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Root-Associated Bacterial and Fungal Community Profiles of *Arabidopsis thaliana* Are Robust Across Contrasting Soil P Levels

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ABSTRACT

Plant survival depends on the ability of roots to sense and acquire nutrients in soils, which harbor a rich diversity of microbes. A subset of this microcosm interacts with plant roots and collectively forms root-associated microbial communities, termed the root microbiota. Under phosphorus-limiting conditions, some plants can engage in mutualistic interactions, for example with arbuscular mycorrhizal fungi. Here, we describe how *Arabidopsis thaliana*, which lacks the genetic capacity for establishing the aforementioned symbiosis, interacts with soil-resident bacteria and fungi in soil from a long-term phosphorus fertilization trial. Long-term, contrasting fertilization regimes resulted in an ~6-fold and ~2.4-fold disparity in bioavailable and total phosphorous, respectively, which may explain differences in biomass of *A. thaliana* plants. Sequencing of marker genes enabled us to characterize bacterial and fungal communities present in the bulk soil, rhizosphere, and root compartments. Phosphorus had little effect on alpha- or beta-diversity indices, but more strongly influences bacterial and fungal community shifts in plant-associated compartments compared with bulk soil. The significant impact of soil P abundance could only be resolved at operational taxonomic unit level, and these subtle differences are more pronounced in the root compartment. We conclude that despite decades of different fertilization, both bacterial and fungal soil communities remained unexpectedly stable in soils tested, suggesting that the soil biota is resilient over time to nutrient supplementation. Conversely, low-abundance, root-associated microbes, which collectively represent 2 to 3% of the relative abundance of bacteria and fungi in the roots, exhibited a subtle, yet significant shift between the two soils.

In soils, P availability varies spatially and temporally, correlates well with soil age, and is dependent on other edaphic parameters such as pH and preexisting P forms (Izquierdo et al. 2013; Oburger et al. 2011). Optimal plant growth is rarely achieved in nature as a result of inadequate levels of bioavailable P (P_a) (Ågren et al. 2012). In fact, P_a is the only form accessible to plants and constitutes a menial, insufficient fraction of the total P present in soils (Turner et al. 2007). Plant fitness costs induced by P limitation likely resulted in a selection pressure for the evolution of efficient nutrient acquisition strategies (Efeyan et al. 2015; Laliberté et al. 2013), such as the secretion of organic acids, specialized root morphology, and the establishment of symbiotic associations with soil-resident microorganisms (Bao et al. 2014; Barberon et al. 2016; Kiers et al. 2011; Marschner et al. 2011).

The most studied symbiotic relationship between plants and microorganisms in P limiting conditions is the interaction with arbuscular mycorrhizal fungi (AMF). Although AMF species are cosmopolitan (Smith and Smith 2011), their relationships with plants are not universal with members of the family Brassicaceae completely lacking the genetic components required for AMF

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formation. The interaction of Brassicaceae with other soil microbes, such as *Colletotrichum tofieldiae*, might have replaced AMF to improve plant fitness during P deprivation (Hiruma et al. 2016). In agriculture, supplemental P fertilization is relied upon to achieve optimal yield in economically important crops, such as oil seed rape by overcoming natural P limitations, even though less than half of applied fertilizers are finally acquired and utilized by the plant (Foley et al. 2011; Smil 2000). Even though *Arabidopsis thaliana* has no agricultural production value, it serves as an important model of the family Brassicaceae. Therefore, we utilized this species to explore how a Brassicaceae member would recruit its symbiotic microbiota in different P fertilization regimes.

In terrestrial ecosystems, microbial communities may reach densities of up to 10^9 cells per gram of soil (Gans et al. 2005; Roesch et al. 2007). Next-generation sequencing technologies allows for culture-independent identification and quantification of individual microbial members, termed operational taxonomic units (OTUs), for microbial community-scale research. Enrichment of soil-derived OTUs associated within roots is indicative of colonization and proliferation of the corresponding microbes in this host organ, resulting in a distinctive and stable root microbiota (Bulgarelli et al. 2012; Lundberg et al. 2012; Schlaeppi et al. 2014) that can positively affect plant growth and health (Berendsen et al. 2012; Haney et al. 2015). The main drivers influencing root microbiota profiles are soil characteristics (e.g., soil geochemistry and soil biome), root-associated compartments (rhizosphere, rhizoplane, and root endosphere), and plant genotype (Bonito et al. 2014; Bulgarelli et al. 2012; Coleman-Derr et al. 2016; Lundberg et al. 2012; Tkacz et al. 2015). The role of P in shaping microbial communities has mainly been investigated in grasslands, pastures, or forests over very short timescales (Adair et al. 2013; Leff et al. 2015; Liu et al. 2012; Tan et al. 2013; Wakelin et al. 2012). In these P supplementation studies, it is difficult to distinguish whether alterations in microbial community composition are a direct result of P amendments or are indirectly caused by an accompanying shift in plant species composition. Furthermore, it is unclear whether these shifts are permanent, or only short-term perturbations which eventually stabilize over time. We therefore decided to take an approach using agricultural plots with the same crop rotation, yet under continuous, contrasting long-term P fertilization regimes to dissect microbial community composition at two distinct P levels in the model plant *A. thaliana*.

To answer our question of how different P levels affect both the soil and root-inhabiting microbiota of a Brassicaceae species, we collected soils from a long-term P fertilization experiment, ongoing since 1949 (Gransee and Merbach 2000; Merbach et al. 2000). Experiments were then conducted under controlled environmental conditions using the sampled soils in the presence and absence of the plant host. At harvest, fresh biomass weights were taken of the rosettes, while roots were handled such that the rhizosphere (RH; soil firmly adhering to roots) and endophytic compartment (RT) constituted two distinct root-associated fractions. Amplicon profiling targeting the bacterial 16S rRNA gene and fungal internal transcribed spacer 1 (ITS1) region were employed to study the effects of soil P availability on the microbial soil and root-associated community structure.

MATERIALS AND METHODS

Soils and sampling from natural site. Soils used for this experiment were obtained from the long-term P fertilization field ("Field D") trial at the Julius Kühn Experimental Station at Martin Luther University of Halle-Wittenberg (51°29′45.6′′N, 11°59′33.3′′E) (Gransee and Merbach 2000; Merbach and Deubel 2007; Merbach et al. 2000). In this trial, three phosphate fertilizer forms are applied on 6 × 5 m plots: superphosphate, basic slag, and alkali sinter phosphate (the last form was terminated in 1995). Plots are used for crop production with a 6-year crop rotation (alfalfa–2 years, potato, winter rye, sugar beet, and spring barley) and are fertilized in autumn according to the application regiment. Samples of 16 soils under superphosphate fertilization regime with P levels P1 and P3 (0 and 45 kg P ha^{-1} $year^{-1}$, respectively) situated in two strips (Supplementary Fig. S1 provides field design information) were taken from unplanted fields using a soil core (10 cm diameter × 15 cm depth). Strips were harvested independently in the middle of March (strip 1) and beginning of April (strip 2). Approximately 2 cm of the topsoil was discarded and the remaining lower 13 cm of soil was transported and stored at 4°C until use. Soils were homogenized with a mesh sieve wire (5×5 mm^2) and about 220 g of soil was added to a pot ($7 \times 7 \times 7$ cm^3).

Soil analysis. Soils were analyzed by Labor für Boden- und Umweltanalytik (Eric Schweizer AG, Thun, Switzerland) for structural composition, pH value, and nutrient content. Available macronutrients were determined by extraction with pure water (1:10 dry soil: H_2O). Total nutrient content was ascertained using extraction with ammonium acetate and EDTA (1:10 dry soil: 0.5 M ammonium acetate + 0.02 M EDTA, pH 4.65).

Plant material and growth conditions. *A. thaliana* ecotype Col-0 was exclusively used for testing root-associated and root-inhabiting bacterial communities. Seeds were surface sterilized (20 min 70% EtOH, 10 s 100% EtOH) before being planted directly onto moist soil. Sown seeds were stratified for 3 days at 4°C before being placed in a greenhouse under short-day conditions (6/18 day-night cycle; 19 to 21°C) for 7 weeks (first experiment: soil samples from strip 1) and 8 weeks (second experiment: soil samples from strip 2). Germinating seedlings were thinned in order to keep four plants per pot.

Experimental design and sample preparation. An experiment was designed using six pots for each of the 16 field plots, whereby three had no plant host and three were sown with sterile *A. thaliana* seeds at a rate of four plants per pot. To isolate compartments of interest, a fractionation protocol was used as previously detailed (Dombrowski et al. 2017; Schlaeppi et al. 2014). At harvest, roots from a single pot were severed about 5 mm below the hypocotyl and processed to obtain rhizosphere (RH; defined for these experiments as soil particles strongly adhering within about 1 mm from roots) and endosphere (RT) compartments. Freshly harvested roots were washed twice with 2.5 ml of PBS-S buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0, 0.02% Silwet L-77). RH pellet samples were obtained from particles from the first wash after centrifugation (20 min at 1,500 rpm) and removal of supernatant. RT compartments were obtained by placing roots in fresh PBS-S buffer for sonication (10×: 30 s pulsation, 30 s pause) to remove tightly adhering soil particles and epiphytic microbes. Bulk soil (BS) samples were taken by weighing about 250 mg of unplanted soil (1 cm below the soil surface). All samples were frozen with liquid nitrogen and kept at −80°C until processed.

DNA extraction and sequence preparation. DNA was extracted from bead-homogenized samples using FastDNA Spin Kit for soil (MP Biomedicals, Solon). RH and BS samples were homogenized once in the presence of MT and sodium phosphate buffers, whereas RT samples were homogenized twice (once in the absence and again after addition of buffers). Samples were eluted in nuclease-free water and DNA concentration of eluate was determined via Quanti-iT PicoGreen dsDNA Assay Kit (Invitrogen, Life Technologies, Hercules, CA) by mean of three measurements (3 cycles: 30 s at 25°C) of fluorescence absorption (iQ 5 real-time PCR; Bio-Rad Laboratories, Hercules, CA).

Samples were diluted to concentrations of 3.5 ng μl^{-1} of DNA with nuclease-free water for the first PCR reaction to amplify the V5 to V7 variable region of the 16S rRNA gene. Reactions were

prepared in triplicate in 25-μl volumes consisting of 10 ng of DNA template, 1× incomplete buffer, 0.3% bovine serum albumin, 2 mM $MgCl_2$, 200 μM dNTPs, 300 nM 799F (5′-AACMGGATTAGATACCCKG-3′; Dombrowski et al. 2017), 300 nM 1193R (5′-ACGTCATCCCCACCTTCC-3′; Dombrowski et al. 2017) and 2 U of DFS-Taq DNA polymerase (Bioron, Ludwigshafen, Germany) and the thermal cycling conditions were preheating: 2 min at 94°C, 25 cycles: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and termination: 10 min at 72°C. The same concentrations were used to amplify the fungal ITS1 region using ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′; Gardes and Bruns 1993) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′; White et al. 1990). Enzymatic cleanup (24.44 μl: 20 μl of template, 20 U of exonuclease I, 5 U of Antarctic phosphatase, 1× Antarctic phosphatase buffer; New England Labs, Frankfurt, Germany) rendered purified PCR product for second PCR after enzyme cleanup (30 min at 37°C, 15 min at 85°C; centrifuge 10 min at 4,000 rpm). During the second PCR (preheating: 2 min at 94°C; 10 cycles: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and termination: 10 min at 72°C), triplicate reactions were prepared using 3 μl of template and sample-specific barcoded primers (5′-AATGATACGGCGACCACCGAGATCTACACGACTGGCG-799F/ITS1F-3′; 5′-CAAGCAGAAGACGGCATACGAGAT-BARCODE(12-NT)-1193R/ITS2-3′) were attached to samples allowing for pooled sequencing. For the bacterial amplicons, bands of approximately 500 bp were excised from gels after gel electrophoresis (1.5% agarose in Tris-acetate-EDTA buffer; 2 h at 75 V) and purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). For fungal amplicons, PCR reactions were purified using with Agencourt AMPure XP purification kit (Beckman Coulter, Krefeld, Germany).

DNA concentration of gel- or bead-purified samples was measured with PicoGreen as mentioned previously, and approximately 30 ng of barcoded DNA from each sample was pooled before purification of the final library with Agencourt AMPure XP purification kit.

Sequencing of libraries was performed with Illumina MiSeq desktop sequencer (v3 chemistry, 2× 300 bp). Libraries were diluted to 4 nM and treated with an equal amount of 200 mM NaOH and HT1 buffer before being spiked with 10% PhiX DNA to impede luminescent saturation of clusters during sequencing. Final loading concentration of libraries was targeted at 16 pM. Sequencing data are uploaded and available within The European Nucleotide Archive (ENA) with accession number PRJEB23949.

Computational analyses. Bacterial sequence data were processed using a combination of QIIME (Caporaso et al. 2010a) and USEARCH (Edgar 2010) pipelines. First, forward and reverse reads were joined (*fastq_join*; USEARCH) with default parameters. Any reads not passing the joining command were removed from further analysis. Next, barcodes were extracted from the reads (*extract_barcodes.py*; QIIME). After demultiplexing and quality control (*split_libraries_fastq.py*; QIIME, Phred quality threshold: 30), sequences were dereplicated (*derep_fulllength*; USEARCH) and singletons were removed. Sequences were *de novo* clustered into OTUs as having ≥97% sequence identity using UPARSE (Edgar 2013) (*cluster_otus*; USEARCH). Final OTU sequences were aligned using PyNAST (Caporaso et al. 2010b) (*align_seqs.py*; QIIME) after removal of any chimeric sequences (*uchime_ref*; USEARCH) and an OTU table was generated (*usearch_global*; USEARCH) with read frequencies. Taxonomic assignment was accomplished using the UCLUST algorithm (Edgar 2010) and greengenes database (DeSantis et al. 2006) (*assign_taxonomy.py*; QIIME).

Fungal sequence data were processed in a similar way. In addition to the joined paired reads (*fastq_join*; USEARCH), all forward reads were kept as well. Both joined and all forward reads, were demultiplexed and quality controlled (*split_libraries_fastq.py*; QIIME, Phred quality threshold: 30). After this, all forward reads that had also a joined read counterpart were removed. All downstream analysis was done with the remaining forward reads and joined reads. Next, all sequences were trimmed to an equal length of 220 nucleotides. The trimmed sequences were dereplicated (*derep_fulllength*; USEARCH) and singletons were removed. For the remaining sequences, the ITS1 region was extracted using ITSx (Bengtsson-Palme et al. 2013). Sequences without a detectable ITS region were discarded. The ITS sequences were de-novo clustered using UPARSE (Edgar 2013) at a similarity level of 97% (*cluster_otus*; USEARCH). Chimeras were removed (*uchime_ref*; USEARCH) using a dedicated ITS database (Nilsson et al. 2015). All OTUs were compared with a eukaryotic ITS database using BLAST (Altschul et al. 1990). This eukaryotic database was established by downloading eukaryotic ITS region sequences from the NCBI RefSeq server (Pruitt et al. 2007). All OTUs that did not have any hit or only hits within nonfungal sequences were removed from the dataset. Taxonomic classification of OTUs was done by RDP classification (Wang et al. 2007), using the WarCup fungal ITS training set (Deshpande et al. 2016). Out of the remaining OTUs, the final OTU table was generated (*usearch_global*; USEARCH).

α-Diversity indices (Shannon and number of observed OTUs) were determined after normalizing to a depth of 1,000 reads (*alpha_diversity.py*; QIIME). β-Diversity measures (Bray-Curtis and weighted and unweighted UniFrac) were calculated using a CSS-normalized OTU table (*beta_diversity.py*; QIIME).

All statistical comparisons were computed with analysis of variance (ANOVA) using Tukey's honest significant difference *posthoc* test to determine pairwise comparisons. Principle coordinate analysis (PCoA) was done by classical multidimensional scaling of β-diversity distance matrices using the *cmdscale* function in R. Canonical analysis of principle coordinates (CAP) (Anderson and Willis 2003) was computed with the *capscale* function implemented in the *vegan* package (Oksanen et al. 2015) in R, by constraining for 'compartment', 'strip', or 'P level' conditioning for all remaining environmental variables. Statistical significance was determined by a permutation-based ANOVA test implemented in *vegan* (*anova.cca*) using 5,000 permutations. To correlate soil geochemical parameters to changes in community composition, a permutational multivariate analysis of variance using distance matrices (adonis, implemented in *vegan*) was applied.

Relative abundances of taxonomic contributions were characterized in R by selecting the six most abundant phyla and 30 most abundant families/genera based on calculated means at the respective taxonomic rank across all samples in a single compartment.

Significantly enriched OTUs of different P levels were determined with the *edgeR* package (Robinson et al. 2010) by first calculating normalization factors (*calcNormFactors*) and then estimating the negative binomial dispersion parameter (*estimateGLMCommonDisp*) as well as an empirical Bayes estimate of the dispersion with reads specified by a log-linear model (*estimateGLMTagwiseDisp*). Afterward, data were fit to a negative binomial generalized linear model (*glmFit*) and reads were tested for likelihood ratios for trends (*glmLRT*) to determine significant enrichment of OTUs at different P levels (*decideTestsDGE*), accounting for false discover rates (FDR corrected P values, where $P < 0.05$). Plots in figures were generated with ggplot2 (Wickham 2009).

RESULTS

P fertilization at the experimental site has been imposed in incremental P regimes since 1949 in either annual or triennial treatments, such that the mean annual fertilization rate of superphosphate is 0, 15, and 45 kg ha^{-1}. Plots used in our experiments encompass the lowest and highest P treatment, each replicated in two strips separated about 40 m apart, hereafter designated as P1 and P3 treatments, respectively.

The plots of the fertilization experiment are used for a continual crop rotation system (Merbach and Deubel 2007). Taken together, our single soil source set-up has the advantage of removing biases due to differences in host plants, fertilization schemes, fertilization sources, soil types, and biogeography.

Various macro- and micronutrients, and other soil characteristics were measured for a full soil geochemistry analysis and the differences in soil geochemistry were assessed by two-way ANOVA for P level and strip. We noted variation in factors pH, K_a, Ca_a, Mg_t, K_t, Ca_t, Mg_t, B, and Mn between replicate strips at the experimental site, regardless of P level with the most striking difference for pH (0.75 $\log_{10}$ unit difference; $P < 0.001$) (Supplementary Tables S1 and S2). This observation and independent cultivation of *A. thaliana* plants in soils from the two strips prompted us to introduce the strips as an additional variable in the analysis to estimate local fluctuations in soil geochemistry. Clear differences in terms of median P_a levels between P1 and P3 treatments exist in strips 1 and 2 ($P < 0.05$ and 0.1; respectively) (Fig. 1A) and, similarly, total P (P_t) is significantly increased in P3 soils of both strips ($P < 0.001$) (Fig. 1B). Compared with strip 1, median P_a values are slightly lower in strip 2 for both P1 and P3 treatments (Fig. 1A). Conversely, median P_t values are elevated for both treatments in strip 2, indicating slightly higher immobilization rates of P fertilizer in strip 2.

On the basis of yield data of crop plants grown before in the plots, we expected that the differences of P_a would also affect rosette biomass of *A. thaliana*. At harvest, rosette biomass was measured from pots containing three to four *A. thaliana* plants. Indeed, plants grown at P1 level display a reduced biomass at the end of the growing period in comparison with P3 plants (Fig. 1C). The largest effect was observed with soils from strip 1 ($P < 0.001$). Plants grown in P1 soils of strip 2 also exhibited moderately reduced median rosette biomass compared with P3 plants ($P < 0.1$).

To understand the overall influence of soil P as an edaphic factor on community structure, we harvested three compartments, BS,

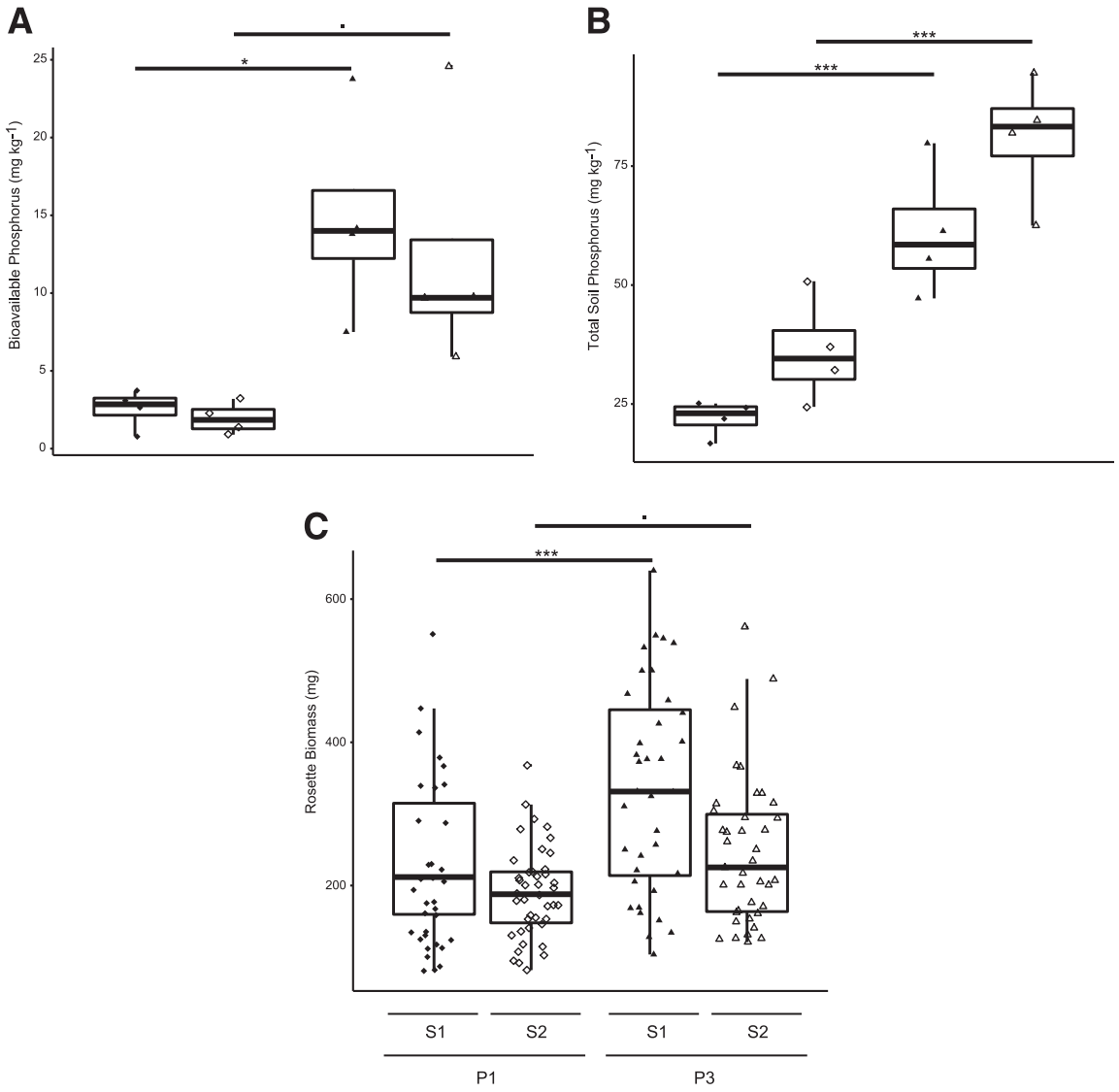

Fig. 1. P nutrient content of soils and rosette biomass at harvest are significantly higher in P3 soils. **A,** P_a (mg kg^{-1}) amount is reduced in P1 soils compared with P3 soils ($n = 8$, respectively) in both strips. **B,** P_t (mg kg^{-1}) extracted from P1 is significantly less compared with P3 soils ($n = 8$, respectively) in both strips. **C,** Rosette biomass (mg) at harvest from P1 ($n = 76$) and P3 ($n = 75$) pots indicates that plants accumulate significantly more biomass in P3 pots compared with P1 pots. Graphs are shown with samples split within P levels by strip and are distinguishable by solid (S1, strip 1) and open (S2, strip 2) shapes. Analysis of variance tests determined significance, and differences between levels of the same strip are labeled with bars (signifying comparisons) and significance values (·, $P < 0.1$; *, $P < 0.05$; and ***, $P < 0.001$).

RH, and RT from the two experiments using a previously described fractionation protocol (Dombrowski et al. 2017; Schlaeppi et al. 2014). Samples from each compartment were used in a two-step barcoded Illumina sequencing method to amplify the V5 to V7 variable region of the 16S rRNA gene for bacteria (Dombrowski et al. 2017) and the ITS1 region for fungi (Anderson and Cairney 2004; Gardes and Bruns 1993). Individual OTUs were identified by clustering sequences at ≥97% identity (Schloss and Handelsman 2006). Median final sequence counts of samples were 62,901 and 56,236 for bacterial and fungal sequences, respectively (Supplementary Table S3) after sequence filtering and quality control (see methods). Alpha-diversity (within-sample diversity) (Whittaker 1972) was determined using Shannon index. For both microbial kingdoms, we observed a progressive reduction in alpha-diversity from BS to RH to RT compartments. In addition, fungal alpha-diversity was generally lower than the bacterial one within each compartment. However, the tested soil P levels had no significant effect on alpha-diversity in each of the three compartments (Fig. 2). Nevertheless, alpha-diversity of bacterial communities for BS is significantly higher in strip 2 compared with strip 1, while the opposite trend was observed for fungal alpha-diversity (Fig. 2A and B). Transitioning from BS to RH and RT abolishes the significant differences that were attributable to the strip location (Fig. 2C to F). Thus, small differences in soil geochemical composition can inversely impact the bacterial and fungal soil biome, while the impact of the host eliminates this difference in root-associated compartments.

Beta-diversity (between-sample diversity) was measured for the fungal and bacterial communities by quantifying differences in overall community structure between samples with Bray-Curtis distances. Compartment and strip location represented the two main factors determining community structure. PCoA confirms that for bacterial communities, compartment alone explains over one-third (36.39%) of the variation in data, clearly separating the data points for each compartment. Separation along the second coordinate explains differences between the two strips (Fig. 3A). Similar tendencies are observed for fungal communities, where compartment and strip location accounted for the largest portion of variance (Fig. 3B). In addition, delineation of compartment-specific fungal communities was found to be less pronounced, indicating a less stringent role of the host in structuring root-associated fungal communities. We used a CAP method to quantify the percentage of variance explained by the factor compartment and strip: for both microbial kingdoms, compartment is the main factor influencing community structure, while strip accounts for about half of that effect (Supplementary Fig. S2). These observations are in line with previous publications when considering the two strips as different soil types. The differences in community structure between the strips can be explained by the differences in pH as tested by permutational multivariate analysis of variance (adonis test). Other soil factors were not significant except for available potassium (Supplementary Table S4).

To quantify the impact of soil P level on bacterial and fungal community variation, CAP analysis constraining for P level was applied and the significance was determined by a permutation-based ANOVA test. Across all compartments, P was observed to exert a subtle, yet significant effect on the community structure of both microbial kingdoms. We first estimated the effect of soil P level for the entire dataset and then assessed its impact on each compartment (Fig. 4). Only a very small fraction of the overall variance of the data were attributable to P fertilization for both bacteria (Fig. 4A; 1.29% of the variance; $P < 0.001$) and fungi (Fig. 4B; 1.61%; $P < 0.001$). Within a compartment, BS communities were collectively the least affected by the amount of P (3.53 and 4.13% explained variance for bacterial and fungal communities, respectively) (Fig. 4C and D). For the fungal communities, the impact of P levels increased in both root-associated compartments up to 4.5% of the explained variance (RH, $P = 0.009$; and RT, $P = 0.011$; Fig. 4F and H). A similar increase up to 4.21% of the explained variance was detected for bacterial assemblages, although its significance was found to be low (RH, $P = 0.061$; and RT, $P = 0.13$; Fig. 4E and G).

We were further interested in exploring whether any response to P was distinguishable at different taxonomic levels (i.e., at which taxonomic rank any shifts in community structure became evident as a result of P fertilization). Relative abundances were taken for different samples at phylum and family level for bacteria and at phylum and genus level for fungi. At the phylum level, none of the tested bacterial phyla showed a significant difference between the soil P levels in any compartment, whereas clear differences were seen in taxonomic composition as communities transition toward the plant–soil interface (e.g., rhizosphere) and finally colonize the plant root (Fig. 5A). There is a progressive and marked increase in the relative abundance of *Proteobacteria* and concomitant depletion of *Acidobacteria* from BS to RT, whereas *Actinobacteria* remained largely stable over the three compartments (Fig. 5A). At the family level, bacterial community structure was little affected by soil P levels with *Flavobacteriaceae* significantly ($P = 0.04$) enriched at high P level in the RH (Fig. 5B).

Fungal community structures appeared more stable across the different compartments, especially at the phylum level (Fig. 5C). The only exception is an outlier BS sample, which is completely dominated by a member of the Zygomycota. In the RT compartment, fungal community structure is more variable compared with BS and RH at the genus level, but no genera are enriched for a specific P level (Fig. 5D). At genus rank, *Spizellomyces* in BS became depleted in RH and RT compartments.

Quantitative differences based on contrasting P levels only became evident at the OTU level in each compartment. A progressive increase in the number of differentially abundant OTUs (identified by fitting a negative binomial generalized linear model to the OTU abundances) was detected from BS to RH to RT for both bacteria and fungi (45 bacterial and 36 fungal root-enriched OTUs, respectively) (Fig. 5E) and this was accompanied by an increase of their proportion in the respective root-associated compartments up to 3.5% for fungi and 2.5% for bacteria under low soil P. The aggregated relative abundance of the differentially abundant OTUs across all compartments for fungi was 7.1% and exceeded that of bacteria (3.2%), suggesting that the bacterial assemblages are more resilient to changes in soil P. Based on the distribution at phylum level, one can observe that a phylogenetic signature in the OTUs responding to altering soil P availability is lacking (Fig. 5E). Earlier extensively studied microbes involved in aiding plant P nutrition such as *Pseudomonas*, *Bacillus*, or *Colletotrichum,* are not found in the assigned OTUs (Alori et al. 2017). Differentially abundant OTUs that were independently identified in root-associated compartments of plants grown in the two strips (nine bacterial OTUs and seven fungal OTUs; Supplementary Tables S5 and S6) could represent community members that robustly respond to soil P contrasts despite local variation in soil geochemical composition. Differentially abundant OTUs unique to each strip might either reflect the differences in soil geochemical composition or represent stochastic fluctuations in low abundance OTUs between samples.

DISCUSSION

Although *A. thaliana* is a valuable molecular-genetic model for deciphering fundamental plant traits under laboratory conditions and for phenotypic variation studies in field experiments using natural genetic variation, little is known of how this plant copes with

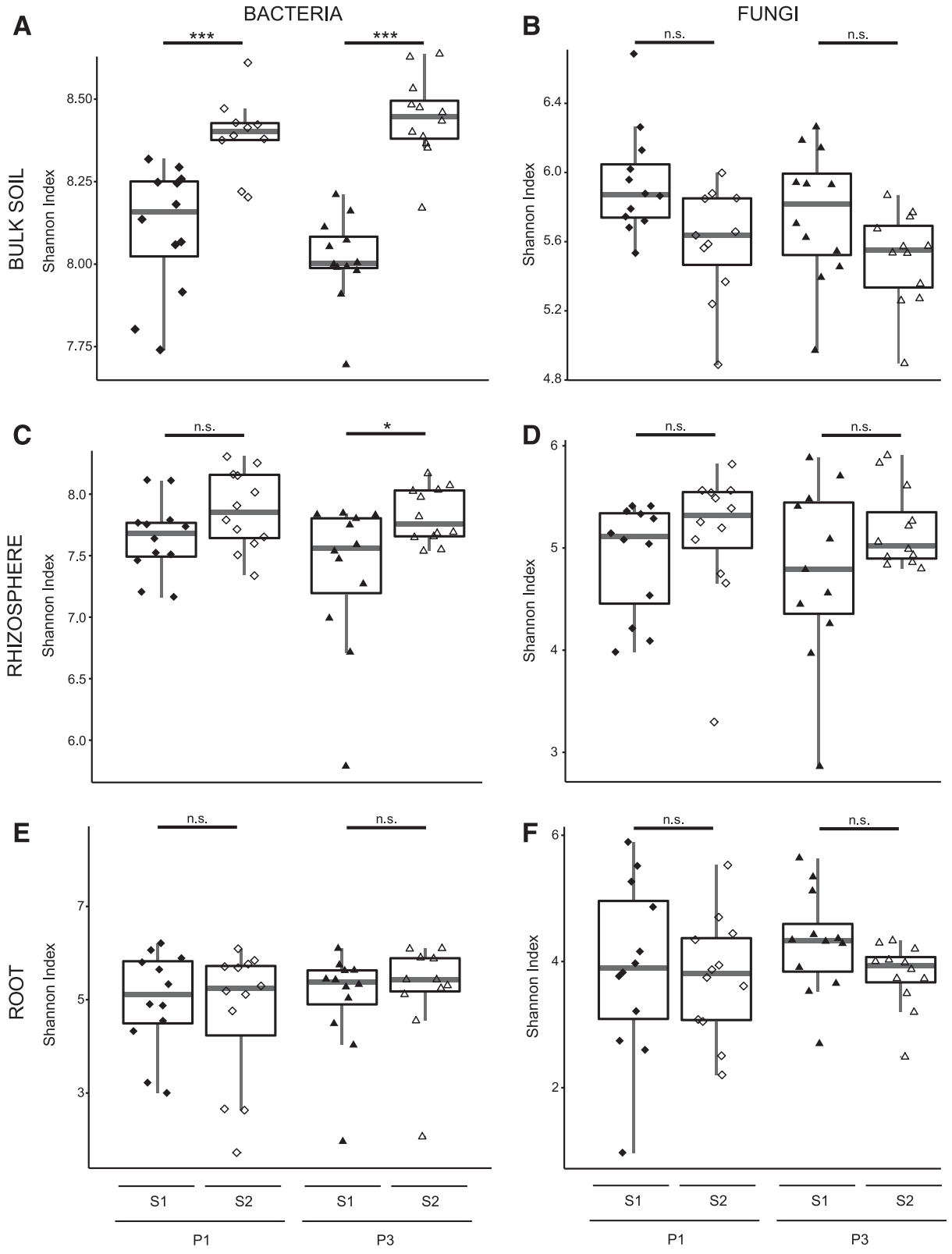

Fig. 2. Alpha-diversity of bacterial and fungal communities, as measured by Shannon index, is influenced by compartment and soil geochemistry, but not by P level. **A,** Bacterial alpha-diversity in bulk soil (BS) is significantly different between strips at the same P level and **B,** fungal alpha-diversity shows the opposite trend in BS, though these differences are not significant. **C,** Effects of soil geochemistry on bacterial alpha-diversity are reduced in the rhizosphere (RH), but may still play a significant role, whereas **D,** fungal alpha-diversity is not significantly impacted by soil type in the RH. **E,** Bacterial and **F,** fungal alpha-diversity contained within RT compartment was the lowest of all compartments and appears robust against P fertilization, remaining stable across levels and both strips. Samples are split by strip within P levels and are distinguishable by solid (S1, strip 1) and open (S2, strip 2) shapes. Compartments are ordered top to bottom: bulk soil, rhizosphere, and root. Sample sizes consist of 8 biological × 3 technical replicates for each P level. Analysis of variance testing determined significance, and differences between strips of the same level are labeled with bars and significance values (n.s., not significant; *, $P < 0.05$; and ***, $P < 0.001$).

fluctuating nutritional conditions in natural soils (Krämer 2015; Provart et al. 2016). Because of this and the fact that *A. thaliana* is generally considered a pioneer plant that can thrive in marginal soils (Mitchell-Olds and Schmitt 2006), it was essential to test whether P_a and P_t would affect rosette biomass at the tested soil P levels. Although considerable variation exists in the rosette biomass data, perhaps due to soil heterogeneity and positional effects during growth, the median rosette biomass was consistently reduced in the low P soils. This supports the use of these soils as a tool for studying the impact of nutritional constraints, exemplified here by contrasting soil P, on *Arabidopsis* growth, the root microbiota and their interaction under otherwise controlled environmental conditions. We also observed substantial differences in several soil geochemical parameters between the two sampled field strips (<40 m apart from each other) pointing to the importance of taking into account local spatial heterogeneity of soil parameters in future field experiments.

Despite more than 60 years of continuous P fertilization of the tested soil, both the soil-resident bacterial and fungal assemblages were similar despite ~6-fold difference in soil P_a and ~2.4-fold difference in soil P_t. Although P amendment of the soils included superphosphate application only, the observed effect is not limited to P_a changes, but indirectly affected also various immobile P pools as evidenced by a marked increase in soil P_t (absolute changes of P_a and P_t levels of 11 and 42 mg kg^{-1}, respectively). Our findings of subtle changes in microbial assemblages contrast with previous work on soil P availability reporting major and similar

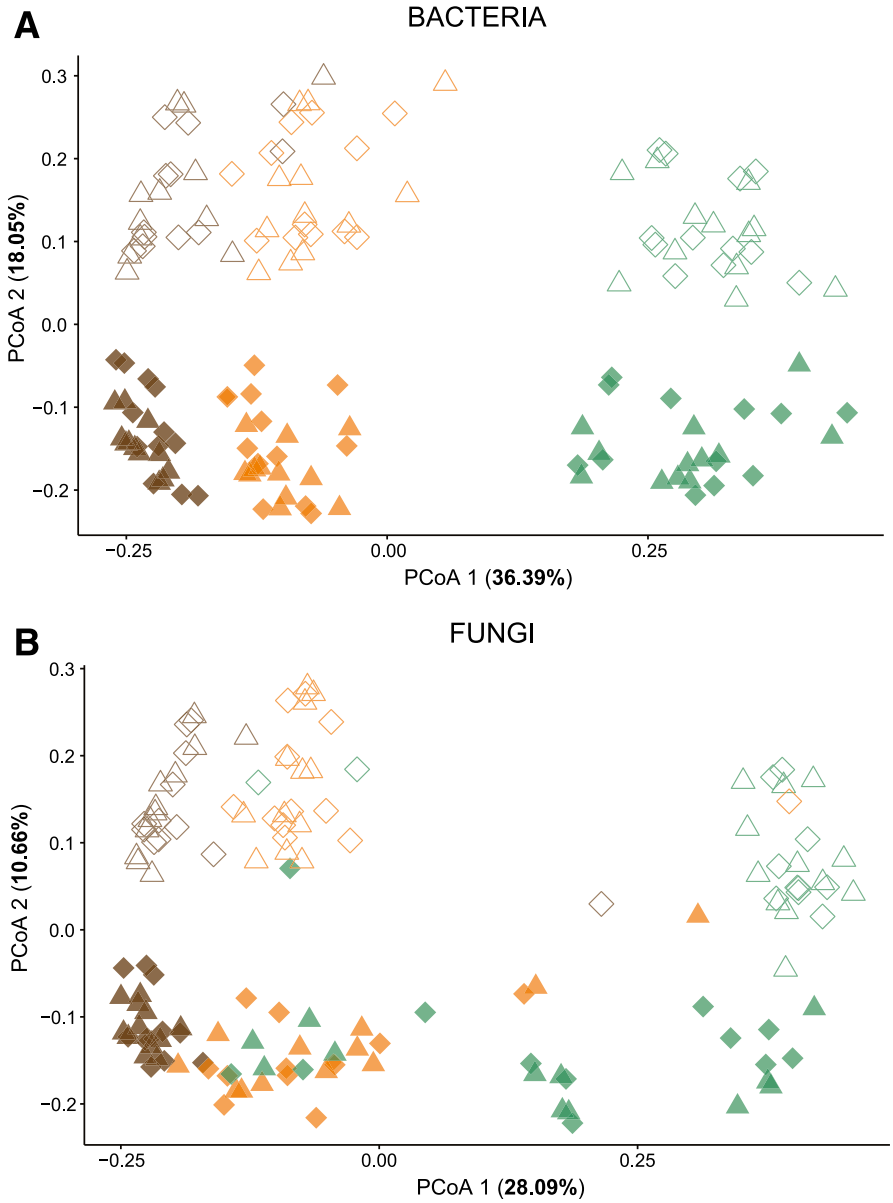

Fig. 3. Principal coordinates analysis (PCoA) of beta-diversity, measured by Bray-Curtis distances of bacterial and fungal communities, reveals that compartment and soil geochemistry are main drivers of microbial community structures. **A,** PCoA plot of bacterial beta-diversity driven highly by both the plant and soil type, explaining over 50% of the observed variance, though P level appears to have little impact on overall beta-diversity. **B,** PCoA of fungal beta-diversity shows the same trends as that seen for bacteria; however, clustering of samples was less concise for fungi particularly in strip 1. Furthermore, less than 40% of the observed variance can be explained by the plant and soil type. Brown, orange, and green represent soil, rhizosphere, and root samples, respectively. Closed and open symbols indicate strip 1 and strip 2, respectively. Diamonds and triangles indicate P1 and P3 level, respectively.

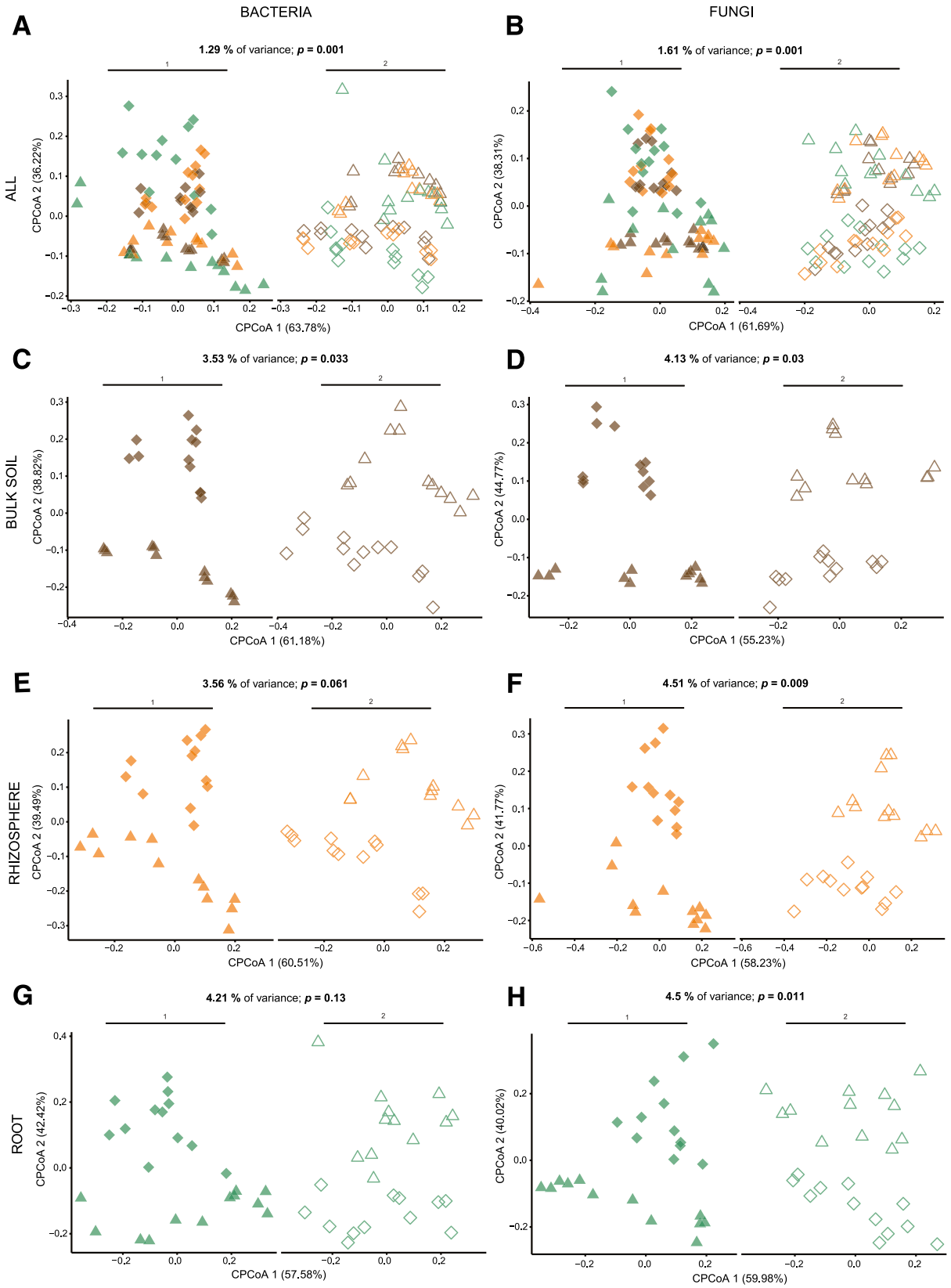

Fig. 4. Canonical analysis of principle coordinates reveals that P levels explain a small, yet significant portion of observed variation in community structures. **A,** Differences in P levels explain 1.29% of variance in bacterial community structures across all compartments, and **B,** 1.61% of variance in fungi. Separation of individual compartments demonstrates that P level explains the least variance for **C,** bacteria and **D,** fungi in bulk soil. The variance explained by P level in the rhizosphere increases for **E,** bacteria and is the highest and most significant for **F,** fungi. **G,** The highest variance explained by P level for bacteria occurs inside roots and **H,** variance explained by P level in the roots for fungi is slightly less than in the rhizosphere. Brown, orange, and green represent soil, rhizosphere, and root samples, respectively. Closed and open symbols indicate strip 1 and strip 2, respectively. Diamonds and triangles indicate P1 and P3 level, respectively.

shifts in microbial communities of grassland and forest soils irrespective of variation in geographical sites and climates (Adair et al. 2013; Leff et al. 2015; Liu et al. 2012; Tan et al. 2013; Wakelin et al. 2012). However, these studies have only investigated changes shortly after P addition and do not address potential long-term adaptation, including resilience of soil biomes. Furthermore, previous work could not distinguish whether the observed community shifts are directly caused by changes in soil P or, indirectly, are influenced by

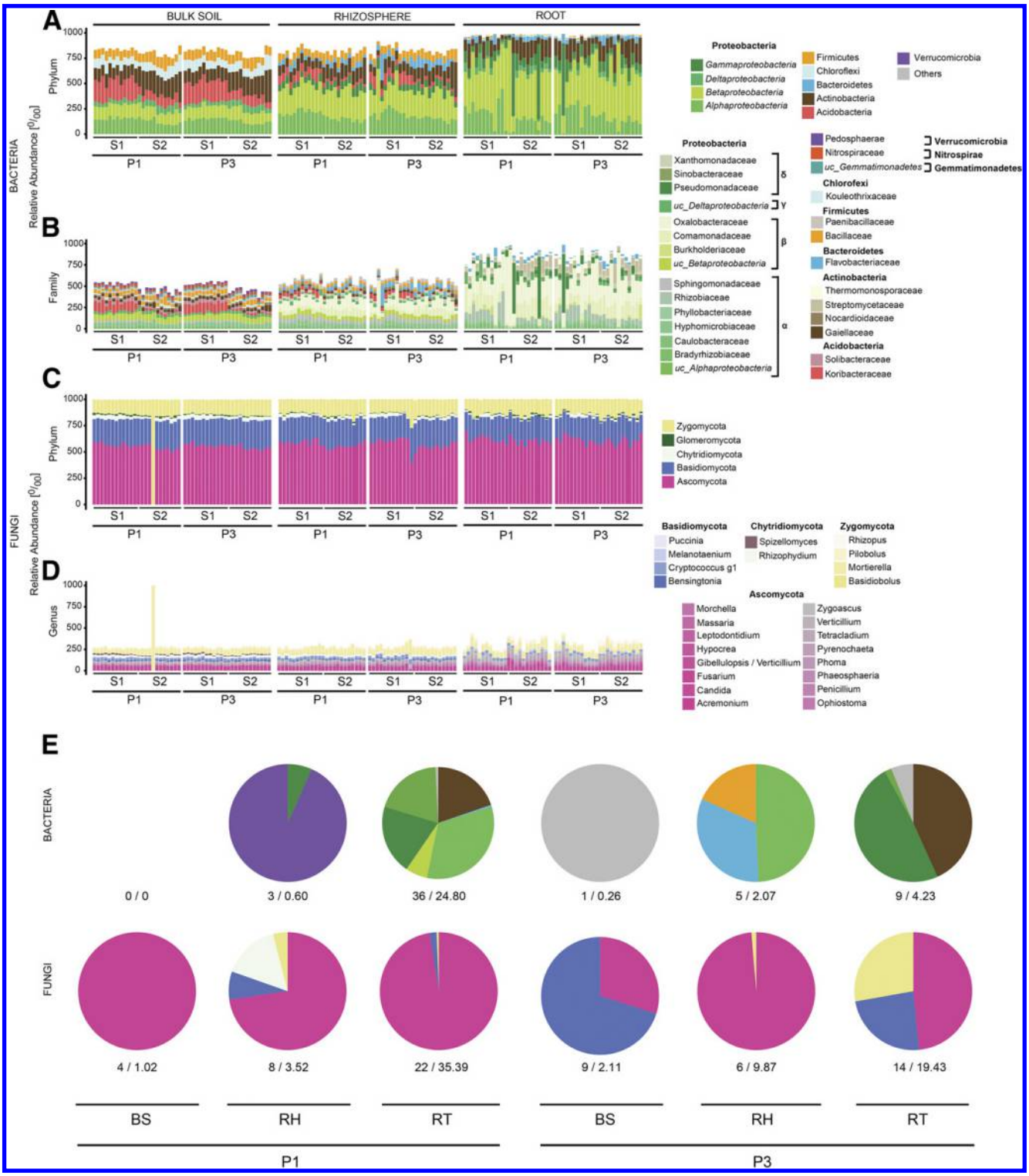

Fig. 5. Effects of P level cannot be resolved by relative abundances of bacteria and fungi at higher taxonomic levels. **A,** Relative abundances of the five most abundant phyla present in bulk soil, rhizosphere, and roots (left to right) in bacteria. **B,** Relative abundances of the 15 most abundant families present in each of the three compartments (left to right) in bacteria. **C,** Relative abundances of the 5 most abundant phyla found within the three compartments (left to right) for fungi. **D,** Relative abundances of the 15 most abundant genera in each of the three compartments (left to right) for fungi. **E,** The taxonomic distribution at phylum levels of operational taxonomic units (OTUs) enriched under P1 or P3 level. The color coding is similar to panel A and C. The numbers under the pie charts refer to the number of OTUs and the total aggregated relative abundance presented in each pie chart.

preceding shifts in plant communities. For example, it has been noted that sites exhibiting the largest effects of P addition on microbial diversity and composition are also those most affected by changes in plant diversity (Leff et al. 2015). With our experimental design, we can directly quantify the effects of soil P on the soil biota and uncouple these from potentially confounding effects of changes in plant diversity. We found that differences in soil P contribute only to 3.5 and 4.1% of the variation in soil bacterial and fungal communities, respectively, suggesting that the soil biome is largely resilient to long-term P fertilization. However, we cannot rule out that the specific long-term crop rotation at the experimental site has contributed to a change of the soil biome via a feedback of the root microbiota. Irrespective of soil P levels, we noted in soil an inverse relationship in alpha-diversity between bacterial and fungal assemblages (Fig. 2A and B). An increase in bacterial diversity between strip 1 and 2 correlates with a decreased diversity for soil fungi, suggesting reciprocal interkingdom interactions that might balance overall microbial soil diversity. Although soil pH differs in the two tested strips by 0.75 $\log_{10}$ units and soil pH is known to differentially affect bacterial and fungal communities (Rousk et al. 2010), we cannot rule out that variation in other edaphic factors contributes to the observed differences. Besides microbial diversity, pH also significantly explains the differences in microbial community structure between strip 1 and 2.

The subtle differences in soil fungal and bacterial assemblages, explained by P availability, were linked to slightly larger differences in root-associated community profiles for both microbial kingdoms (4.21% of the variance for bacteria and 4.5% for fungi). Taxa contributing to this variance were significantly detected only at the OTU level, but not at higher taxonomic ranks, implying that there is no specific taxonomic lineage of bacteria or fungi responding to a P-deprived nutritional level that constrains *A. thaliana* growth. Assuming that the differentially abundant root-associated OTUs have a beneficial role for plant growth, their growth promoting activities can be mediated by a small subset of microbiota members with broad taxonomic affiliation. Alternatively, these OTUs might have no direct impact on plant growth, but their differential enrichment could be merely an indirect consequence of plant P starvation response (PSR). Higher plants including *A. thaliana* have evolved a sophisticated PSR system (Chiou and Lin 2011) that, at least under in vitro conditions, adjusts many cellular responses, including root and shoot growth, under P-deprived conditions. In our study, the P_a in soil of low P (P1; 2.3 mg of P_a kg^{-1} of soil) is probably below the P_i concentration used in typical PSR experiments (50 μM P_i or 4.8 mg of P_i kg^{-1} of medium) (Hiruma et al. 2016) and the accompanying PSR might have induced host-dependent changes in the root-associated microbiota. However, we do not know whether the chosen *A. thaliana* accession for our experiments, Col-0, is adapted to fluctuations in soil P levels. The experiments described here can be extended in future to a wide range of *A. thaliana* ecotypes to explore natural genetic variation in the host in relation to soil P levels, root microbiota composition, and plant growth.

To our knowledge, these are the first experiments that comprehensively examine the impact of P availability on long-term effects to the soil biome and its consequences on the root-associated bacterial and fungal assemblages. We have shown that P has little effect on the soil-resident bacteria and fungi across different P treatments after over 60 years of contrasting fertilization regimes. Conversely, the most significant effect of P is inside the root, demonstrating that plants grown in different P conditions have slight, but significant differences to their root-colonizing bacterial and fungal community composition. Furthermore, the cumulative change in relative abundance for fungal root-associated consortia is more than double compared with the bacterial assemblages (7.1% versus 3.2%, respectively), which may indicate a preference toward root colonization of soil-resident fungi in *A. thaliana* growing in soils with reduced P availability. Due to constraints on the marker genes used for community profiling (bacterial 16S rRNA gene versus fungal ITS region), a precise comparison of changes in community structures between the two microbial kingdoms was not possible, due to a potentially different taxonomic resolution of bacterial and fungal OTUs.

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