes described in prior research (Asplund et al., 2019; Bárta et al., 2017; Felsmann et al., 2015; Nacke et al., 2016; Uroz et al., 2016). Several of these broad taxonomic groups were previously shown to be impacted by reduced throughfall in beech and spruce forest (Felsmann et al., 2015), and other forest types (Bastida et

al., 2017; Bouskill et al., 2013). Our results demonstrate that soil bacterial communities in species-specific spruce root zones have a higher resistance to the impacts of drought and to seasonal reductions in soil moisture than in beech root zones (Figure 4; Figure S10). This difference corresponded with a higher proportion of drought-tolerant bacteria in the spruce microbiome than in beech, and higher proportions of drought-sensitive bacteria in the beech microbiome. While this is the first time such broad differences in moisture sensitivity have been reported, prior research has shown that the mineral weathering activity of rhizobacteria isolated from beech was higher during wetter periods while the reverse was true for those isolate from spruce (Collignon et al., 2011).

Prevailing differences in soil conditions between spruce and beech stands likely help explain the disparity in drought tolerance and sensitivity in their respective soil microbiomes. Physiological differences in rooting depth result in consistently drier conditions in shallow soils in spruce stands relative to beach (Allen et al., 2019; Zwetsloot and Bauerle, 2021). Our findings indicate that this effect is large enough to select for higher proportion of drought stress-tolerant bacteria in spruce soils. Additionally, soil acidification was more pronounced in spruce root zones relative to beech (Figure S1), as indicated by the high relative abundance of acidophilic indicator taxa (Acetobacterales, Frankiales and Acidobacteriales), as previously shown (Sridhar et al., 2022a). Our observations were consistent with current understanding about difference in soil development under beech and spruce stands caused by root and litter chemistry, nutrient leaching and uptake, and mineralization and nitrification rates (Cremer and Prietzel, 2017). Spruce roots and litter contain significantly higher concentrations of polyphenols than beech (Kuiters and Denneman, 1987; Zwetsloot and Bauerle, 2018), which are generally toxic to soil heterotrophs (Adamczyk et al., 2013; Chunmei et al., 2010; Inderjit et al., 2009; Metsämuuronen and Sirén,

2019). The high acidity and polyphenol content of spruce soils create adverse growing conditions, which retard decomposition (Albers et al., 2004; Berger et al., 2004) and reduce soil respiration and biomass in spruce stands relative to mixed-species and beech stands (Borken et al., 2002; Borken and Beese, 2005; Lu and Scheu, 2021). These adverse conditions, along with consistently lower shallow soil moisture levels in spruce stands, may select for stress-tolerant populations, which may better endure water stress. This theory is supported by the overlapping physiological stress response to acidity, low osmolarity, and desiccation observed in model bacteria (Ait-Ouazzou et al., 2012; Hengge-Aronis, 2002; Spector and Kenyon, 2012). It is also anecdotally supported by the enrichment of the thermophilic, acid-tolerant genus *Acidothermus* by throughfall exclusion in spruce and mixed root zones (Figure S14), which are characteristic of droughted and arid soils (Eppard et al., 1996; Lacerda-Júnior et al., 2019).

The prevalence of drought-sensitive taxa in beech-specific soils could also reflect differences in the degree of rhizosphere activity between beech and spruce. Beech produce more fine root mass (Finér et al., 2007), apparent at all our sites ($\bar{x}$ = 1.4 to 2.1-fold higher), and sustain more microbial biomass and higher soil respiration rates than spruce (Borken et al., 2002; Borken and Beese, 2005; Lu and Scheu, 2021). Thus, the apparent sensitivity of beech-associated taxa may reflect the inability of beech roots to sustain basal levels of microbial activity during drought. The potential diminished influence of beech on the soil microbiome during drought was evident in the decline of microbial populations indicative of higher trophic complexity, including members of the Vermiphilaceae, endosymbionts of amoeba (Delafont et al., 2015), and Candidatus *Xiphinematobacter*, symbionts of nematode (Rius et al., 2021; Vandekerckhove et al., 2000). While we cannot disentangle the relative contributions of roots versus litter to differences in the soil microbiomes between spruce and beech, root traits have a far greater importance in explaining

the physicochemical properties of species-specific and mixed forest soils than litter traits (Cesarz et al., 2013; Gillespie et al., 2021).

4.2 Effects of root mixing on the impacts of reduced soil moisture

Most beech- and spruce-associated fungi or bacteria occurred at an intermediate relative abundance in mixed root zones. This result indicates that the influence of each tree follows a gradient and that taxa associated with either tree species can co-occur. These observations are consistent with prior reports of intermediate heterotrophic activity (Borken et al., 2002; Borken and Beese, 2005), microbial biomass (Lu and Scheu, 2021), litter decomposition rates (Albers et al., 2004), and mineral weathering (Cremer and Prietzel, 2017) in mixed root zones. Thus, the hybrid soil microbiome in mixed root soils reflects the combined, but weakened, influences of each tree.

The resistance of several beech and spruce-associated bacteria to throughfall exclusion was enhanced in mixed root zones (Figure 5AB). These populations included members of Acidobacteria (*Bryobacter* and *Occallatibacter*, both spruce-associated rhizobacteria), which are reputed for their production of extracellular polymeric substances, which confer stress tolerance to cells and may influence soil moisture dynamics (Foesel et al., 2016; Kielak et al., 2016; Kulichevskaya et al., 2010). In contrast, several members of Xanthobacteraceae (*Bradyrhizobium* and *Rhodoplanes*) were more susceptible to throughfall exclusion in mixed root zones (Figure 5C). Xanthobacteraceae are known to increase in abundance in proximity to beech trunks (Nacke et al., 2016), suggesting that their capacity to resist drought may depend on the density of beech roots or litter. Conversely, several spruce-associated taxa exhibited enhanced resistance to drought exclusively in mixed roots zones (Figure 5D), suggesting some populations may benefit from the reduced competition where drought-sensitive, beech-associated taxa are diminished. Our explanations for trends in mixed root zones remain to be tested, but these observations illustrate

the range of interactions occurring where the influence of trees and, by extension, their microbiomes mix.

4.3 Effects of reduced soil moisture on root-associated taxa

The rhizosphere activity of spruce and beech varies by season and moisture availability, with the highest activity in spring (Calvaruso et al., 2014) and diminished photosynthate-derived microbial rhizosphere biomass during drought (Ruehr et al., 2009). We hypothesized that reductions in soil moisture would disproportionately impact root-associated taxa, like EMF, which are important contributors to nutrient acquisition by spruce and beech (Brzostek et al., 2013; Calvaruso et al., 2014; Collignon et al., 2011; Gan et al., 2021; Nicolitch et al., 2016). Contrary to expectations, we did not observe any compositional changes in soil EMF populations from throughfall exclusion, seasonal variation, or across the precipitation gradient. The lack of shift in EMF populations is not without precedent in spruce and beech forests (Gorfer et al., 2021; Nickel et al., 2018; Žifčáková et al., 2015). The apparent resiliency of EMF to changes in community composition may reflect their direct connection to live roots and a privileged access to root exudates relative to bacteria, consistent with the more severe impacts in bacterial populations we observed and reported elsewhere (de Vries et al., 2018; Fuchslueger et al., 2014). Access to exudates and shelter within the root might explain why endophyte populations (*Phialocephala*) were among the few fungi favored by throughfall exclusion (Figure S3).

Rhizobacteria were among the most affected by throughfall exclusion and seasonal and latitudinal variation in soil moisture. Members of the Rhizobiales and Burkholderiaceae were greatly diminished by throughfall exclusion in both beech and spruce root zones (Figure 6; Figure S15). These drought-sensitive populations were dominated by members of *Bradyrhizobium* and *Rhodoplanes* (Xanthobacteraceae) and *Caballeronia* and *Paraburkholderia* (Burkholderiaceae) which are consistently associated with the rhizosphere of European beech and Norway spruce

(Colin et al., 2017; Uroz et al., 2016) and are common root- and mycorrhizae-associated bacteria isolated from forest soils (Burke et al., 2008; Izumi et al., 2007, 2006; Kataoka et al., 2008; Tanaka and Nara, 2009; Uroz et al., 2012; Wilhelm et al., 2020). The apparent drought sensitivity of these rhizobacteria has implications for soil nutrient cycling and plant nutrition, given their involvement in priming decomposition (Wilhelm et al., 2021; Zwetsloot et al., 2020), phosphate solubilization in the beech and spruce rhizosphere (Lepleux et al., 2012; Nicolitch et al., 2016), and endophytic nitrogen fixation (Puri et al., 2020).

5. Conclusions

We conclude that any potential complementation of soil microbiome function during drought is likely to confer greater benefits to beech than spruce, given the higher proportion of drought-favored spruce-associated taxa and their sustained resistance in mixed root zones. The enhanced drought tolerance of beech in mixed stands with spruce has been reported (Schäfer et al., 2017), though the benefits of mixing are more commonly reported to favor Norway spruce (del Río et al., 2014; Ding et al., 2017; Rötzer et al., 2017). Thus, it remains to be seen what functions the complementation of soil microbiomes might have in the drought resistance of mixed beech-spruce forests. The impact of reduced soil moisture was greater on rhizobacteria than EMF populations, with a higher proportion of EMF occurring in the beech soil microbiome. Thus, the likeliest form of complementarity in mixed-root zones might correspond with the enrichment of drought-tolerant populations by spruce and the sustenance of EMF activity by beech.

Forests and their soil microbiome are complex adaptive systems in which legacy and context shape biological responses to water limitation (Bouskill et al., 2013). Yet, the moisture sensitivity of major drought-affected populations, such as members of the Rhizobiales, were consistent across sites and season, providing evidence for the potential widespread occurrence of phenomena reported here. Future research is needed to understand the ecological and environmental drivers of

differences across sites and to test the functional consequences of complementation between microbiomes of beech and spruce, and other abundant tree species, in mixed root zones.

6. Acknowledgements

We thank Tommi Schieder at Cornell University for assisting with DNA extractions, and Laura Pohlenz together with the class of 2017/18 Ecology Gap Year Volunteers at Helmholtz Center Munich for assisting in soil and root sample processing. This work was supported by the German Research Foundation (grant DFG PR555/2-1) and by the U.S. Department of Energy, Office of Biological & Environmental Research Genomic Science Program under award number DE-SC0016364. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the United States Department of Energy.

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Data Availability Statement

All sample metadata is provided in Table S5. All analyses can be reproduced with scripts included in the Supplementary Data package available through the Open Science Foundation (doi: 10.17605/OSF.IO/DN9CH). Raw sequencing data was archived with the European Nucleotide Archive under the BioProject accession: PRJEB36981.

Author Contributions

RCW performed the data analysis, research, and writing. JMU contributed to data analysis and research. Field sampling was performed by JMU, TLB, MG, FW, and KP and sample processing (soil parameters, root biomass) by JMU and FW. Amplicon sequencing libraries were prepared by RCW, JMU, and FW. TLB, KP, and DHB guided all research efforts, including analyses and writing. The authors declare we have no competing or conflicts of interest.

Tables

Table 1. The top environmental and soil microbiome predictors of soil water content were identified, and ranked, using Boruta random forest-based feature selection. The relative abundance pattern of aggregated counts of Rhizobiales was ranked as the best predictor of SWC, followed by soil layer and precipitation gradient site. Fungal and bacterial count data, aggregated by Order, were included in feature selection, but only bacteria had predictive value. Bacterial orders that had significant Pearson's correlations with SWC content in both layers ($r > |0.2|$ and $p_{adj} < 0.05$) and were among the top 20 most important features are displayed (full list in Table S10). The family Gimesiaceae is historically known as 'Planctomycetaceae.'

Figures

Figure 1. A schematic overview of the experimental design used to examine the effects of natural and experimental variation in soil moisture on the soil microbiome of species-specific and mixed-species stands of European beech and Norway spruce. In (A), samples were collected in spring, summer, and fall and at sites spread across a natural precipitation in Bavaria, Germany. In (B), at Kranzberg, the mid-point of the gradient, samples were taken from a five-year old throughfall exclusion experiment where rain-out roofs had been used to reduce soil moisture during the growing season. Each symbol accurately reflects the distribution of trees species (full details in Rötzer *et al.* 2017). In (C), at all sites, and in the throughfall exclusion plots at KROOF, samples were taken from species-specific or mixed-species tree root zones. Ten soil cores (30 cm deep) were randomly sampled from five to six plots for each tree root zone at each site and during every season and subdivided into upper and lower soil layers before being composited.

Figure 2. The composition of soil bacterial and fungal communities at Kranzberg differed primarily by soil layer and tree root zone, as evident in (A) the grouping of samples by principal coordinates analysis and (B) the proportion of variance explained (R^2) in a PERMANOVA analysis based on the Bray-Curtis dissimilarity in community composition.

Figure 3. Beech and spruce root zones had marked differences in the taxonomic composition of soil bacteria and fungi and rhizobacteria. In (A), the bar plots provide a summary of the relative proportions of indicator OTUs for beech- and spruce-associated bacteria (n = 506) and fungi (n = 63) according to the ratio of their aggregated relative abundance at rank Order. The subset of tree-associated taxa affected by drought are labeled on the y-axis. In (B), the taxonomic profile or rhizobacteria associated with either beech or spruce according to indicator analysis using 16S rRNA gene amplicon data generated from root material.

Figure 4. The resistance (R) of soil bacterial communities to the effects of throughfall exclusion in soil moisture was greatest in spruce root zones at Kranzberg. Differences in R among root zones (B) corresponded with the relative abundance of drought-favored and drought-sensitive populations. Pairwise differences in resistance among tree root zones sites were tested using TukeyHSD ($p_{adj} < 0.05$). In (B), the effects of throughfall exclusion ($p_{excl.}$) were significant for all groups. Any significant differences between tree root zone were denoted by bars with asterisk and interactions between throughfall treatment and tree root zone were denoted by asterisk.

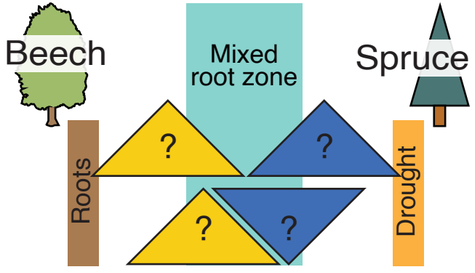
Figure 5. The responses of beech- and spruce-associated OTUs to throughfall exclusion were significantly altered in soil microbiome of mixed root zones at Kranzberg. In (A), the relative abundance of several drought-sensitive beech-associated OTUs (n = 10) were largely undiminished in mixed root zones. In (B), several drought-sensitive spruce-associated OTUs (n = 5) had enhanced resistance to throughfall exclusion in mixed root zones. Several OTUs that were not significantly affected by throughfall exclusion in species-specific root zones showed, in (C), an increased susceptibility (n = 8) or, in (D), an increased resilience (n = 4) in mixed zones. These trends were consistent with trends in relative abundances between spring (wettest) and fall (driest season) at Kranzberg (lower panel of C and D). Individual OTU exhibited significant interactions between throughfall exclusion and tree root zone (Table S8 and Table S9) but were displayed in aggregate. Any significant differences between tree root zone were denoted by bars with asterisk and interactions between throughfall treatment (or season) and tree root zone were denoted by asterisk.

Figure 6. The relative abundance of four major bacterial orders followed changes in soil water content caused by throughfall exclusion plots, seasonal variation, and across the latitudinal precipitation gradient. In (A), the soil water content in the upper soil layer was reduced by throughfall exclusion (box plot), and across seasons (x-axis) and gradient sites (lines). In (B), the relative abundances of the four bacterial Orders identified in feature selection as predictive of SWC (Table 1). The same trends were evident in lower layer soils, though less pronounced (Figure S13). Significant differences between mean SWC or relative abundance between seasons were denoted by bars with asterisks. Significant interactions between season and site were denoted by colored asterisks. Lettering denotes significant differences among sites according to TukeyHSD ($p_{adj} < 0.05$).

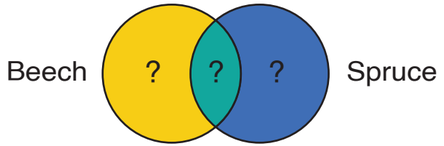
Figure 7. Shifts in the relative abundance of rhizobacteria in response to (A) throughfall exclusion or (B) seasonal differences between spring and fall revealed the putative partitioning of roots between soil layers. Spruce-associated rhizobacteria (upper panel) tended to occur at higher proportions in the upper soil layer, while beech-associated rhizobacteria (lower panel) tended to be more abundant in the lower layer. Significant differences between soil layer means were denoted by bars with asterisk and interactions between throughfall treatment (or season) and soil layer were denoted by asterisk.

Graphical Abstract

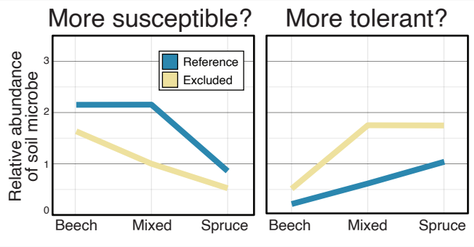
Research questions



Will complementation in the drought response of microbiomes occur?



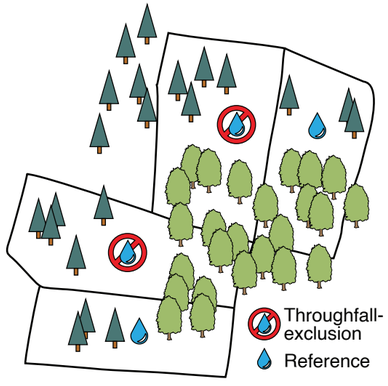
Do tree-associated microbes overlap?

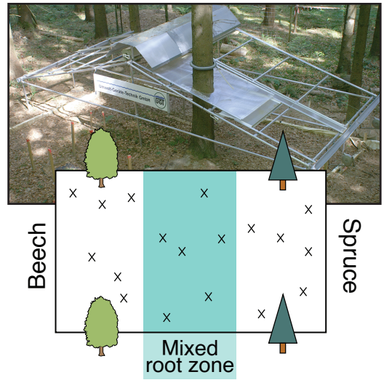


Does the drought response of soil microbes differ in mixed root zones?

Experimental design

Dataset 1. Throughfall exclusion experiment





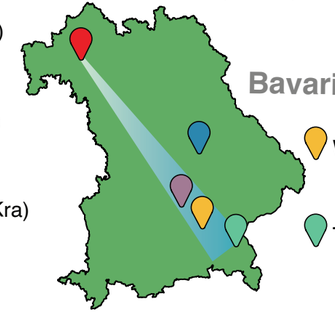
Dataset 2. Latitudinal precipitation gradient

(same plot design as dataset 1)

Arnstein (Arn)
MAP: 654 mm
MAT: 9.5 °C

Kelheim (Kel)
MAP: 713 mm
MAT: 8.5 °C

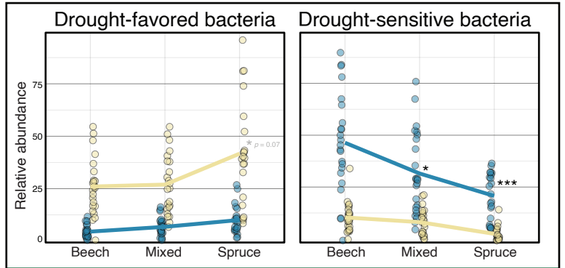
Kranzberg (Kra)
MAP: 854 mm
MAT: 8.2 °C



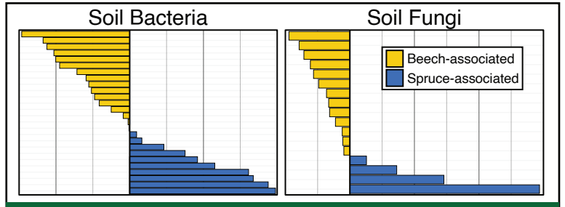
Wasserburg (Was)
MAP: 858 mm
MAT: 8.8 °C

Traunstein (Tra)
MAP: 962 mm
MAT: 9.1 °C

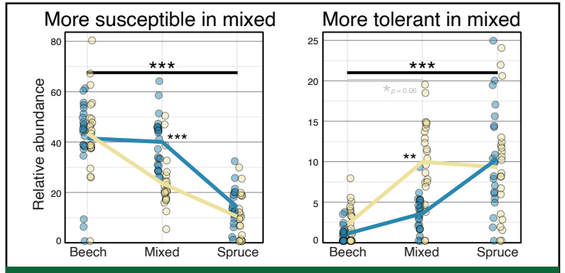
Results



The propotion of drought-tolerant vs. sensitive bacteria differed by tree species



Bacteria and fungi differed by tree species



Complementation in mixed zones can occur

Highlights

- Long-term reduction in moisture had little impact on mycorrhizal population structure
- More drought-sensitive bacteria were present in beech relative to spruce root zones
- Mixed-species root zones were a hybrid of beech- and spruce-associated microbiomes
- Several bacterial populations were more resistant to drought in mixed root zones
- Complementation in the drought resistance of tree-associated microbiomes can occur

The effects of mixed-species root zones on the resistance of soil bacteria and fungi to long-term experimental and natural reductions in soil moisture

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Running title: Drought-microbiome effects in mixed rooting zones

Abstract

Mixed forest stands tend to be more resistant to drought than species-specific stands partially due to complementarity in root ecology and physiology. We asked whether complementary differences in the drought resistance of soil microbiomes might contribute to this phenomenon. We experimented on the effects of reduced soil moisture on bacterial and fungal community composition in species-specific (single species) and mixed-species root zones of Norway spruce and European beech forests in a 5-year-old throughfall-exclusion experiment and across seasonal (spring-summer-fall) and latitudinal moisture gradients. Bacteria were most responsive to changes in soil moisture, especially members of Rhizobiales, while fungi were largely unaffected, including ectomycorrhizal fungi (EMF). Community resistance was higher in spruce relative to beech root zones, corresponding with the proportions of drought-favored (more in spruce) and drought-sensitive bacterial taxa (more in beech). The spruce soil microbiome also exhibited greater resistance to seasonal changes between spring (wettest) and fall (driest). Mixed-species root zones contained a hybrid of beech- and spruce-associated microbiomes. Several bacterial populations exhibited either enhanced resistance or greater susceptibility to drought in mixed root zones. Overall, patterns in the relative abundances of soil bacteria closely tracked moisture in seasonal and latitudinal precipitation gradients and were more predictive of soil water content than other environmental variables. We conclude that complementary differences in the drought resistance of soil microbiomes can occur and the likeliest form of complementarity in mixed-root zones coincides with the enrichment of drought-tolerant bacteria associated with spruce and the sustenance of EMF by beech.

Key words: plant–soil interactions**,** forest soil microbiome, drought resistance, precipitation gradient, beech-spruce forest, and root complementarity.

1. Introduction

Plant species diversity positively correlates with ecosystem productivity (Hooper and Vitousek, 1997; Liang et al., 2016; Tilman, 2001) and with increased resistance to extremes in water availability, at least in grasslands (Craven et al., 2016; Isbell et al., 2015). Forest ecosystems are vulnerable to the increasing frequency, intensity, and duration of drought caused by changing precipitation patterns (Dai, 2013; IPCC, 2018). However, it remains to be proven whether forests with higher plant diversity or functional richness are more resistant to drought than single species ('species-specific') plantations (García-Valdés et al., 2021). The general relationship between plant species diversity and productivity is, at least, partially due to the effects of biotic feedbacks between plants and soil microorganisms (Hendriks et al., 2013; Schnitzer et al., 2011). Yet, to date, research into diversity-productivity relationships in forests has been primarily focused on aboveground parameters, e.g. annual growth (Paquette and Messier, 2011; Pretzsch et al., 2020, 2010). More recently, belowground parameters were found to differ between species-specific and mixed-species forest stands, including tree root lifespan dynamics (Zwetsloot et al., 2019) and root niche partitioning during drought (Altinalmazis-Kondylis et al., 2021; Zwetsloot and Bauerle, 2021). These observations raise questions about the potential influence of other belowground biotic interactions on the drought resistance of mixed forest stands.

Belowground interactions between root systems of European beech (henceforth 'beech') and Norway spruce (henceforth 'spruce'), a common forest type in Central Europe, can differ between monoculture and mixed-species forests (Schmid and Kazda, 2002). Spruce and beech exhibit anatomical (tracheid vs. xylem vessel elements) and physiological (isohydric vs. anisohydric) differences that effect their hydrological processes and drought response (Lyr et al., 1992; Pretzsch et al., 2013) and their root systems can interact in complementary ways that reduce competition and facilitate access to soil moisture. These effects are evident in the differences

between species-specific and mixed root zones, where beech roots exhibit lower mortality than in monospecific root zones, due to decreased intraspecific competition (Zwetsloot et al., 2019), and where spruce roots maintain higher fine root mass during chronic drought (Zwetsloot and Bauerle, 2021). This complementarity is due to several aspects of root ecophysiology, which include: (i) differences in rooting depth, with beech colonizing lower soil layers in mixed root zones (Bolte and Villanueva, 2006), and (ii) diverging responses in fine root growth during drought, where spruce become more dormant (cease growth and enhance suberization), while beech maintain a degree of root activity, producing new, albeit shorter lived, fine root mass (Nikolova et al., 2020; Rötzer et al., 2017). Beech and spruce also select for broadly different soil microbiomes (Bárta et al., 2017; Uroz et al., 2016). Complementarity in the function and drought-tolerance between their associated microbiomes might contribute to the altered plant drought responses where roots mix.

Root-associated microbes support the nutritional needs of trees (Brzostek et al., 2013; Calvaruso et al., 2014; Gan et al., 2021; Nicolitch et al., 2016), and provide protection from phytopathogens (Lehr et al., 2008; Terhonen et al., 2016) and the effects of mild drought (Pena et al., 2013; Shi et al., 2002). Prior research has shown that the soil microbiome in mixed-species forests can have greater functional and metabolic diversity than species-specific forests (Beugnon et al., 2021; Prada-Salcedo et al., 2021). Thus, complementarity in the function and drought-tolerance of soil microbiomes associated with beech and spruce might confer benefits during drought. Several observations indicate the potential unevenness in the susceptibility or tolerance of members of the beech- and spruce-associated microbiomes. The spruce soil microbiome is characterized by a higher relative abundance of Actinobacteria, Armatimonadetes, and Acidobacteria (and a higher denitrification potential) and greater proportion of ectomycorrhizal fungi (EMF) relative to saprotrophic fungi than the beech soil microbiome, which has higher

proportions of Alphaproteobacteria, Planctomycetes, and Verrucomicrobia and different dominant EMF taxa (*Scleroderma*, *Russula* and *Laccaria*) than spruce (*Boletus* and *Thelephora*) (Bárta et al., 2017; Felsmann et al., 2015; Nacke et al., 2016; Uroz et al., 2016). Populations of Alphaproteobacteria and Planctomycetes (beech-associated) are generally more sensitive to drought, while Armatimonadetes and Actinobacteria (spruce-associated) are more tolerant (Bastida et al., 2017; Bouskill et al., 2013; Chodak et al., 2015; Curiel Yuste et al., 2014; Evans et al., 2014; Felsmann et al., 2015; Hartmann et al., 2017; Preece et al., 2019; Zeng et al., 2016; Zhou et al., 2018). EMF also differ in their drought tolerance with more sensitive (ex. within *Laccaria* and *Lactarius*) and more tolerant (ex. within *Russula*, *Cenococcum*, and *Scleroderma*) species among those that associate with beech and spruce (Coleman et al., 1989; Di Pietro et al., 2007; Ortega et al., 2004). Disparities in the drought tolerance or susceptibility of beech- and spruce-associated microbiomes have yet to be experimentally tested.

With our study, we investigated whether the effects of experimental and natural reductions in soil moisture on the structure of beech and spruce microbiomes differed in species-specific (single species) versus mixed-species root zones. Drought-affected bacteria and fungi were identified in a 5-year-old throughfall exclusion experiment and changes in these populations were profiled in seasonal and latitudinal gradients in soil moisture and precipitation, respectively (Figure 1). The composition of bacterial and fungal communities and root-associated 'rhizobacteria' (rhizoplane and root endophytes) were determined with 16S rRNA gene and ITS region amplicon sequencing. We expected EMF and rhizobacterial populations to be most sensitive to reductions in soil moisture given the associated reduction in fine root mass (Zwetsloot and Bauerle, 2021). We hypothesized that the impact of reduced soil moisture would affect beech and spruce microbiomes differently, and that these responses would be further altered in soils where roots

mixed. Specifically, our experiment tested the null hypothesis that the responses of beech- and spruce-associated bacteria and fungi would not differ in mixed-species root zones relative to species-specific zones. We then tested whether similar differences occurred in response to seasonal variation and latitudinal differences in soil moisture. Our experiment advances an understanding of the effects of drought and natural gradients in soil moisture on beech and spruce soil microbiomes and establishes the potential for complementation to occur where root systems mix.

2. Methods

2.1 Study overview and experimental design

In 2013, a latitudinal precipitation gradient was established in Bavaria, Germany (Pretzsch et al., 2014). The gradient is composed of five forest sites containing 60 to 90-year-old Norway spruce (*Picea abies* [L.] Karst) and European beech (*Fagus sylvatica* L.) which are (from driest to wettest): Arnstein, Kelheim, Kranzberg, Wasserburg, and Traunstein (Figure 1A). All five sites are similar in mean annual temperature (MAT) and soil type (Cambisol and Luvisol), but differ in their mean annual precipitation (MAP; Table S1). Relative differences in total annual precipitation were consistent among sites during the duration of the experiment (Table S2). However, periods of drought occurred throughout Germany in summer in 2015 and 2018 (Schuldt et al., 2020). Individual sites consist of a species-specific spruce and beech root zone, as well as a mixed spruce and beech root zone with varying degrees of mixing (Table S3). A throughfall-exclusion experiment was established at the middle site of the precipitation gradient, (Kranzberg Forest Roof Experiment or 'KROOF'; Figure 1B) and is composed of six drought plots, each with a species-specific beech and spruce and mixed-species stand covered by a throughfall exclusion roof, and six corresponding uncovered reference plots, with corresponding stand compositions. At the time of the last sampling in 2018, seasonal throughfall had been excluded for five years, resulting in an overall reduction of ~70% soil moisture across time and significantly greater pre-dawn water stress

for beech ($\bar{x}$ = -0.66 ± 0.2 MPa water potential) and spruce ($\bar{x}$ = -0.98 ± 0.3 MPa) compared to reference plots (Grams et al., 2021).

2.2 Soil and root sampling

Soils from each root zone were sampled during the fall of 2017 (November), and the spring (April), summer (July), and fall of 2018 (October) at all five sites and from the throughfall exclusion experiment. During each sampling, ten soil cores (1.6 cm in diameter and 30 cm long) were harvested from five plots (approximately 10 m^2) randomly located within in each tree root zone and/or throughfall exclusion treatment plot. The O_l (litter layer) was manually removed, and each soil core was divided into upper (0-8 cm deep) and lower soil layers (9-30 cm) comprised of the $O_f + {}_hA_h$ and A_lB_v, respectively (KA5 classifications; Nickel et al., 2018). The upper and lower layers of ten cores were separately composited to yield 5 replicates per tree root zone per site and/or per throughfall exclusion treatment. Five grams of the upper and lower composite sample (excluding roots and soil particles > 2 mm) was weighed into Whirl-pak® bags in the field (Nasco Sampling, Madison, WI, USA), and transported on dry ice to the Technische Universität München (TUM) campus in Freising, where they were dried at 60 ºC to a constant weight. Oven drying was used to minimize the period in which cells are active and has negligible impact on bacterial and fungal community composition (Castaño et al., 2016; Tzeneva et al., 2009). Dried samples were shipped to Cornell University (Ithaca, NY, USA) and stored at room temperature until DNA was extracted within two months. Over short periods, changes in microbiome composition due to air drying and storage are minor (Clark and Hirsch, 2008; Lauber et al., 2010; Tzeneva et al., 2009). The remaining soil samples were stored at TUM at 4 ºC for additional analyses and to determine fine root biomass (mg) ($\leq$ 1 mm diameter), also dried to 60 ºC to a constant weight. Prior to the onset of KROOF, in 2013, fine root samples were taken from upper and lower layers in species-specific spruce and beech root zones for the purpose of identifying root-associated bacteria

('rhizobacteria') associated with each tree (n = 20), as opposed to 'soil bacteria,' which we use to refer to the amplicon data generated from whole soil cores. Roots were thoroughly cleaned in tap water, identified under a stereomicroscope and frozen until DNA was extracted. The bacterial populations identified via this method correspond to a combination of 'rhizoplane' and root endophyte populations.

2.3 Analyses of soil properties

The remaining soil from each sample was used to measure soil water content (SWC), pH, and soil organic matter (SOM). SWC was averaged from triplicate measurements using 3 g of fresh soil, which was calculated as the percentage mass lost after drying for 24 hours at 105℃. Soil pH was measured using a VWR Scientific Products model 2000 pH meter, in a 1:10 (w/v) dilution with distilled deionized water, after the sample was shaken for one minute and allowed to settle for 10 minutes. SOM was estimated using loss on ignition method, according to (Howard and Howard, 1990), by weighing 2 g of soil onto clay trays that were put in a muffle furnace where temperature was slowly increased by 10 ºC increments until a final temperature of 550 ºC was reached and maintained for 3 h.

2.4 Analyses of soil bacterial and fungal communities and rhizobacteria

DNA was extracted from 0.25 g of soil sample using the Qiagen PowerSoil kits (Germantown, Maryland, USA) according to the manufacturer's protocol with a bead beating treatment of 3 min at 5.5 $m \cdot s^{-1}$ (Bio-spec, Bartellsville, OK, USA). DNA quantification and PCR amplification of bacterial (V4 region of 16S rRNA gene; 515f/806r) and fungal phylogenetic gene markers (ITS1; nBITS2f/58A2r) was performed as previously described (Sridhar et al., 2022). Duplicate PCR reactions per sample were pooled prior to Illumina MiSeq (2 x 250 bp; v2) sequencing, which was performed at Cornell Biotechnology Resource Center (Ithaca, NY) using dual-indexed bar-coded primers (Koechli et al., 2019; Kozich et al., 2013). Seventy-five ITS

sequencing libraries from the Kranzberg throughfall-exclusion experiment were discarded due to poor sequencing quality. These were discarded at random without impacting the balance of our experimental design (Table S4). To identify rhizobacteria, DNA was extracted from 0.35-0.45 g of root using the PowerSoil kit as previously described (Nickel et al., 2018) and 16S rRNA amplicon libraries were prepared targeting rhizobacteria using the same methods (details in SI) except that the V3-V4 region was targeted using primers optimized for plant DNA-rich samples (335f/769r), as previously described (Dorn-In et al., 2015). All sample metadata is provided in Table S5, and raw sequencing data was archived with the European Nucleotide Archive under the BioProject accession: PRJEB36981 (data reference, see Wilhelm et al., 2022).

Sequencing data was processed using QIIME2 (v. 2020.2; Bolyen et al., 2019) with a dependency on DADA2 (v. 1.10; Callahan et al., 2016) to assign sequences to operational taxonomic units (i.e., amplicon sequence variants). Taxonomic classification was performed using the QIIME2 'q2-feature-classifier' trained on the Silva database (v. 132; Quast et al., 2013) and UNITE database (v. 7.2; Nilsson et al., 2019) for bacteria and fungi, respectively. OTUs found in the non-template controls and in low abundance were removed, namely those present in fewer than three samples, or at a total relative abundance < 0.01%. All counts were normalized by proportion of total reads and presented as counts per thousand reads. R package *phyloseq* (v. 1.34; McMurdie and Holmes, 2013) was used to characterize the communities and estimate diversity parameters on rarified libraries (n_{bact} = 15,300 and n_{fungi} = 2,544 reads per sample). Fungal taxonomic classifications were used to identify EMF, endophytic, and saprotrophic fungi using the FUNGuild database (v. 1; Nguyen et al., 2016). Samples from throughfall-exclusion plots were removed during all analyses of seasonal or latitudinal effects.

2.5 Bioinformatic and statistical analyses

The differential abundance of OTUs between reference and throughfall exclusion plots (i.e., 'drought-affected') were identified using the *R* software package *indicspecies* (v. 1.7.9; Cáceres and Legendre, 2009). Indicator species analysis was performed independently for soil layers, season, and forest type, then combined and de-duplicated. Non-significant indicator OTUs ($p_{adj} < 0.05$) and those with low indicator values (< 0.35) were excluded. Alpha-diversity was measured as species richness, Shannon diversity, and Pielou's evenness. Beta-diversity was assessed using Bray-Curtis dissimilarity and differences in community composition were visualized using principal coordinates analysis and tested with PERMANOVA ($n_{perm} = 999$) using 'adonis' from the *R* package *vegan* (v. 2.5.7; Oksanen et al., 2015). Resistance (R) to change in microbiome composition due to throughfall exclusion was measured as the Bray-Curtis dissimilarity between reference and exclusion plots (D), such that R = 1 – D. A lower R value indicates a greater dissimilarity between reference and throughfall exclusion plots, indicating a greater drought effect (De Vries and Shade, 2013). R was calculated for the average of all permutations of replicates between reference and exclusion plots. The environmental and microbiome features that were most predictive of SWC were identified using random forest-based feature selection implemented in the *R* package *Boruta* (v. 7.0; Kursa and Rudnicki, 2010). Features included in model selection were environmental (soil layer, root zone, site, season, year) and microbiome (aggregated counts at the taxonomic rank of Order scaled with the 'scale' function in *R*). Subsequently, Pearson's correlations between selected bacterial and fungal features and SWC were performed using 'rcorr' from the *R* package *Hmisc* (v. 4.5; Harrell and Dupont, 2015). Differences in the relative abundance of OTUs among tree root zones were tested with ANOVA ('aov' function in *R*). The main effects and interactions between throughfall exclusion, season, or tree root zone on the relative abundances of OTUs, were determined by fitting to fixed effects

linear models ('lm' function). P-values were adjusted for multiple test correction according to the Benjamini and Hochberg false discovery rate. Only the most abundant OTUs (> 0.05% of sample reads) were included in statistical testing. Significant effects are denoted by asterisk: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All analyses can be reproduced with scripts included in the Supplementary Data package available through the Open Science Foundation (doi: 10.17605/OSF.IO/DN9CH).

3. Results

Our initial analyses were performed to establish the drought susceptible and tolerant (section 3.1) and spruce- and beech-associated soil microbiome and rhizobacteria (section 3.2) prior to testing whether the response of these groups differed in species-specific versus mixed root zones (section 3.3). Additional analyses were performed to characterize the natural variation (seasonal and precipitation gradient) in drought-affected populations among tree root zones and soil layers (section 3.4).

3.1.1 General effects of throughfall exclusion on soil properties and the soil microbiome

Across the full 5-year experimental period, throughfall exclusion resulted in a 70% reduction in SWC, on average, during the growing season (Grams et al., 2021) and, at the time of our sampling, the throughfall exclusion plots had significantly lower SWC in upper ($\bar{x}$ = -27.8%) and lower soil layers ($\bar{x}$ = -22.4%) across all seasons (Figure S1). Upper layers had higher average SWC though the extent of moisture reduction was comparable in both layers (t = -3.9 vs. -3.2, respectively). Throughfall exclusion significantly decreased fine root mass, with a greater effect on upper layers ($\bar{x}$ = -51.1%; t = -4.2; $p < 0.001$) compared to lower layer soils ($\bar{x}$ = -29.8%; t = -3.2; $p < 0.001$). Exclusion had minor effects on soil pH (+1% in upper layer; $p = 0.03$); and had mostly insignificant effects on SOM and DNA yield (Figure S1). Throughfall exclusion accounted for a relatively low proportion of variation in the beta-diversity of soil bacterial and fungal

communities (Figure 2AB), which was primarily attributable to differences among soil layer and tree root zone. Communities from mixed root zones exhibited an intermediate similarity between species-specific root zones (Figure 2A).

3.1.2 Drought-affected soil bacteria and fungi

The differences in the relative abundance of OTUs between reference and throughfall excluded plots were used to identify 'drought-favored' or 'drought-sensitive' bacterial and fungal populations. The soil microbiome at Kranzberg was comprised of 10,357 bacterial and 3,282 fungal OTUs. Of these, a total of 369 bacterial and 23 fungal OTUs were indicative of throughfall exclusion (Table S6). More OTUs were drought-sensitive (n_{bact} = 194 and n_{fungi} = 15) than drought-favored (n_{bact} = 175 and n_{fungi} = 8). More drought-affected OTUs were observed in upper (n = 215) versus lower soil layers (n = 130), but OTUs did not differ in their likelihood of being drought-affected based on their soil layer association (Fisher's test; p = 0.8).

Drought-favored fungi were primarily classified as Ascomycota (80%) and belonged to genera designated as saprotrophic (*Pseudogymnoascus*, *Niesslia*, and *Ciliolarina*). Two of the eight drought sensitive fungal OTUs were classified to genera of EMF (*Inocybe* and *Lactarius*). However, the overall relative abundance of EMF was largely unaffected by throughfall exclusion (Figure S2A). Fungal endophytes (genus *Phialocephala*) were significantly more abundant in throughfall-excluded upper soils (Figure S3A) and were relatively more abundant in spruce root zones (Figure S3B).

Throughfall exclusion affected a phylogenetically diverse group of bacteria, but most of the affected OTUs were classified to Planctomycetes, Alphaproteobacteria and Actinobacteria (Figure S4; complete list in Table S6). Actinobacteria were near uniformly drought-favored (primarily classified as Pseudonocardiales, Solirubrobacterales, and Micrococcales), while Planctomycetes were primarily drought-sensitive (Pirellulales, Gemmatales, and

Planctomycetales), though certain clades of Planctomycetes also contained a high proportion of drought-favored taxa (Tepidisphaerales and Isosphaerales). Within Alphaproteobacteria, several orders were favored by throughfall exclusion, namely populations of Caulobacterales (*Phenylobacterium*), Elsterales, and Sphingomonadales (*Sphingomonas*) while others were sensitive, namely populations of Rhizobiales (*Roseiarcus*, *Rhodoplanes*, and *Bradyrhizobium*) and Reyranellales (*Reyranella*). Other major bacterial groups that were drought sensitive included: Deltaproteobacteria (Myxococcales), Verrucomicrobia (Pedosphaeraceae and Xiphinematobacteriaceae), and Dependentiae (Vermiphilaceae).

3.2.1 Beech- and spruce-associated soil bacteria and fungi and rhizobacteria

Soil microbiome composition significantly differed between beech and spruce root zones, though the most abundant bacterial and fungal OTUs (≥ 0.2% of total reads) were present in all root zones (97% of bacteria and 52% of fungi; Figure S5). The relative abundances of beech- and spruce-associated OTUs in mixed zones were characteristically intermediate between the relative abundance in the species-specific zones (Figure S6). The spruce-associated microbiome was dominated by several orders of Actinobacteria (Frankiales and Solirubrobacterales) and Acidobacteria (Acidobacteriales and Solibacterales; Figure 3A) and had a higher Ascomycota:Basidiomycota ratio compared to beech (Figure S7). The beech microbiome had a higher proportion of Proteobacteria (Alpha-, Gamma- and Delta-), Verrucomicrobia and Planctomyces and a higher proportion of EMF (Figure S2B). Beech-associated taxa were significantly more likely to be drought-sensitive than spruce-associated taxa (Fisher's Exact, O.R. = 23.8; $p < 0.001$), with spruce-associated taxa tending to increase in relative abundance in throughfall excluded plots.

Rhizobacterial populations associated with beech and spruce roots were identified using indicator analysis. Beech-associated rhizobacterial populations were enriched in

Alphaproteobacteria from the family Xanthobacteraceae relative to spruce, which were enriched in Acidobacteriales (*Acidipila*, *Granulicella* and *Occallatibacter*) and Frankiales (*Acidothermus*; Figure 3B, complete list in Table S7). Bacterial species richness and evenness were significantly higher in beech than spruce root zones, while no differences were evident for fungi (Figure S8).

3.2.2 Corresponding differences in plant and soil properties among root zones

Trends in the soil microbiome corresponded primarily with differences in fine root mass and soil organic matter content in root zones. The upper soil layer in spruce root zones had a significantly higher percentages of soil organic matter (2-fold) and SWC than beech, except for at Arnstein, which received the lowest precipitation (Figure S9A). In contrast, the beech root zone had significantly higher fine root mass in both the upper (66%) and lower layers (60%) at all sites, while mixed zones had intermediate root mass. In all sites, fine root mass followed seasonal trends in SWC between spring and fall, increasing in wetter sites (Wasserburg and Traunstein) and declining in drier sites (Arnstein and Kranzberg), with the trend most pronounced in beech stands (Figure S9B). Soil pH was significantly more acidic in spruce ($\bar{x}$ = 4.04) than beech ($\bar{x}$ = 4.30), with intermediates values in mixed root zones ($\bar{x}$ = 4.21).

3.3.1 Contrasting the effects of soil moisture on microbiomes among root zones

We tested for the generalized effects of soil moisture reduction on soil bacteria and fungi among root zones using the community resistance (R) metric. In our study, lower R values indicate a greater change in community composition resulting from reduced soil moisture (i.e., a lower resistance). On average, R values for bacterial communities differed by tree root zone, but not for fungal communities. Bacterial communities in the spruce root zones had higher R values than in the beech root zones, though this difference was only significant in the lower soil layer (Figure 4A). Differences in R among root zones corresponded with the proportion of drought-favored (higher in spruce) and drought-sensitive bacterial OTUs (higher in beech; Figure 4B). The spruce

soil microbiome also exhibited greater R values in relation to seasonal changes between spring (wettest) and fall (driest) at Kranzberg and, in this case, the mixed root zone also exhibited significantly higher R than species-specific beech (Figure S10A). These differences also corresponded to significant seasonal increases in drought-favored and drought-sensitive taxa (Figure S10B). R values for fungal communities were variable and did not significantly differ among tree root zones (Figure S11A). Yet, the relative abundance of drought-favored fungal OTUs was also significantly higher in spruce root zones (Figure S11B).

3.3.2 Contrasting responses in the soil microbiome of species-specific and mixed root zones

The effects of throughfall exclusion on several abundant beech- and spruce-associated bacteria were significantly altered in the mixed-species root zone. Several beech- and spruce-associated OTUs that were affected by throughfall exclusion in species-specific root zones exhibited enhanced tolerance in mixed root zones (Figure 5AB; Table S8). Conversely, several OTUs that were unaffected by throughfall exclusion in species-specific root zones exhibited different responses in mixed-species root zones. These could be divided into beach-associated OTUs, that exhibited a greater susceptibility (Figure 5C), and spruce-associated OTUs, that exhibited a greater resistance, in mixed-species root zones (Figure 5D; Table S9). These trends were evident when profiling the same populations across seasonal differences in SWC at Kranzberg (lower panels in Figure 5C and 5D), and at other sites, though not uniformly (Figure S12). There were no significant interactions between root mixing and throughfall exclusion for soil fungi.

3.4.1 Seasonal and latitudinal trends in drought-affected populations

Complementary to the throughfall exclusion experiment, we profiled changes in the soil microbiome of species-specific and mixed-species root zones across seasonal and latitudinal gradients in soil moisture and precipitation, respectively (Figure 1A). A random forest, decision-

tree-based feature selection was used to identify the environmental variables and microbial taxa that were most predictive of soil water content in these gradient (Table 1). Several bacterial orders were selected as predictors of SWC, and the abundance patterns of Rhizobiales outranked all other features in importance, including environmental parameters (Table 1). No fungal taxa were selected as predictors of SWC. The abundance patterns of the top microbiome-based predictors of SWC closely tracked seasonal and latitudinal differences in precipitation in upper (Figure 6) and lower soil layers (Figure S13) and were among the same groups impacted by throughfall exclusion. Among the OTUs identified as drought-affected in data from the throughfall exclusion experiment, the number that significantly differed among high and low precipitation sites in the latitudinal gradient was lowest in spring (n = 92), when SWC was highest, and progressively increased in summer (n = 133) and fall (n = 220).

EMF did not exhibit a clear relationship to SWC as their relative abundance did not vary significantly by season or site position in the precipitation gradient. The exception was at Arnstein during the summer, when increased relative abundance of EMF coincided with some of the lowest SWC values measured (Figure S2B). The EMF taxa that increased in Arnstein during the summer included taxa from the genera: *Amphinema*, *Clavulina*, *Otidea*, *Sebacina*, *Tricholoma*, *Inocybe*, and *Lactarius*.

3.4.2 Moisture related shifts in rhizobacterial abundance between soil layers

Reductions in soil moisture can cause stratification of roots in spruce, to upper soil layers, and beech, to lower soil layers, according to observed root behavior (Bolte and Villanueva, 2006). We found that the relative abundance of spruce- and beech-associated rhizobacteria tended to differ between upper and lower soil layers correspondingly, particularly at Kranzberg (Figure 7). The enrichment of spruce-associated rhizobacteria in upper layer soils was even more pronounced during fall, when SWC was lowest, though this interaction was only significant at Kranzberg and

Wasserburg (Figure 7). In contrast, beech-associated rhizobacteria were more prevalent in the lower soil layer, and no shift was seen due to throughfall exclusion or season. Additionally, at Kranzberg, the seasonal shift to the upper soil layer was most pronounced in mixed root zones ($t_{interact.}$ = 8.1; $p < 0.001$) compared to species-specific spruce (t = 3.9) or beech root zones (t = 3.6). No shifts in spruce- and beech-associated fungi were evident, though we did not obtain fungal sequence data from rhizoplane / endophyte samples.

4. Discussion

Our study reveals major differences in the response of Norway spruce and European beech soil microbiomes to changes in soil moisture and their interplay in mixed root zones. A significantly higher proportion of beech-associated taxa were sensitive to reductions in soil moisture compared to the more drought-tolerant populations associated with spruce. Furthermore, several populations of rhizobacteria and soil bacteria had increased resistance or susceptibility to drought in mixed-species root zones relative to species-specific. These findings lead us to reject the hypothesis that the response of microbial populations to changes in moisture would not differ in mixed-species root zones, allowing for the possibility that complementation can occur. Here, we discuss the possible reasons why drought may impact spruce- and beech-associated soil microbiomes differently, and the potential consequences of the phenomena observed in mixed-species root zones.

4.1 Explaining the contrasting effects of reduced soil moisture on beech and spruce microbiomes

Taxa that were consistently impacted by experimental or natural reductions in soil moisture were broadly characteristic of the spruce and beech microbiomes described in prior research (Asplund et al., 2019; Bárta et al., 2017; Felsmann et al., 2015; Nacke et al., 2016; Uroz et al., 2016). Several of these broad taxonomic groups were previously shown to be impacted by reduced throughfall in beech and spruce forest (Felsmann et al., 2015), and other forest types (Bastida et

al., 2017; Bouskill et al., 2013). Our results demonstrate that soil bacterial communities in species-specific spruce root zones have a higher resistance to the impacts of drought and to seasonal reductions in soil moisture than in beech root zones (Figure 4; Figure S10). This difference corresponded with a higher proportion of drought-tolerant bacteria in the spruce microbiome than in beech, and higher proportions of drought-sensitive bacteria in the beech microbiome. While this is the first time such broad differences in moisture sensitivity have been reported, prior research has shown that the mineral weathering activity of rhizobacteria isolated from beech was higher during wetter periods while the reverse was true for those isolate from spruce (Collignon et al., 2011).

Prevailing differences in soil conditions between spruce and beech stands likely help explain the disparity in drought tolerance and sensitivity in their respective soil microbiomes. Physiological differences in rooting depth result in consistently drier conditions in shallow soils in spruce stands relative to beach (Allen et al., 2019; Zwetsloot and Bauerle, 2021). Our findings indicate that this effect is large enough to select for higher proportion of drought stress-tolerant bacteria in spruce soils. Additionally, soil acidification was more pronounced in spruce root zones relative to beech (Figure S1), as indicated by the high relative abundance of acidophilic indicator taxa (Acetobacterales, Frankiales and Acidobacteriales), as previously shown (Sridhar et al., 2022a). Our observations were consistent with current understanding about difference in soil development under beech and spruce stands caused by root and litter chemistry, nutrient leaching and uptake, and mineralization and nitrification rates (Cremer and Prietzel, 2017). Spruce roots and litter contain significantly higher concentrations of polyphenols than beech (Kuiters and Denneman, 1987; Zwetsloot and Bauerle, 2018), which are generally toxic to soil heterotrophs (Adamczyk et al., 2013; Chunmei et al., 2010; Inderjit et al., 2009; Metsämuuronen and Sirén,

2019). The high acidity and polyphenol content of spruce soils create adverse growing conditions, which retard decomposition (Albers et al., 2004; Berger et al., 2004) and reduce soil respiration and biomass in spruce stands relative to mixed-species and beech stands (Borken et al., 2002; Borken and Beese, 2005; Lu and Scheu, 2021). These adverse conditions, along with consistently lower shallow soil moisture levels in spruce stands, may select for stress-tolerant populations, which may better endure water stress. This theory is supported by the overlapping physiological stress response to acidity, low osmolarity, and desiccation observed in model bacteria (Ait-Ouazzou et al., 2012; Hengge-Aronis, 2002; Spector and Kenyon, 2012). It is also anecdotally supported by the enrichment of the thermophilic, acid-tolerant genus *Acidothermus* by throughfall exclusion in spruce and mixed root zones (Figure S14), which are characteristic of droughted and arid soils (Eppard et al., 1996; Lacerda-Júnior et al., 2019).

The prevalence of drought-sensitive taxa in beech-specific soils could also reflect differences in the degree of rhizosphere activity between beech and spruce. Beech produce more fine root mass (Finér et al., 2007), apparent at all our sites ($\bar{x}$ = 1.4 to 2.1-fold higher), and sustain more microbial biomass and higher soil respiration rates than spruce (Borken et al., 2002; Borken and Beese, 2005; Lu and Scheu, 2021). Thus, the apparent sensitivity of beech-associated taxa may reflect the inability of beech roots to sustain basal levels of microbial activity during drought. The potential diminished influence of beech on the soil microbiome during drought was evident in the decline of microbial populations indicative of higher trophic complexity, including members of the Vermiphilaceae, endosymbionts of amoeba (Delafont et al., 2015), and Candidatus *Xiphinematobacter*, symbionts of nematode (Rius et al., 2021; Vandekerckhove et al., 2000). While we cannot disentangle the relative contributions of roots versus litter to differences in the soil microbiomes between spruce and beech, root traits have a far greater importance in explaining

the physicochemical properties of species-specific and mixed forest soils than litter traits (Cesarz et al., 2013; Gillespie et al., 2021).

4.2 Effects of root mixing on the impacts of reduced soil moisture

Most beech- and spruce-associated fungi or bacteria occurred at an intermediate relative abundance in mixed root zones. This result indicates that the influence of each tree follows a gradient and that taxa associated with either tree species can co-occur. These observations are consistent with prior reports of intermediate heterotrophic activity (Borken et al., 2002; Borken and Beese, 2005), microbial biomass (Lu and Scheu, 2021), litter decomposition rates (Albers et al., 2004), and mineral weathering (Cremer and Prietzel, 2017) in mixed root zones. Thus, the hybrid soil microbiome in mixed root soils reflects the combined, but weakened, influences of each tree.

The resistance of several beech and spruce-associated bacteria to throughfall exclusion was enhanced in mixed root zones (Figure 5AB). These populations included members of Acidobacteria (*Bryobacter* and *Occallatibacter*, both spruce-associated rhizobacteria), which are reputed for their production of extracellular polymeric substances, which confer stress tolerance to cells and may influence soil moisture dynamics (Foesel et al., 2016; Kielak et al., 2016; Kulichevskaya et al., 2010). In contrast, several members of Xanthobacteraceae (*Bradyrhizobium* and *Rhodoplanes*) were more susceptible to throughfall exclusion in mixed root zones (Figure 5C). Xanthobacteraceae are known to increase in abundance in proximity to beech trunks (Nacke et al., 2016), suggesting that their capacity to resist drought may depend on the density of beech roots or litter. Conversely, several spruce-associated taxa exhibited enhanced resistance to drought exclusively in mixed roots zones (Figure 5D), suggesting some populations may benefit from the reduced competition where drought-sensitive, beech-associated taxa are diminished. Our explanations for trends in mixed root zones remain to be tested, but these observations illustrate

443 the range of interactions occurring where the influence of trees and, by extension, their
444 microbiomes mix.

445 *4.3 Effects of reduced soil moisture on root-associated taxa*

446 The rhizosphere activity of spruce and beech varies by season and moisture availability, with
447 the highest activity in spring (Calvaruso et al., 2014) and diminished photosynthate-derived
448 microbial rhizosphere biomass during drought (Ruehr et al., 2009). We hypothesized that
449 reductions in soil moisture would disproportionately impact root-associated taxa, like EMF, which
450 are important contributors to nutrient acquisition by spruce and beech (Brzostek et al., 2013;
451 Calvaruso et al., 2014; Collignon et al., 2011; Gan et al., 2021; Nicolitch et al., 2016). Contrary to
452 expectations, we did not observe any compositional changes in soil EMF populations from
453 throughfall exclusion, seasonal variation, or across the precipitation gradient. The lack of shift in
454 EMF populations is not without precedent in spruce and beech forests (Gorfer et al., 2021; Nickel
455 et al., 2018; Žifčáková et al., 2015). The apparent resiliency of EMF to changes in community
456 composition may reflect their direct connection to live roots and a privileged access to root
457 exudates relative to bacteria, consistent with the more severe impacts in bacterial populations we
458 observed and reported elsewhere (de Vries et al., 2018; Fuchslueger et al., 2014). Access to
459 exudates and shelter within the root might explain why endophyte populations (*Phialocephala*)
460 were among the few fungi favored by throughfall exclusion (Figure S3).

461 Rhizobacteria were among the most affected by throughfall exclusion and seasonal and
462 latitudinal variation in soil moisture. Members of the Rhizobiales and Burkholderiaceae were
463 greatly diminished by throughfall exclusion in both beech and spruce root zones (Figure 6; Figure
464 S15). These drought-sensitive populations were dominated by members of *Bradyrhizobium* and
465 *Rhodoplanes* (Xanthobacteraceae) and *Caballeronia* and *Paraburkholderia* (Burkholderiaceae)
466 which are consistently associated with the rhizosphere of European beech and Norway spruce

(Colin et al., 2017; Uroz et al., 2016) and are common root- and mycorrhizae-associated bacteria isolated from forest soils (Burke et al., 2008; Izumi et al., 2007, 2006; Kataoka et al., 2008; Tanaka and Nara, 2009; Uroz et al., 2012; Wilhelm et al., 2020). The apparent drought sensitivity of these rhizobacteria has implications for soil nutrient cycling and plant nutrition, given their involvement in priming decomposition (Wilhelm et al., 2021; Zwetsloot et al., 2020), phosphate solubilization in the beech and spruce rhizosphere (Lepleux et al., 2012; Nicolitch et al., 2016), and endophytic nitrogen fixation (Puri et al., 2020).

5. Conclusions

We conclude that any potential complementation of soil microbiome function during drought is likely to confer greater benefits to beech than spruce, given the higher proportion of drought-favored spruce-associated taxa and their sustained resistance in mixed root zones. The enhanced drought tolerance of beech in mixed stands with spruce has been reported (Schäfer et al., 2017), though the benefits of mixing are more commonly reported to favor Norway spruce (del Río et al., 2014; Ding et al., 2017; Rötzer et al., 2017). Thus, it remains to be seen what functions the complementation of soil microbiomes might have in the drought resistance of mixed beech-spruce forests. The impact of reduced soil moisture was greater on rhizobacteria than EMF populations, with a higher proportion of EMF occurring in the beech soil microbiome. Thus, the likeliest form of complementarity in mixed-root zones might correspond with the enrichment of drought-tolerant populations by spruce and the sustenance of EMF activity by beech.

Forests and their soil microbiome are complex adaptive systems in which legacy and context shape biological responses to water limitation (Bouskill et al., 2013). Yet, the moisture sensitivity of major drought-affected populations, such as members of the Rhizobiales, were consistent across sites and season, providing evidence for the potential widespread occurrence of phenomena reported here. Future research is needed to understand the ecological and environmental drivers of

differences across sites and to test the functional consequences of complementation between microbiomes of beech and spruce, and other abundant tree species, in mixed root zones.

6. Acknowledgements

We thank Tommi Schieder at Cornell University for assisting with DNA extractions, and Laura Pohlenz together with the class of 2017/18 Ecology Gap Year Volunteers at Helmholtz Center Munich for assisting in soil and root sample processing. This work was supported by the German Research Foundation (grant DFG PR555/2-1) and by the U.S. Department of Energy, Office of Biological & Environmental Research Genomic Science Program under award number DE-SC0016364. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the United States Department of Energy.

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Data Availability Statement

All sample metadata is provided in Table S5. All analyses can be reproduced with scripts included in the Supplementary Data package available through the Open Science Foundation (doi: 10.17605/OSF.IO/DN9CH). Raw sequencing data was archived with the European Nucleotide Archive under the BioProject accession: PRJEB36981.

Author Contributions

RCW performed the data analysis, research, and writing. JMU contributed to data analysis and research. Field sampling was performed by JMU, TLB, MG, FW, and KP and sample processing (soil parameters, root biomass) by JMU and FW. Amplicon sequencing libraries were prepared by RCW, JMU, and FW. TLB, KP, and DHB guided all research efforts, including analyses and writing. The authors declare we have no competing or conflicts of interest.

Tables

Table 1. The top environmental and soil microbiome predictors of soil water content were identified, and ranked, using Boruta random forest-based feature selection. The relative abundance pattern of aggregated counts of Rhizobiales was ranked as the best predictor of SWC, followed by soil layer and precipitation gradient site. Fungal and bacterial count data, aggregated by Order, were included in feature selection, but only bacteria had predictive value. Bacterial orders that had significant Pearson's correlations with SWC content in both layers ($r > |0.2|$ and $p_{adj} < 0.05$) and were among the top 20 most important features are displayed (full list in Table S10). The family Gimesiaceae is historically known as 'Planctomycetaceae.'

Figures

Figure 1. A schematic overview of the experimental design used to examine the effects of natural and experimental variation in soil moisture on the soil microbiome of species-specific and mixed-species stands of European beech and Norway spruce. In (A), samples were collected in spring, summer, and fall and at sites spread across a natural precipitation in Bavaria, Germany. In (B), at Kranzberg, the mid-point of the gradient, samples were taken from a five-year old throughfall exclusion experiment where rain-out roofs had been used to reduce soil moisture during the growing season. Each symbol accurately reflects the distribution of trees species (full details in Rötzer *et al.* 2017). In (C), at all sites, and in the throughfall exclusion plots at KROOF, samples were taken from species-specific or mixed-species tree root zones. Ten soil cores (30 cm deep) were randomly sampled from five to six plots for each tree root zone at each site and during every season and subdivided into upper and lower soil layers before being composited.

Figure 2. The composition of soil bacterial and fungal communities at Kranzberg differed primarily by soil layer and tree root zone, as evident in (A) the grouping of samples by principal coordinates analysis and (B) the proportion of variance explained (R^2) in a PERMANOVA analysis based on the Bray-Curtis dissimilarity in community composition.

Figure 3. Beech and spruce root zones had marked differences in the taxonomic composition of soil bacteria and fungi and rhizobacteria. In (A), the bar plots provide a summary of the relative proportions of indicator OTUs for beech- and spruce-associated bacteria (n = 506) and fungi (n = 63) according to the ratio of their aggregated relative abundance at rank Order. The subset of tree-associated taxa affected by drought are labeled on the y-axis. In (B), the taxonomic profile or rhizobacteria associated with either beech or spruce according to indicator analysis using 16S rRNA gene amplicon data generated from root material.

Figure 4. The resistance (R) of soil bacterial communities to the effects of throughfall exclusion in soil moisture was greatest in spruce root zones at Kranzberg. Differences in R among root zones (B) corresponded with the relative abundance of drought-favored and drought-sensitive populations. Pairwise differences in resistance among tree root zones sites were tested using TukeyHSD ($p_{adj} < 0.05$). In (B), the effects of throughfall exclusion ($p_{excl.}$) were significant for all groups. Any significant differences between tree root zone were denoted by bars with asterisk and interactions between throughfall treatment and tree root zone were denoted by asterisk.

Figure 5. The responses of beech- and spruce-associated OTUs to throughfall exclusion were significantly altered in soil microbiome of mixed root zones at Kranzberg. In (A), the relative abundance of several drought-sensitive beech-associated OTUs (n = 10) were largely undiminished in mixed root zones. In (B), several drought-sensitive spruce-associated OTUs (n = 5) had enhanced resistance to throughfall exclusion in mixed root zones. Several OTUs that were not significantly affected by throughfall exclusion in species-specific root zones showed, in (C), an increased susceptibility (n = 8) or, in (D), an increased resilience (n = 4) in mixed zones. These trends were consistent with trends in relative abundances between spring (wettest) and fall (driest season) at Kranzberg (lower panel of C and D). Individual OTU exhibited significant interactions between throughfall exclusion and tree root zone (Table S8 and Table S9) but were displayed in aggregate. Any significant differences between tree root zone were denoted by bars with asterisk and interactions between throughfall treatment (or season) and tree root zone were denoted by asterisk.

Figure 6. The relative abundance of four major bacterial orders followed changes in soil water content caused by throughfall exclusion plots, seasonal variation, and across the latitudinal precipitation gradient. In (A), the soil water content in the upper soil layer was reduced by throughfall exclusion (box plot), and across seasons (x-axis) and gradient sites (lines). In (B), the relative abundances of the four bacterial Orders identified in feature selection as predictive of SWC (Table 1). The same trends were evident in lower layer soils, though less pronounced (Figure S13). Significant differences between mean SWC or relative abundance between seasons were denoted by bars with asterisks. Significant interactions between season and site were denoted by colored asterisks. Lettering denotes significant differences among sites according to TukeyHSD ($p_{adj} < 0.05$).

Figure 7. Shifts in the relative abundance of rhizobacteria in response to (A) throughfall exclusion or (B) seasonal differences between spring and fall revealed the putative partitioning of roots between soil layers. Spruce-associated rhizobacteria (upper panel) tended to occur at higher proportions in the upper soil layer, while beech-associated rhizobacteria (lower panel) tended to be more abundant in the lower layer. Significant differences between soil layer means were denoted by bars with asterisk and interactions between throughfall treatment (or season) and soil layer were denoted by asterisk.

Feature	Rank	Imp.	Order	Family	Correlation with SWC r_{upper}	r_{lower}
Environmental	**2**	**21.7**	Soil layer		-	-
	4	**17.7**	Site		-	-
	13	10.5	Season		-	-
	28	6.9	Tree root zone		-	-
	37	6.0	Year		-	-
Microbiome	**1**	**24.2**	Rhizobiales		0.39 ***	0.33 ***
				Beijerinckiaceae	0.38 ***	0.19 **
				Xanthobacteraceae	0.25 ***	0.31 ***
				KF-JG30-B3	-0.33 ***	-0.11 *
	6	15.7	Planctomycetales		0.35 ***	0.24 ***
				Gimesiaceae	0.49 ***	0.20 **
	9	11.2	Gaiellales		-0.23 ***	-0.23 ***
	11	10.6	Caulobacterales		-0.32 ***	-0.22 ***
				Caulobacteraceae	-0.33 ***	-0.24 ***
	15	9.6	Tepidisphaerales		-0.35 ***	-0.11 *
				WD2101 soil group	-0.34 ***	-0.11 *

Drought sensitive Drought favored

Table 1. The top environmental and microbiome predictors of soil water content were identified, and ranked, using Boruta random forest-based feature selection. The relative abundance pattern of aggregated counts of Rhizobiales was ranked as the best predictor of SWC, followed by soil layer and precipitation gradient site. Fungal and bacterial count data, aggregated by Order, were included in feature selection, but only bacteria had predictive value. Bacterial orders that had significant Pearson's correlations with SWC content in both layers ($r > |0.2|$ and $p_{adj} < 0.05$) and were among the top 20 most important features are displayed (full list in Table S8). The family Gimesiaceae is historically known as 'Planctomycetaceae.'

Figure 1.

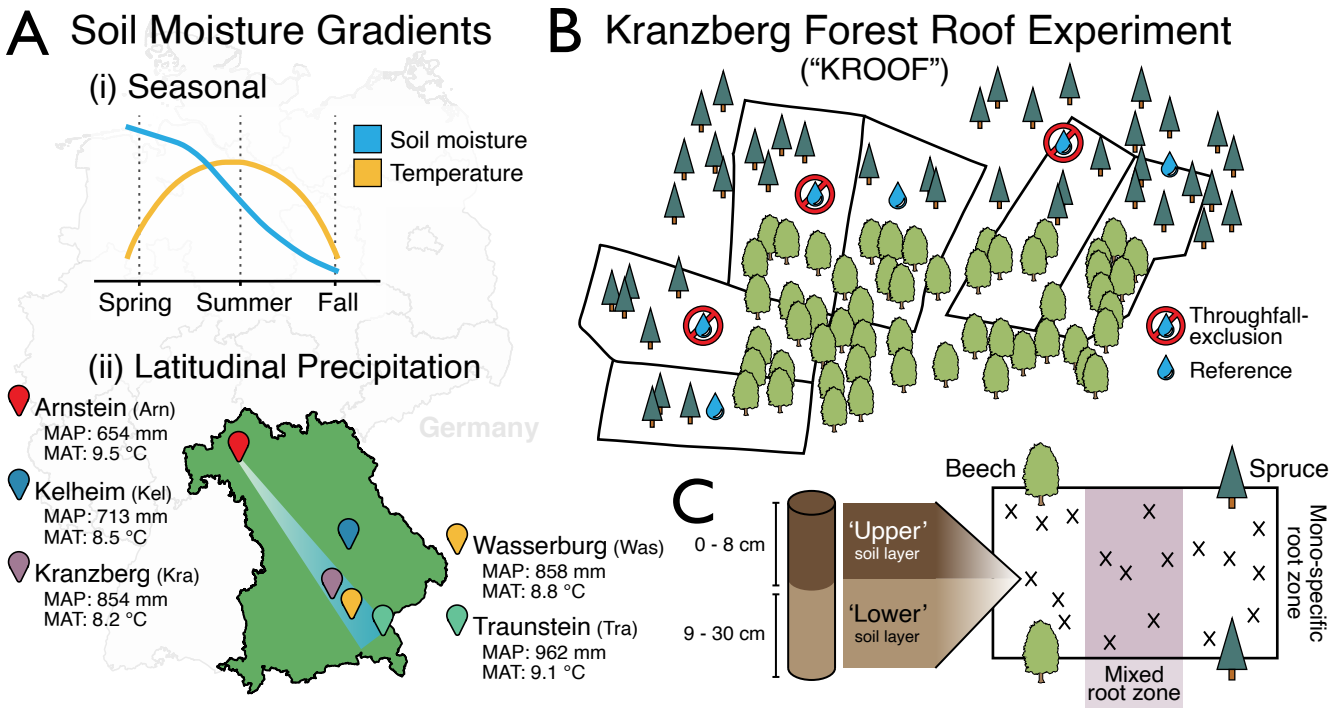
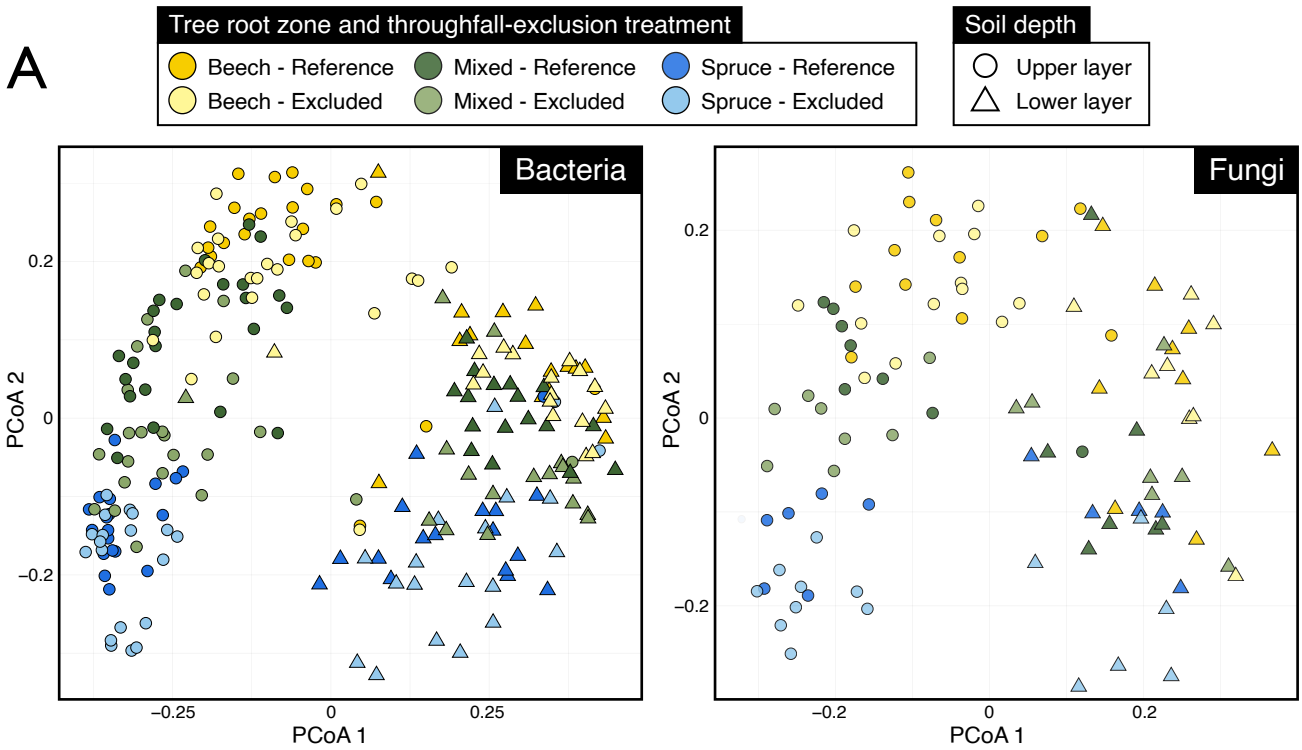


Figure 2.

A



B

Bacteria	df	R^2	F	*p*	
soil depth	1	0.17	65.3	0.001	***
root zone	2	0.09	17.1	0.001	***
year	1	0.059	22.6	0.001	***
season	2	0.048	9.1	0.001	***
replicate (plots)	6	0.033	2.1	0.001	***
thr. exclusion	1	0.011	4.4	0.001	***
residual	222	0.59			
total	235	1			

Fungi	df	R^2	F	*p*	
soil depth	1	0.089	11	0.001	***
replicate (plots)	5	0.07	1.8	0.001	***
root zone	2	0.066	4.1	0.001	***
year	1	0.026	3.2	0.001	***
thr. exclusion	1	0.021	2.6	0.002	**
season	1	0.014	1.7	0.006	**
residual	87	0.82			
total	98	1			

PERMANOVA Results

Figure 3.

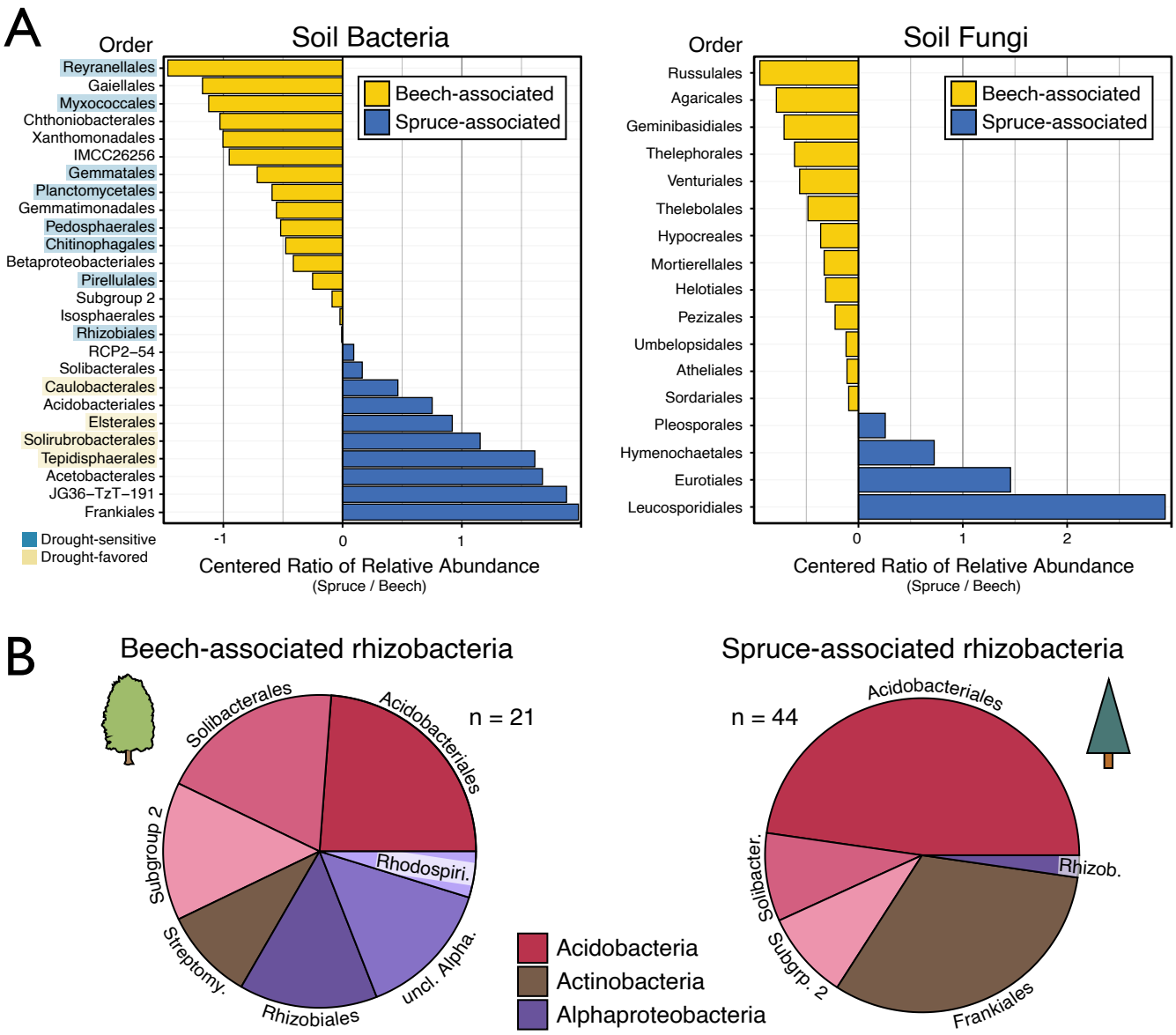
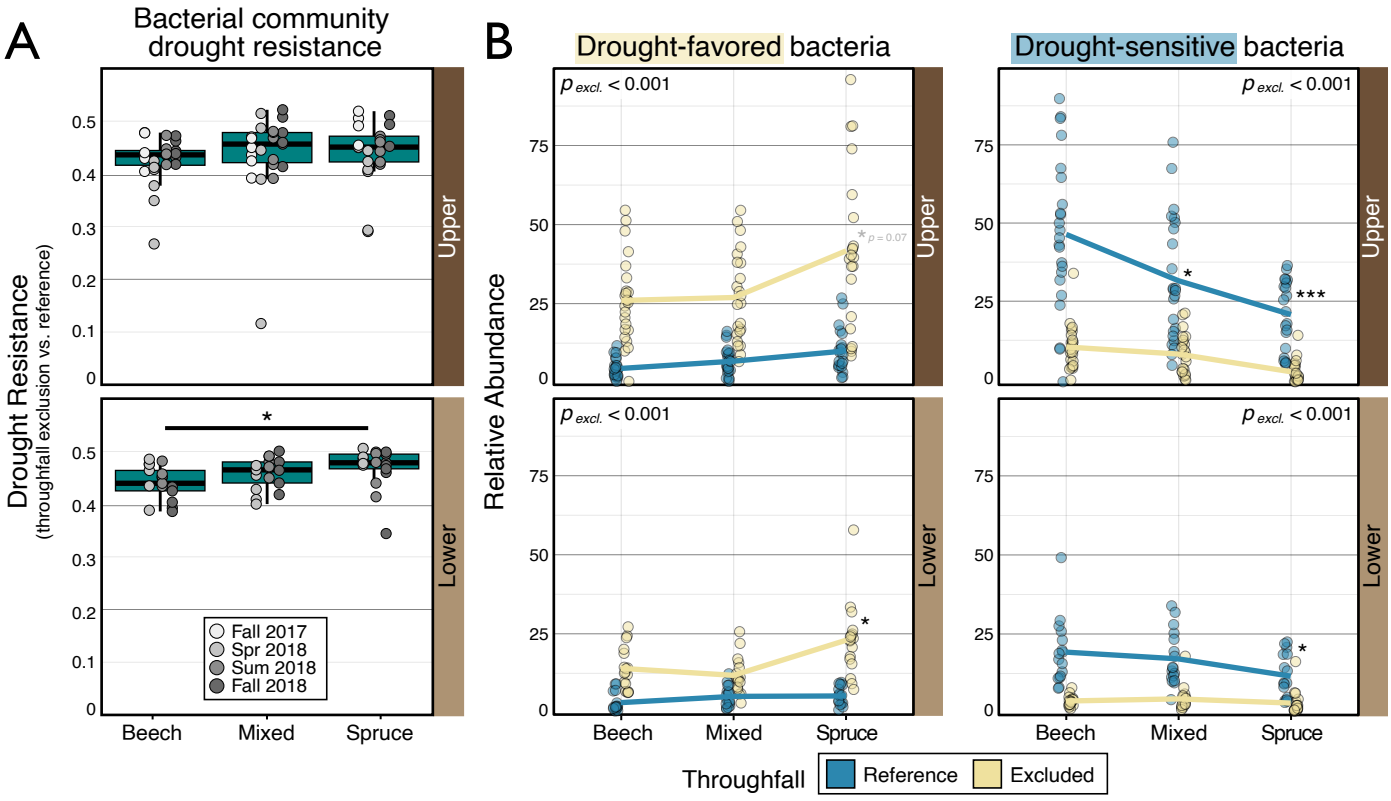


Figure 4.



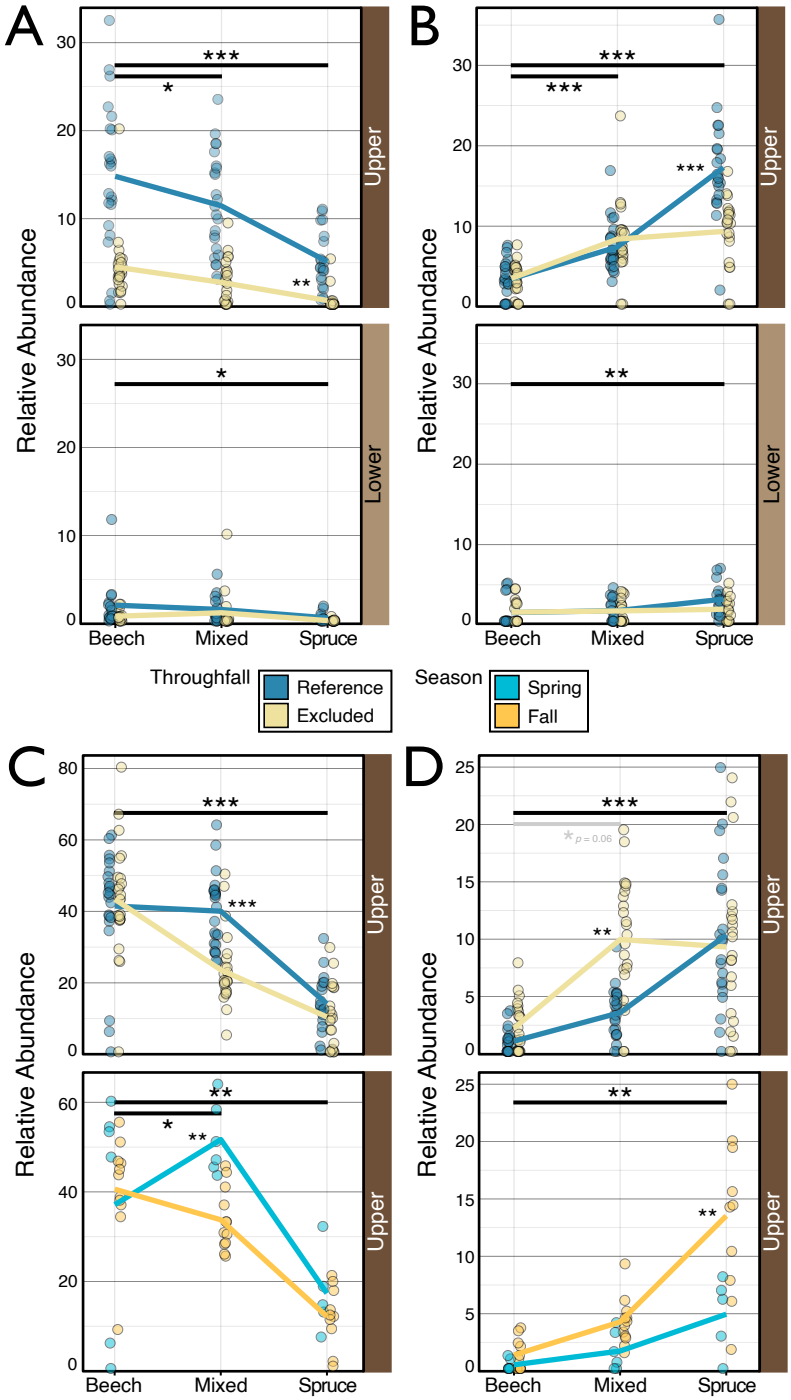


Figure 5.

Figure 6.

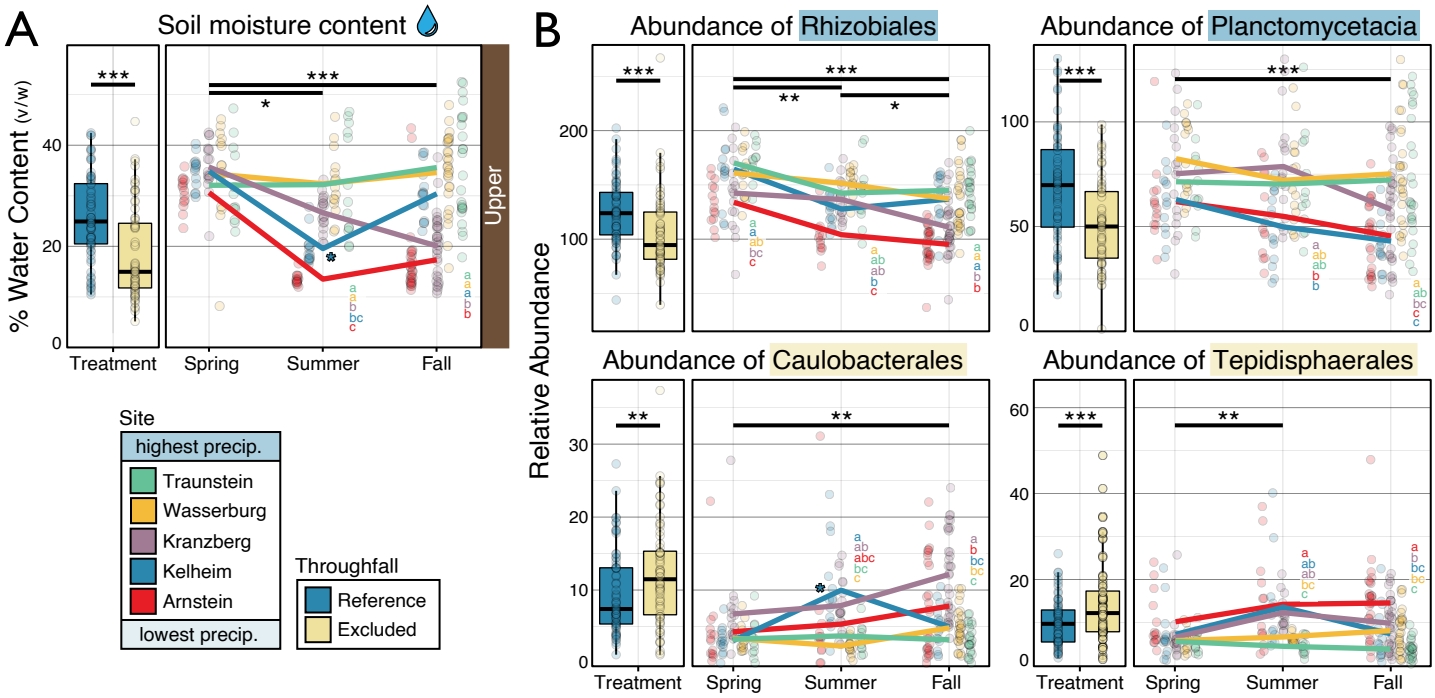
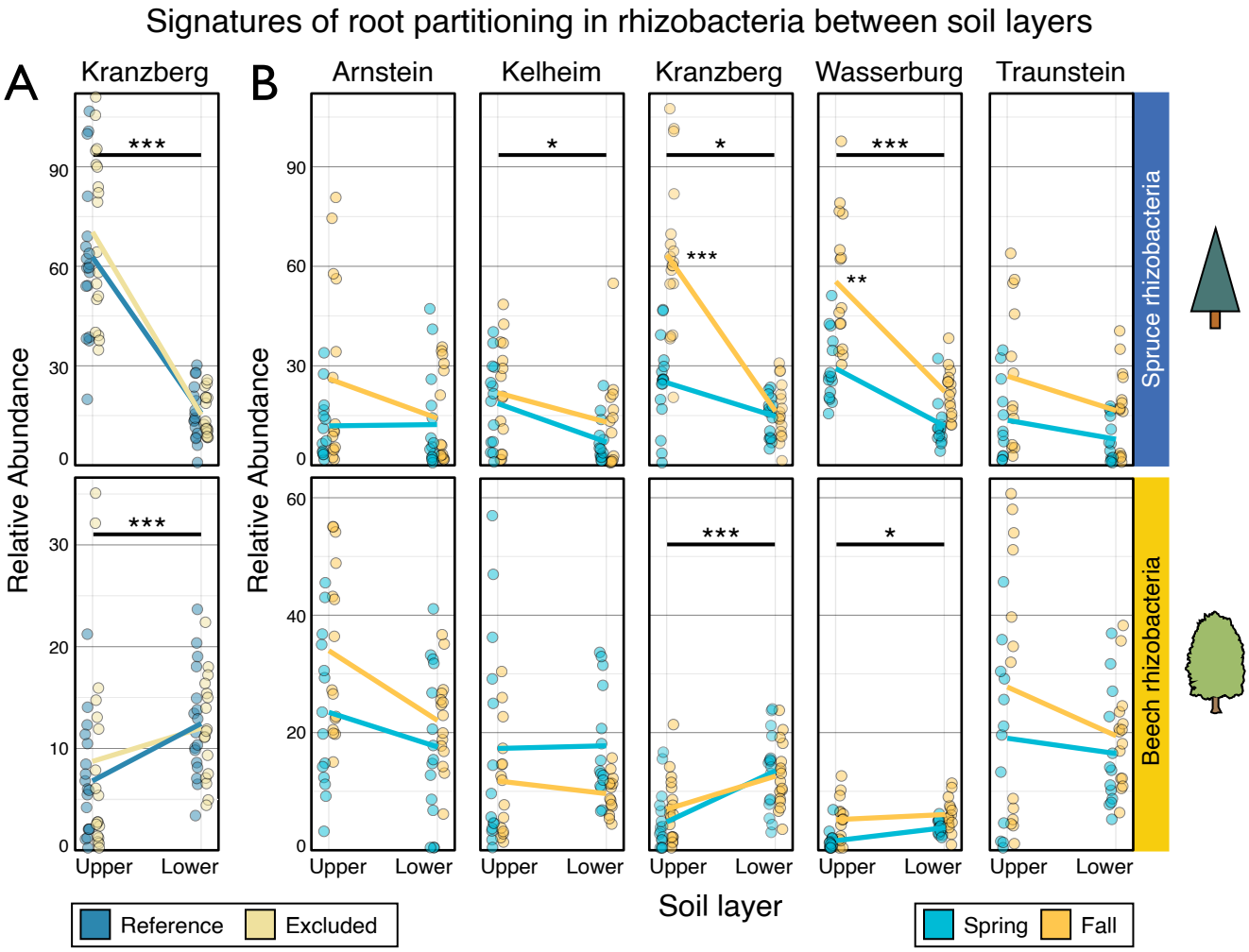


Figure 7.



Supplementary Tables

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Supplementary Material

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author Contributions

RCW performed the data analysis, research, and writing. JMU contributed to data analysis and research. Field sampling was performed by JMU, TLB, MG, FW, and KP and sample processing (soil parameters, root biomass) by JMU and FW. Amplicon sequencing libraries were prepared by RCW, JMU, and FW. TLB, KP, and DHB guided all research efforts, including analyses and writing.