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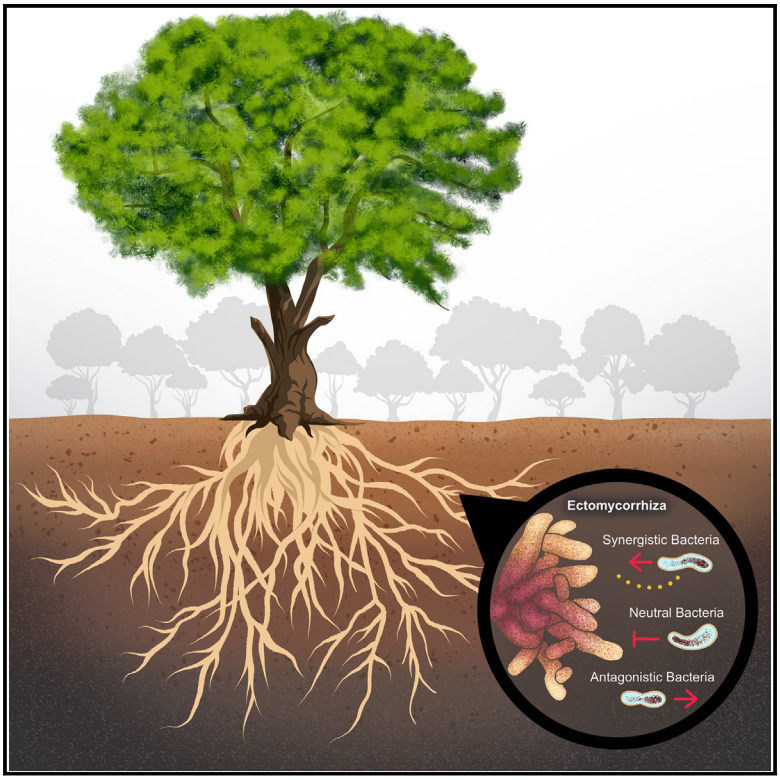
Positive interactions between mycorrhizal fungi and bacteria are widespread and benefit plant growth

Graphical abstract



Authors

Louis Berrios, Jay Yeam, Lindsey Holm, ..., Mei Lin Chin, Terry W. Henkel, Kabir G. Peay

Correspondence

berriosl@stanford.edu

In brief

Berrios et al. combine large-scale environmental sampling efforts and small-scale plant growth experiments to capture the direction, strength, and stability of bacteria-ectomycorrhiza interactions across landscape scales. These analyses suggest that multipartite interactions are a rule and not an exception in plant microbiomes.

Highlights

- Ectomycorrhizas select a small group of bacteria across a climate-latitude gradient
- Positive bacteria-ectomycorrhiza interactions are linked to plant and fungal growth
- Cytokinin and betalain biosynthesis may drive bacteria-ectomycorrhiza relationships

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Article

Positive interactions between mycorrhizal fungi and bacteria are widespread and benefit plant growth

Louis Berrios,[1,6,*] Jay Yeam,[1] Lindsey Holm,[2] Wallis Robinson,[3] Peter T. Pellitier,[1] Mei Lin Chin,[4] Terry W. Henkel,[4] and Kabir G. Peay[1,5]

[1]Department of Biology, Stanford University, Stanford, CA 94305, USA
[2]PO Box 702, Arcata, CA 95518, USA
[3]Forestry and Forest Health Program, University of California Cooperative Extension Humboldt and Del Norte Counties, Eureka, CA 95503, USA
[4]Department of Biological Sciences, California State Polytechnic University, Humboldt, Arcata, CA 95521, USA
[5]Department of Earth System Science, Stanford University, Stanford, CA 94305, USA
[6]Lead contact
*Correspondence: berriosl@stanford.edu
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SUMMARY

Bacteria, ectomycorrhizal (EcM) fungi, and land plants have been coevolving for nearly 200 million years, and their interactions presumably contribute to the function of terrestrial ecosystems. The direction, stability, and strength of bacteria-EcM fungi interactions across landscapes and across a single plant host, however, remains unclear. Moreover, the genetic mechanisms that govern them have not been addressed. To these ends, we collected soil samples from Bishop pine forests across a climate-latitude gradient spanning coastal California, fractionated the soil samples based on their proximity to EcM-colonized roots, characterized the microbial communities using amplicon sequencing, and generated linear regression models showing the impact that select bacterial taxa have on EcM fungal abundance. In addition, we paired greenhouse experiments with transcriptomic analyses to determine the directionality of these relationships and identify which genes EcM-synergist bacteria express during tripartite symbioses. Our data reveal that ectomycorrhizas (i.e., EcM-colonized roots) enrich conserved bacterial taxa across climatically heterogeneous regions. We also show that phylogenetically diverse EcM synergists are positively associated with plant and fungal growth and have unique gene expression profiles compared with EcM-antagonist bacteria. In sum, we identify common mechanisms that facilitate widespread and diverse multipartite symbioses, which inform our understanding of how plants develop in complex environments.

INTRODUCTION

Ectomycorrhizal (EcM) fungi and bacteria have outsized impacts on the functions of forests,[1,2] yet the structure of their relationships across soil niches and landscapes remains poorly understood.[3] Research has shown that EcM fungi form a keystone symbiosis with ~60% of all trees on Earth,[4] supplying trees with up to 80% of their nitrogen and 70% of phosphorus requirements.[5] Likewise, bacteria colonize the roots of every known plant species,[6] and though their ecological roles are diverse, some bacteria enhance plant fitness.[7–9] The partnerships between EcM fungi and bacteria have received considerable attention, but a lack of field-to-lab investigations has impeded progress.[10–13] For instance, small-scale investigations (e.g., greenhouse experiments) have suggested that a small group of "mycorrhizal helper bacteria" (MHBs) promote mycorrhizal-plant symbioses, enhance mycorrhizal colonization, and buffer adverse conditions for mycorrhizal growth.[14–18] Landscape-scale environmental sampling studies have suggested that EcM fungi may maintain or exclude populations of bacteria in their fruiting bodies (i.e., mushrooms) and around their hyphae.[19,20] Nonetheless, it remains unclear whether ectomycorrhizas (i.e., mycorrhizal roots) enrich for bacteria that enhance plant and EcM fungi fitness across regional landscapes. Therefore, integrating small- and large-scale approaches may help us understand the mechanistic and evolutionary processes that govern a potentially widespread, tripartite symbiosis with consequences for critical ecological processes.[21]

To date, a few themes characterize the research on MHBs and EcM fungi-tree symbioses. Inoculation-based approaches, for instance, have been used to investigate how MHBs affect mycorrhizas and seedlings. In such studies, presumed MHBs have been isolated from bulk soils of EcM tree stands, fruiting bodies (i.e., mushrooms), or the hyphal mantles of ectomycorrhizas. Reports have shown that MHBs can double the percent colonization of EcM fungi on roots and increase plant biomass.[22–25] The bacteria used in these inoculation-based experiments are usually species in the genera *Pseudomonas*, *Bacillus*, and *Streptomyces* because they can be cultured easily (see Frey-Klett et al.[17] for a review), but other genera such as *Bradyrhizobium*,[26] *Burkholderia*, and *Paenibacillus* may also have MHB members.[22] However, many of these

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inoculation-based experiments failed to account for the relative abundance of tested strains in the focal system (e.g., forests or farmlands) or used sterile soils instead of live soils. Each of these approaches can restrict our understanding of how interkingdom and multipartite interactions contribute to plant productivity and ecosystem processes because complex, microbial communities can affect bipartite interactions. Likewise, a single species of EcM fungi has often been used, despite known differences in EcM life history traits,[27,28] resource acquisition strategies,[29,30] and the diversity among EcM genera in forest soils.[31–33] Therefore, while providing some key insights, small-scale experiments have not elucidated the broader ecological functions and impacts of naturally occurring bacteria-EcM fungi-tree symbioses.

Recent investigations have deployed next-generation sequencing to understand bacteria-EcM fungi-tree symbioses within local landscapes. But these investigations tend to focus on bacterial associations with mushrooms or cannot distinguish between transient and EcM-specialized bacteria.[20,34] For instance, while Nguyen and Bruns[35] found that the bacterial genus *Burkholderia* dominated the EcM-colonized roots of Northern California pine species, they did not determine bacterial abundance in bulk soils—a soil compartment harboring immense microbial diversity. In addition, whether EcM-specialized bacteria inhabit ectomycorrhizas across the latitudinal range of the plant host or whether MHB-like bacteria form tripartite symbioses that impact plant biomass, EcM colonization, and microbial community members was unaddressed. Considering these investigations, we hypothesized that (1) diverse bacteria specialize on EcM roots compared with other soil compartments, (2) these relationships would be consistent across the plant host range and diversity of EcM fungal communities, and (3) EcM-specialized bacteria would have synergistic effects on plant and fungal performance.

To test these hypotheses, we collected 90 soil cores from three distinct regions that span the upper, middle, and lower climate-latitudinal range of Bishop pine (*Pinus muricata* D. Don). After fractionating the soils into three compartments (bulk soil, rhizosphere, and endosphere; STAR Methods), we had a total of 270 soil and root samples. We used DNA sequencing to compare the community composition of bacteria and fungi across soil compartments and geographic regions and then used greenhouse experiments with live soils, RNA sequencing (RNA-seq), and controlled inoculations of EcM fungi to investigate how the belowground interactions between bacteria and EcM fungi inform our understanding of Bishop pine development. Consistent with our hypotheses, our data show that (1) ectomycorrhizas enrich for *Burkholderia* and *Bradyrhizobium* species across the range of Bishop pine, and (2) larger, transcriptionally active populations of these bacteria in and around pine roots are associated with larger pines and higher levels of fungal colonization. In addition, we uncovered transcriptional and metabolic patterns (e.g., genes involved in betalain and cytokinin biosynthesis) that clarify the role(s) of *Bradyrhizobium* and *Burkholderia* species during tripartite symbioses. To our knowledge, this is the first field-to-lab report to demonstrate that frequent bacteria-fungi symbioses in nature underpin a widespread mutualism and contribute to above- and belowground forest dynamics.

RESULTS

Regional factors and proximity to ectomycorrhizas shape bacterial communities

To determine the community composition of bacteria across soil compartments and regions, we first derived alpha and beta diversity estimates. Alpha diversity estimates suggested that bacterial communities differ significantly across regions ($F_{2,\ 217} = 14.75$; $p = 3.92 \times 10^{-7}$) and soil compartments ($F_{2,\ 217} = 173.2$; $p = 2.0 \times 10^{-16}$). Bacterial richness was highest in the bulk soil and lowest in the endosphere (Figure S1). Beta diversity estimates further highlighted the differences among microbial communities as a function of both compartment and region (Figure 1A). Regional differences explained the most community variation for bacteria ($F_{2,\ 217} = 31.54$; $r^2 = 0.214$; $p = 0.001$). However, we detected evidence that the mycorrhizosphere naturally selects bacteria despite regional differences (e.g., rainfall and soil chemistry). For example, bulk soil and endosphere communities differed significantly ($F_{1,\ 144} = 5.99$; $r^2 = 0.04$; $p = 0.003$ false discovery rate [FDR]). Rhizosphere and endosphere communities also differed significantly ($F_{1,\ 145} = 2.82$; $r^2 = 0.02$; $p = 0.003$ FDR). However, significant differences were not observed between the bulk soil and rhizosphere communities ($F_{1,\ 144} = 1.88$; $r^2 = 0.01$; $p = 0.060$ FDR). In sum, regional differences strongly shape the structure of bacterial communities, but ectomycorrhizas also filter bacterial communities across regions (Figure 1A, bottom).

Next, we examined the taxonomic variation across compartments and regions. Acidobacteria, Actinobacteria, Bacteroidota, and Proteobacteria comprised ~75% of our dataset. The remaining ~25% of our sequences were spread out among 17 other phyla. At the genus level, we found that *Acidothermus* and *Burkholderia* were among the most abundant genera in the rhizosphere and endosphere compartments and across geographical regions (Figure 1B). DESeq2 confirmed that numerous amplicon sequence variants (ASVs), belonging to >30 bacterial genera, were enriched in the endosphere (Figure S2). For example, members of the bacterial genera *Acidothermus*, *Burkholderia*, and *Bradyrhizobium* were differentially enriched in the endosphere and depleted in the bulk soil. Differences in community composition were less exaggerated across the endosphere-rhizosphere continuum, where only six bacterial genera were differentially abundant. *Acidibacter*, *Acidothermus*, *Actinocatenispora*, *Haliangium*, and *Streptomyces* were enriched in the endosphere, and *Opitutus* favored the rhizosphere.

Although regional factors have strong effects on bacterial community composition, we show that ectomycorrhizas select for a conserved group of bacterial genera across regions. Therefore, these data suggest that the mycorrhizosphere (i.e., the soil directly adjacent to EcM roots and the fungal mantle) impacts the stability of multipartite interactions. However, because our endosphere compartment included Bishop pine rootlets without EcM-colonized roots, the bacteria enriched in the mycorrhizosphere represent a combination of plant-root- and EcM-selected taxa.

Regression models and functional assignments clarify interkingdom relationships

To clarify bacteria-EcM fungi relationships, we generated linear regression models that predict EcM relative sequence

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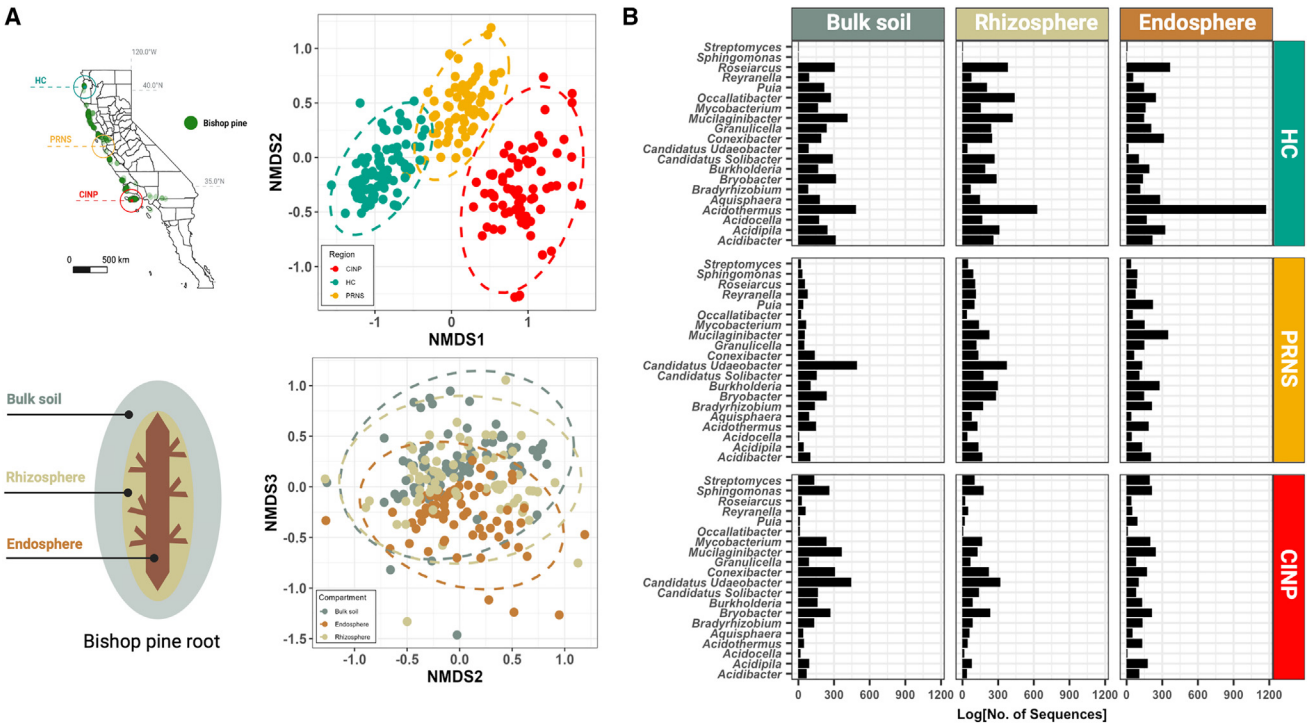

Figure 1. Diversity, taxonomy, and niche enrichments of bacterial communities

(A) Non-metric dimensional scaling (NMDS) of bacterial communities based on soil region (top) and soil compartment (bottom). The permutational analyses of variance (PERMANOVA) results for differences in community composition based on region are $F_{2,\ 217} = 31.54$; $r^2 = 0.214$; $p = 0.001$. The PERMANOVA results for differences in community composition based on soil compartment are $F_{2,\ 217} = 4.699$; $r^2 = 0.0312$; $p = 0.001$. A map of the Bishop pine distribution (green dots) and the sampling locations are shown (top). A schematic showing soil compartments relative to colonized Bishop pine roots is also shown (bottom).

(B) Log-transformed abundances of the top 20 bacterial genera faceted by region and soil compartment. HC, Humboldt County; PRNS, Point Reyes National Seashore; CINP, Channel Islands National Seashore. The sequence data presented here are 16S rDNA sequence data. See Figure S1 for additional diversity estimates.

abundance with and without selected bacterial ASV abundances. The top 20 most abundant bacterial genera (Figure 1B) that were also enriched in the endosphere (Figure S2) across regions were used as predictor variables. Several genera affected model predictions of EcM abundance (Figure 2), but we focused our analyses on genera that were also active and dominant in our RNA-seq experiments (see below for more detail). For example, *Bradyrhizobium* and *Burkholderia* species were positively associated with EcM sequence abundance, and including their ASV abundances in our models enhanced predictions of EcM abundances by ~6% and ~2%, respectively, compared with the models without *Bradyrhizobium* and *Burkholderia* ASV abundances. In contrast, *Mycobacterium* and *Sphingomonas* were negatively associated with EcM abundance, and including their species abundances enhanced our predictions of EcM abundance by an average of 3%. In addition, of the 11 genera that enhanced predictions of EcM relative abundance, *Bryobacter*, *Candidatus* Solibacter, and *Reyranella* were the only three genera that were significantly enriched in the bulk soil relative to the endosphere, which suggests that their interactions with EcM fungi may occur primarily with EcM hyphae or that they share environmental preferences with EcM fungi. Of the 19 genera analyzed, eight genera failed to enhance model predictions (Figure 2) despite their abundance in the endosphere compartment (Figure 1B), suggesting that they are endosphere specialists but not necessarily EcM specialists. Although correlational, these analyses indicate that EcM fungus-bacteria interactions are diverse in nature and have ecologically significant consequences for species abundances.

Next, we used functional annotation of prokaryotic taxa (FAPROTAX)[36] to estimate the biological function(s) of bacterial genera, focusing our attention on the bacteria that were either enriched in the endosphere (Figures 1 and S2) or affected EcM fungi's relative abundances (Figure 2). Although only 37% of bacterial ASVs were assigned to at least one function, our FAPROTAX analysis highlighted important biological functions. For example, *Bradyrhizobium* sp. were the dominant bacteria capable of nitrogen fixation in our dataset, accounting for 48% of all sequences associated with nitrogen fixation. Five other genera (each genus accounting for ≤20% of nitrogen fixer abundance) were also detected. However, these five genera were neither enriched in the endosphere nor were they abundant in our dataset, which suggests that their functions are likely marginal and independent of EcM fungi (Figure S3). Despite its unlikely contribution to EcM fungi abundance (Figure 2), the genus *Acidothermus* accounted for most of the cellulolytic functions assigned by FAPROTAX (~82%). This observation, paired with the enrichment of *Acidothermus* in the endosphere (Figure 1B),

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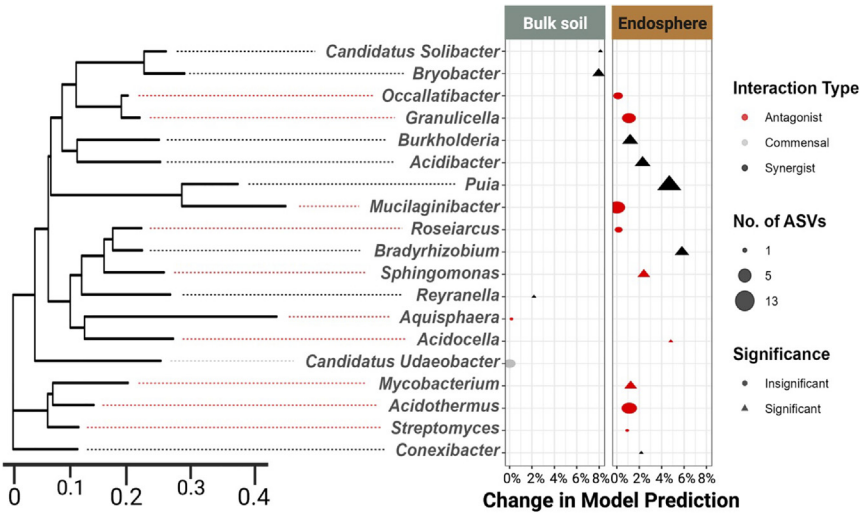

Figure 2. Regression models explaining EcM fungi abundances across landscapes

Phylogeny of representative bacteria enriched in the endosphere or bulk soil compartment (DESeq2, strata = region). The scale bar represents the number of nucleotide substitutions per site (bootstrap = 1,000). Interaction types are defined by pairwise correlation coefficients. The changes in model prediction (y axis) represent the change in proportional reduction in error (PRE) as a function of bacterial presence. Higher numbers indicate greater increases in model precision. Model comparisons were aided by the *supernova* and *stats* R packages, and significance was determined when $p < 0.05$. ASVs are grouped by genus, and the number of differentially abundant ASVs are shown for each genus to represent intra-genus diversity. Genera are faceted by their dominant compartment (differentially abundant according to DESeq2). Each regression model was built using EcM fungi relative abundance as the response variable, with bacterial relative abundance, region, soil compartment, and soil chemistry as predictor variables (see Table S1 for full model outputs). The sequence data presented here are either 16S or ITS1 rDNA sequence data. See Figures S2 and S3 for additional taxa-function data.

suggests that *Acidothermus* species are degrading plant root material yet not necessarily yielding detectable effects on EcM fungal abundance (see discussion for more detail). In addition, although FAPROTAX captured 34 additional biological functions (e.g., nitrate respiration and fermentation), none of these functions informed our understanding of direct bacteria-EcM fungi interactions because the taxa assigned to these functions were neither enriched in the endosphere nor were they associated with EcM fungi. Collectively, our estimates of bacterial function suggest that *Bradyrhizobium* and *Acidothermus* species may play key roles in biological nitrogen and carbon cycling in the mycorrhizosphere, respectively. Moreover, our analyses highlight the limitations of FAPROTAX and demonstrate that higher resolution genetic analyses (e.g., whole genome sequencing and RNA-seq) are required to both test these estimates of function and refine taxa-function relationships.

Synergisms among bacteria, EcM fungi, and plants as indicators of plant performance

To determine how our observed synergisms in the field (Figure 2) function in the context of plant growth, we first used MG-RAST to quantify transcripts in our RNA-seq data to examine the interactions between an EcM fungus (*Suillus pungens*), Bishop pine seedlings, and bacteria in a greenhouse experiment where experimentally induced water stress created large variations in plant and fungal growth (Table S2; see Erlandson et al.[37] for additional details). We chose to focus this analysis on *Bradyrhizobium* and *Burkholderia* species, given their wide distribution (Figure 1), endosphere enrichment (Figure S2), and detectable relationships with EcM fungi abundances in the field (Figure 2). In addition, these two genera were the most abundant and prevalent EcM-synergist taxa throughout our RNA-seq dataset. The abundance of *Bradyrhizobium* RNA inside roots was a strong predictor of overall EcM colonization percentage (SS = 0.170; F value = 12.71; p = 0.007), *S. pungens* sequence abundance (SS = 43.2; F value = 11.02; p = 0.01), and pine biomass (SS = 0.0255; F value = 9.8; p = 0.03; Figure 3A). *Bradyrhizobium* RNA from the soil were also strong predictors of EcM colonization (SS = 0.249; F value = 10.50; p = 0.004), but they failed to inform our understanding of *S. pungens* sequence abundance and pine biomass (Table S2), suggesting that root establishment is key to the synergistic effect(s) of *Bradyrhizobium* species. Similarly, we found that *Burkholderia* RNA abundance inside roots and in soil functioned as significant predictors of EcM colonization and pine biomass but not *S. pungens* sequence abundance (Figure 3; Table S2). Together, these greenhouse data reinforce our field data and demonstrate that both *Bradyrhizobium* and *Burkholderia* species are likely engaged in widespread and common tripartite mutualisms with plants and EcM fungi.

Next, we aligned bacterial transcripts for genera of interest to publicly available and complete genomes that closely matched ASVs from our environmental sequence data. Then we binned bacterial transcripts into their metabolic pathways to investigate conserved mechanisms of tripartite synergistic interactions (STAR Methods). Because we observed strong, synergistic interactions between *Bradyrhizobium*, *Burkholderia*, and EcM fungi and strong, antagonistic interactions between *Mycobacterium*, *Sphingomonas*, and EcM fungi (Figure 2), we contrasted the gene expression profiles for these four bacterial genera. We uncovered clear expression differences between synergistic and antagonistic bacterial genera (Figure 4). Synergistic bacteria expressed genes involved in 16 metabolic pathways that neither antagonistic bacterial genus expressed. Among these pathways, zeatin and sesquiterpenoid biosynthesis have been shown to support positive plant-bacteria interactions.[38,39] Likewise, both synergists and neither antagonist expressed genes involved in the biosynthesis of puromycin—an antibiotic that suppresses bacterial translation—or genes involved in the degradation of 1,4-dichlorobenzene, 2,4-dichlorobenzoate, and bisphenol A (all types of xenobiotics), which suggests that these EcM-synergist bacteria shape microbial species dynamics and buffer abiotic plant and/or fungal stressors (e.g., chemical and oxidative stress).

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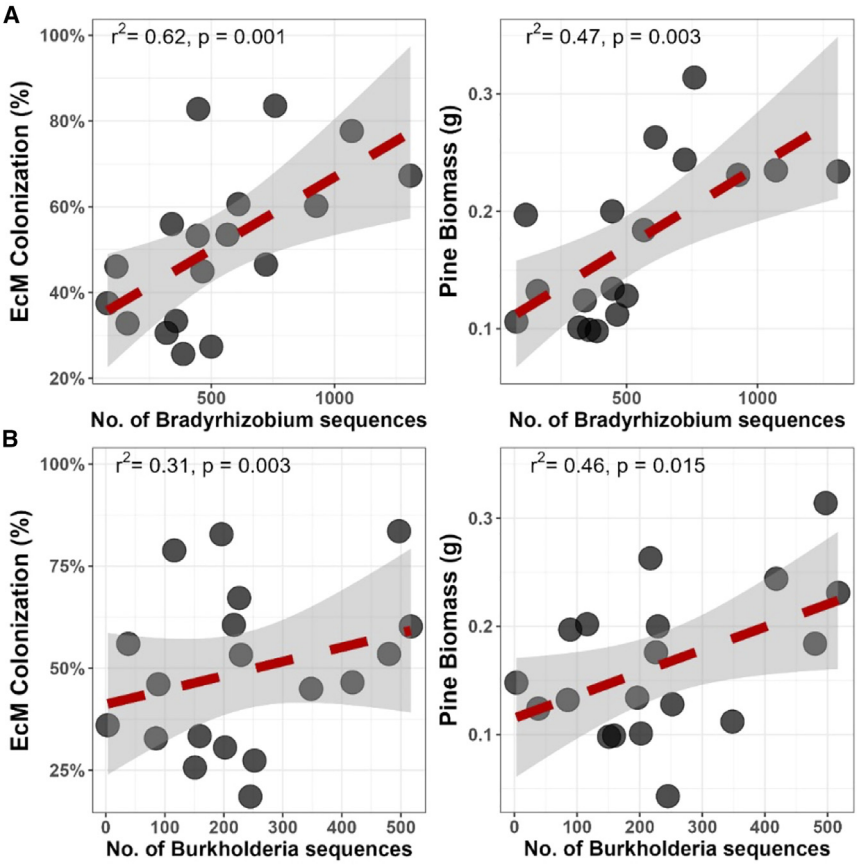

Figure 3. Tripartite synergisms as indicators of plant health

Bradyrhizobium (A) and *Burkholderia* (B) species transcript abundances in the roots correlated with EcM fungi (*Suillus pungens*) colonization percentage (left) and Bishop pine biomass (right). The red dotted lines are the regression trend lines, and the gray area around each trend line is the confidence interval within 95%. Each linear regression model was adjusted for watering regimen effects (STAR Methods), and the model using pine biomass as the response variable was also adjusted for the effects of EcM colonization. The sequence data presented here are either 16S or ITS1 rDNA sequence data derived from our transcriptomic dataset. See Figure S4 for additional transcriptomic data. See also Table S2.

We also observed additional expression patterns that inform our understanding of bacterial lifestyles in roots compared with the surrounding soil (i.e., the rhizosphere). For example, *Bradyrhizobium* species expressed genes that code for enzymes (3-deoxy-D-manno-octulosonic acid transferases) involved in puromycin biosynthesis in the soil but not in the roots, whereas *Burkholderia* species expressed these genes in both compartments. Similarly, both *Bradyrhizobium* and *Burkholderia* species expressed genes required for betalain biosynthesis—involved in oxidative stress reduction—in both the soil and roots, whereas *Mycobacterium* and *Sphingomonas* both had reduced gene expression levels for this pathway and failed to express these genes in plant roots. Other expression patterns were observed that may reveal genus-specific tripartite interactions. For example, *Bradyrhizobium* species expressed genes involved in insect hormone biosynthesis, and *Burkholderia* species expressed genes involved in penicillin and cephalosporin biosynthesis (Figure 4). In addition, while they were the dominant N-fixers identified in the field using FAPROTAX, *Bradyrhizobium* species did not express *nifH* (key for nitrogen fixation) in plant roots or the soil.

In sum, these data indicate that *Bradyrhizobium* and *Burkholderia* species form tripartite synergisms with EcM fungi and Bishop pines (Figure 3; Table S1) and may be indicators of plant and fungal health. The strong interactions among these two bacterial genera and EcM fungi in and around roots are not completely dependent on root colonization, considering their relatively high abundance in soils and soil-mediated effects (Table S3; Figure S4). The mechanisms that *Bradyrhizobium* and *Burkholderia* species use to foster these tripartite interactions converge on two bacterial factors: (1) the expression of genes involved in cytokinin production and (2) the expression of genes involved in oxidative stress management and antibiotic biosynthesis. Given the wide distribution (Figure 1), strength, and evolutionary distance between *Bradyrhizobium* and *Burkholderia* species (Figure 2), our data begin to reveal evolutionarily conserved features of tripartite symbioses.

DISCUSSION

We uncovered strong evidence for microscale and regional patterns of microbial community composition. In line with previous studies,[41–43] our data showed that regional factors shape bacterial and EcM communities, but some bacterial genera are enriched by ectomycorrhizas regardless of landscape variation (Figures 1, S1, and S2). For both bacteria and EcM fungi, species richness was highest in the bulk soil and lowest in the endosphere (Figure S1), which reinforces previous reports of microbial filtering in and around plant roots[44–46] and suggests that some multipartite engagements have been evolutionarily conserved independently of regional drivers of diversity. Although other studies have reported bacterial enrichments in the rhizosphere and endosphere,[47,48] our analyses show that these tri-kingdom relationships can be consistent across the latitudinal species range of the host plant (Figure 1).

The bacterial taxa that we identified in our environmental dataset align with previous observations in pine forests,[35,49] but our sampling approach enabled us to uncover novel findings across regions. Consistent with the findings of Nguyen and Bruns,[35] we found that *Burkholderia* species were enriched in EcM-colonized roots at Point Reyes National Seashore (PRNS). Our analyses expand these findings by showing that *Burkholderia* species are also enriched by EcM fungi in highly

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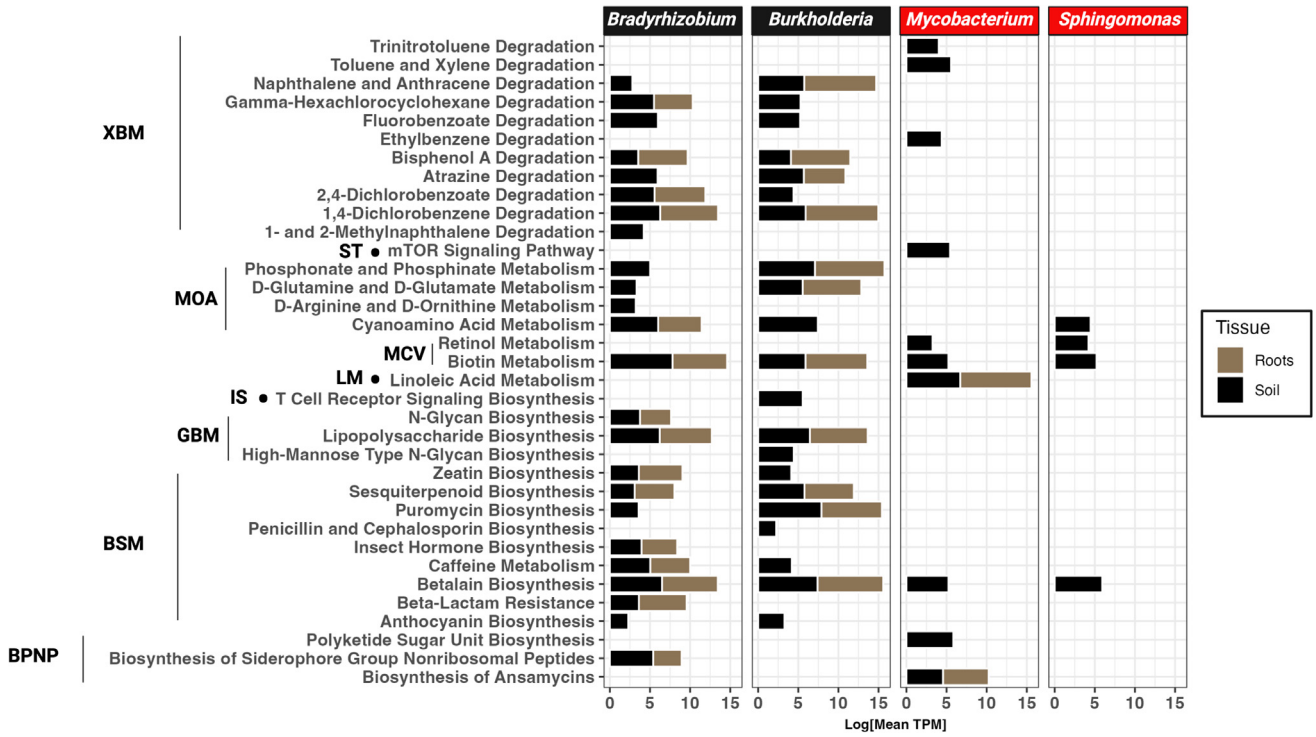

Figure 4. Bacterial transcriptomes mapped to biological pathways

Bacterial genera are grouped by their observed effect on EcM fungi relative abundances (black label = synergist, red label = antagonist) from our environmental dataset (see Figure 2). The pathways shown have (1) unique gene expression profiles to both synergists or both antagonists; (2) unique, expressed genes to a single bacterial genus; (3) genes with ≥2-fold expression differences between synergist and antagonist groups; or (4) categorical tissue-specific differences (e.g., genes expressed in the soil but not in the root or vice versa). The log transformed average transcripts per million (TPM) is shown on the x axis for both tissues (i.e., underground soil compartments; roots, n = 24; soil, n = 31). All transcripts were pseudoaligned using kallisto (Bray et al.[40]), and reference genomes were selected based on availability, completeness, and 16S rDNA sequence similarity. Pathway names are ordered on the y axis by their higher-order pathway classes. Abbreviations for the pathway classes are as follows: BPNPs, biosynthesis of polyketides and non-ribosomal peptides; BSMs, biosynthesis of secondary metabolites; GBM, glycan biosynthesis and metabolism; IS, immune system; LM, lipid metabolism; MCV, metabolism of vitamins and cofactors; MOA, metabolism of other amino acids; ST, signal transduction; XBM, xenobiotics biosynthesis and metabolism. See Figure S4 for a comprehensive illustration of all 134 pathways. See also Table S2.

disparate populations of *P. muricata* (Bishop pine) and that *Burkholderia* species function as predictors of EcM fungi abundance. Moreover, the strong cooccurrences of EcM fungi and *Burkholderia* species at PRNS were consistent over 3 years of sampling.[35] We sampled from PRNS roughly 10 years after these initial reports and found that *Burkholderia* species and EcM fungi still co-occur at PRNS, which demonstrates that these interactions are not restricted to a particular stage of forest succession. Aside from *Burkholderia* species, we also identified 10 other bacterial genera that shape the relative abundance of EcM fungi across those regions (Figure 2). Interaction types (antagonist, commensal, and synergist) were not solely predicated on 16S rDNA phylogenetic relationships. For instance, *Reyranella* and *Sphingomonas* species are closely related, yet *Reyranella* species appear to interact synergistically with EcM fungi, and *Sphingomonas* species appear to have antagonistic effects on EcM fungi (Figure 2). Examples of phylogenetic patterns by EcM interaction-type, however, were observed between *Bryobacter* and *Candidatus* Solibacter and between *Acidibacter* and *Burkholderia*, whereby each of these bacterial genera synergistically paired with EcM fungi. These observations are largely consistent with the notion that evolutionarily conserved lifestyles of microbial interactions can be observed at the genus level.[50] However, strain-specific factors that deviate from genus-level trends also likely contributed to the relationships we observed. Therefore, using whole genome sequencing to determine how diverse and conserved select genetic factors are within and between microbial species remains critical for future explorations.

Biological nitrogen fixation is an integral process for ecosystem functions,[51] and our analyses provide insight into the interkingdom interactions that shape this process. For example, although FAPROTAX identified *Bradyrhizobium* species as the most dominant nitrogen fixers in our amplicon dataset, our transcriptomic dataset suggests that *Bradyrhizobium* species do not fix atmospheric nitrogen under most soil conditions. It is possible that *Beijerinckia*, *Herbaspirillum*, *Methylocapsa*, *Methylocella*, and *Methylocystis* fix nitrogen in these systems,[52] but their aggregated abundance was less than 1% per sample. A collection of low-abundant taxa has nonetheless been postulated as a mechanism to maintain functional flexibility in plant microbiomes.[53] In terms of how nitrogen fixing bacteria (NFB) impact mycorrhizas, most of our knowledge is based on

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observations between arbuscular mycorrhizal fungi (AMF) and NFB. Afkhami and Stinchcombe[54] demonstrated that the nitrogen uptake patterns of AMFs were altered by NFB, which could then alter how nitrogen is transferred from AMF to plant roots.[55] Likewise, others have noted that the synergisms between NFB and AMF can promote plant diversity[56] and enhance crop production.[57] However, ectomycorrhizas have divergent physiologies and nutrient niches compared with AMF,[58] so it is unclear whether we should expect to observe the same relationships with EcM fungi and NFB. In line with this notion, our transcriptomic data suggest that dominant bacteria do not fix nitrogen constitutively within the ectomycorrhizas of Bishop pine.

Aside from helping to clarify the role of nitrogen fixation in these systems, we also implicated *Acidothermus* species as strong competitors for rhizodeposits (i.e., host plant photosynthates). Consistent with the estimates of FAPROTAX, our transcriptomic analyses confirmed that *Acidothermus* species express genes encoding key cellulases to break down cellulose (average TPM of cellulase family glycosylhydrolase = 50.49). The relative enrichment of *Acidothermus* species in the endosphere coupled with their potential cellulolytic activities suggests that they are strong competitors for cellulose rhizodeposits.[59] Such processes may shape interspecies interactions in ways that have gone undetected in our analyses. For instance, pH is a strong driver of microbial community composition,[6] and the acid- and thermostable cellulolytic activity of *Acidothermus* species could provide usable byproducts to neighboring, non-cellulolytic microbes.[60] These observations are particularly relevant in our northernmost sampled region (Humboldt County, HC), which had the lowest soil pH of the three surveyed regions (Table S3) and had a relatively high abundance of *Acidothermus* (Figure 1B). It is unlikely, however, that the cellulolytic activities of *Acidothermus* species provide direct fitness advantages to EcM fungi considering a lack of detectable effects in our models (Figure 2) and the fact that direct transfer of host plant photosynthates are the primary carbon source for EcM fungi.[61,62] As well as their conservation in microbiomes of various *Pinus* species, *Acidothermus* species are elevated in nematode-resistant pines.[49] Such putatively positive roles suggest that *Acidothermus* species serve context-dependent, regulatory functions that link degradation and growth processes in pine forests. Nonetheless, FAPROTAX helped guide our RNA-seq analyses and refined our understanding of the biological mechanisms that govern our focal system.

When we used transcriptomics to investigate how interkingdom interactions influence plant growth, we found that the abundance of transcripts for *Burkholderia* and *Bradyrhizobium* species predicted plant biomass, EcM colonization, and abundance relationships (Figure 3; Table S3). In contrast, other bacterial taxa that either dominated the endosphere compartment in our environmental dataset across regions (Figure 1B) or were enriched in the endosphere were not consistently observed in our RNA-seq dataset, reinforcing the idea that tripartite synergisms among these bacterial taxa and ectomycorrhizas may not occur or are less common (Figure 2). Our analyses also revealed bacterial mechanisms that likely foster these synergistic relationships. For instance, cytokinins are plant hormones that aid cell division and development,[63,64] and genes encoding key enzymes involved in these pathways (tRNA delta(2)-isopentenylpyrophosphate transferase, tRNA adenosine(37)-N6-dimethylallyltransferase MiaA, and glycosyl transferase family 1) were expressed by *Bradyrhizobium* and *Burkholderia* species (EcM synergists) but not *Mycobacterium* and *Sphingomonas* species (EcM antagonists). In addition, antibiotics produced by *Bradyrhizobium* and *Burkholderia* species may function as a mechanism to modify the growth of neighboring microbial taxa. For example, *Bradyrhizobium* species had high expression levels of beta-lactam resistance genes, and *Burkholderia* species expressed genes related to beta-lactam biosynthesis genes. These results suggest that there may be complex interdependencies underlying these species' interactions that merit further analyses.[65] Moreover, EcM synergists had an elevated expression of genes involved in betalain biosynthesis compared with EcM antagonists, and they expressed these genes while inside EcM-colonized roots as well as in the soil. These genes have also been found to facilitate positive bacteria-plant interactions between *Caulobacter* species and *Arabidopsis* plants,[66] but this is the first report to detail their involvement in tripartite synergisms with fungi. Therefore, these genes may be evolutionarily conserved in interkingdom synergisms. However, comparing additional genomes and transcriptomes to pinpoint the generality and specificity of molecular functions will help refine our understanding of the genetic mechanisms involved in tripartite symbioses.

Much debate has surrounded the factors that shape bacterial and EcM fungal communities in nature. Bahram et al.[67] noted that both competition and environmental filtering can affect the abundance, composition, and gene functions of bacterial and fungal communities across the globe. In contrast, others have emphasized the importance of bacterial-fungal mutualisms in soils.[68] Neither study, however, addressed whether ecologically dominant bacteria affect plant and fungal growth. Here, we have shown that common and stable synergisms among bacteria, EcM fungi, and pine trees occur in nature and may influence plant performance. Additionally, evolutionarily conserved bacterial genes may mediate widespread tripartite symbioses. To our knowledge, this is the first report to implicate bacterial-mycorrhizal fungi symbioses as a rule for plant microbiomes and not the exception.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

L.B. designed the experiments, conducted the analyses, and drafted the manuscript. L.B., J.Y., and K.G.P. developed sampling protocols. L.B., T.W.H., and K.G.P. edited the final version of the manuscript. All the authors contributed to field sampling and reviewed the manuscript prior to submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Amplicon datasets	Short Read Archive (SRA)	Bioproject: PRJNA961766
RNA-seq data	Short Read Archive (SRA)	Bioproject: PRJNA771376
Seedling biomass and colonization data	Dryad	https://doi.org/10.5061/dryad.qnk98sfht
Experimental models: Organisms/strains		
Pinus muricata (Bishop pine)	In house	N/A
Suillus pungens FC27	In house	N/A
Sampling sites		
Humboldt County	Humboldt County, California, USA	41°0′N, -124°1′W
Point Reyes National Seashore	Marin County, California, USA	38°04′N, 122°50′W
Channel Islands National Seashore	Santa Barbara County, California, USA	34°0′N, 119°4′W
Software and algorithms		
R statistical software	R project	https://www.r-project.org
Python programming language	Python 3.10	https://www.python.org
Kallisto	Bray et al.[69]	https://github.com/pachterlab/kallisto

RESOURCE AVAILABILITY

Lead contact

Requests for information or datasets should be directed to the lead contact, Louis Berrios (berriosl@stanford.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All datasets are included in the main text or the supplemental text. All sequence files and meta-data are available through the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA: PRJNA771376). Seedling biomass and colonization data are available through Dryad (https://doi.org/10.5061/dryad.qnk98sfht).
- All code generated during for this publication are available at https://github.com/LouisBerrios.
- Amplicon datasets are available through the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA: PRJNA961766).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Site Selections and Sample Collections

All soil and root samples were collected along coastal California within Pinus muricata (Bishop pine) forests. Samples were collected from three regions: Humboldt County (HC), California, United States (41°0′N, -124°1′W), Point Reyes National Seashore (PRNS) in Marin County, California, United States (38°04′N, 122°50′W) and Channel Islands National Park (CINP) in Santa Barbara County, California, United States (34°0′N, 119°4′W). Local plots (n=3) within each region were generally > 1 km apart to maximize spatial variability in fungal communities. The mean annual temperature (MAT) and mean annual precipitation (MAP) for each region are as follows: HC (MAT = 11°C; MAP = 116 cm); PRNS (MAT = 11°C; MAP = 91 cm); CINP (MAT = 16°C; MAP = 36 cm). The average soil pH for each region is as follows: HC (4.5), PRNS (5.2), and CINP (5.8).

METHOD DETAILS

Sample collection and processing

Field sampling was carried out between November 2021 and January 2022. We sampled from three regions that span a climate-latitude gradient in coastal California and covers a large part of the natural range of *Pinus muricata* D. Don (Bishop pine) forests. The

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northernmost region we selected was in Humboldt County (HC), California, United States (41°0′N, -124°1′W). The central and southern regions we selected were Point Reyes National Seashore (PRNS) in Marin County, California, United States (38°04′N, 122°50′W) and Channel Islands National Park (CINP) in Santa Barbara County, California, United States (34°0′N, 119°4′W). Within each region we established local plots characterized as monodominant forests comprised primarily of Bishop pine. While exact plot locations were determined by local accessibility and forest structure, they were generally > 1 km apart to maximize spatial variability in fungal communities. From each region we characterized the bacterial community, fungal community and soil chemistry from a total of 30 composite soil samples. Each composite soil sample consisted of four soil cores (total volume = 4000 cm^3) taken immediately adjacent to each other and pooled together for a single composite sample. Corers were surface sanitized between plots with 70% ethanol (EtOH), and soil washes were performed between each sample collection to limit carryover. Each composite sample was placed into a separate, clean plastic bag, and stored at 4°C within seven hours of collection. Mean annual temperature (MAT) and mean annual precipitation (MAP) for each region are as follows: HC (MAT = 11°C; MAP = 116 cm); PRNS (MAT = 11°C; MAP = 91 cm); CINP (MAT = 16°C; MAP = 36 cm). The average soil pH for each region is as follows: HC (4.5), PRNS (5.2), and CINP (5.8). Soil chemistry analyses were performed at Brookside Laboratories in New Bremen, OH 45869 (see Table S3 for soil chemistry data).

To determine the microscale variation of bacterial and fungal communities, we fractionated each composite soil sample into three operationally defined compartments: bulk soil, rhizosphere, and endosphere (protocol adapted from Nuccio et al.[70]). To do this, the samples were first sieved through a 2 mm gauge (pre-washed with 70% EtOH). The soil that filtered through the 2 mm sieve was classified as bulk soil and was collected into a separate, sterile 50 mL Falcon tube. The soil that remained on top of the sieve was aseptically analyzed using a dissecting microscope to locate Bishop pine roots colonized by EcM fungi. While we chose root segments that had visible EcM colonization, these fractions contained non-mycorrhizal subtending root material and likely some non-mycorrhizal rootlets given that rhizosphere soil necessarily obscured our view of the root surface. This pool of roots was scraped clean of adhered soil with surface-sanitized tweezers and a dissecting needle. The remnant soil was classified as rhizosphere and was collected into a separate, sterile 15 mL Falcon tube. The roots were then vortexed in a sterilized phosphate buffer solution to remove excess soil from the root surface. The cleaned roots were characterized as endosphere and were then pulverized with a mortar and pestle and placed into a sterile 15 mL Falcon tube. While our endosphere communities may include organisms that remain on the rhizoplane (i.e., root surface), we chose not to try and remove them chemically (e.g., using EtOH and/or bleach) as this has been reported to alter the composition of bacterial organisms within roots.[71] Large composite samples were also required to obtain sufficient material for downstream analyses, particularly for the endosphere and rhizosphere compartments. Consequently, our results are likely conservative because they underestimate differentiation across compartments. Nonetheless, our procedure resulted in a total of 3 regions x 30 soil composite samples x 3 compartments, totaling 270 field samples.

DNA extractions, library preparation, and amplicon sequencing

We characterized the field microbial communities by first weighing soil and root material to 0.25 g subsamples for DNA extractions. Soil and root subsamples for DNA extractions were stored at - 20°C until extractions were performed. Some rhizosphere and endosphere samples did not provide enough colonized root material for DNA extractions. However, 243 of 270 potential samples (90%) had sufficient material for DNA extraction. All extractions were performed within one week of sample processing. DNA extractions were conducted using a DNeasy PowerSoil Pro Kit according to the manufacturer's specifications (Qiagen, Carlsbad, CA, USA). DNA extracts functioned as PCR templates to determine both bacterial and fungal communities. First step PCR amplifications were performed by mixing 1 μl of template DNA, 5 μl of MyTaq HS Red Mix, 3.2 μl of PCR-grade H_2O, and 0.4 μl of each primer (515f/806R primer pair to amplify the V4 hypervariable region of the bacterial 16S rRNA gene or the KYO1/KYO2 primer pair to amplify the ITS1 subregion of the fungal rRNA operon). The PCR amplifications occurred in 96-well plates and consisted of 35 cycles with a denaturation temperature of 95°C, an annealing temperature of 50°C (52.5°C for fungi), and an extension temperature of 72°C. Indexed tags for Illumina sequencing were attached during a second step PCR amplification, which consisted of eight cycles with identical temperature settings as the first step PCR. Gel electrophoresis was used to confirm PCR products, and a magnetic bead purification method using Sera-Mag SpeedBeads (MilliporeSigma, Munich, Germany) was used to clean resulting PCR products. A Qubit fluorometer (Thermo Fisher, Waltham, MA, USA) was used to quantify cleaned DNA. Lastly, we pooled our samples into two libraries at equimolar concentrations and submitted them for 2 x 300 Illumina MiSeq sequencing at the Stanford Functional Genomics Facility. Raw sequences are available the National Center for Biotechnology (NCBI) Short Read Archive (SRA: PRJNA961766).

Bioinformatics

We received 14,625,012 demultiplexed reads (avg. per sample = 61,450) for our bacterial dataset and 10,031,548 demultiplexed reads (avg. per sample = 61,923) for our fungal dataset. After filtering, denoising, merging forward and reverse reads, and removing chimeric sequences using the DADA2 workflow,[72] our bacterial dataset consisted of 6,692,319 reads (avg. per sample = 28,119), and our fungal dataset consisted of 8,257,743 reads (avg. per sample = 50,974). The DADA2 workflow accuracy was evaluated by inferring and matching mock community members to their expected sequences (residual error rate = 0%). Taxonomic classifications were assigned to bacteria and fungi using the SILVA and UNITE databases, respectively.[73,74] Sequences assigned to 'chloroplast' and 'mitochondria' were removed from our bacterial dataset. For both our bacterial and fungal datasets, we removed taxa with less than 10 sequences and removed samples with less than 1000 sequences. Samples were rarefied to a read depth of 5,000 using

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the rarefy_even_depth function in the *phyloseq* package in R.[75] After rarefaction, we retained 218 samples in our bacterial dataset and 213 samples in our fungal dataset. Our bacterial dataset consisted of 6,069 amplicon sequence variants (ASVs), and our fungal dataset consisted of 2,769 ASVs.

Functional profiles were assigned for both bacterial and fungal datasets. We used FAPROTAX (Functional Annotation of Prokaryotic Taxa) to assign functional profiles to our bacterial samples.[36] The initial analysis was conducted in Python 3.10,[76] and the following code was used to generate function tables: collapse_table.py -i faprotax.taxon.table.rare3.txt -o functional_table.rare2.tsv -g FAPROTAX.txt -c '#' -d Taxonomy –omit_columns 0 –row_names_are_in Taxonomy -r report2.txt -n columns_after_collapsing -v -s out_subtables/ -f. Sub-tables corresponding to groups were saved to 'out_subtables/'. Read counts were normalized to the total number of functional matches for each sample. Roughly 37% of bacterial ASVs were assigned to at least a single functional group. For fungi, we assigned 62% of all fungal ASVs to guild-level designations using the FungalTraits database.[77]

To identify possible lineages specializing in EcM symbiosis, we conducted phylogenetic analyses of bacterial taxa using MAFFT.[78] Representative bacterial ASVs of genera that were differentially abundant (DESeq2)[79] in the endosphere and bulk soil compartments were aligned (n=19). BMGE (Block Mapping and Gathering with Entropy) was used to select sequence regions that were optimal for phylogenetic inference.[80] The resultant alignment was used to create a tree with the R package *ggtree*.[81]

Plant growth experiments and RNA-seq analyses

We determined genetic mechanisms of bacterial-EcM-plant interactions by analyzing RNA-seq data from a greenhouse experiment with large variations in plant and fungal health. For the experiment, *Pinus muricata* D. Don (Bishop pine) seedlings were grown in live soils (where previous work has shown no pre-existing EcM inoculum)[82] and inoculated with spores of a single EcM fungus (*Suillus pungens*), which is one of the most common EcM fungi that associates with Bishop pine in the field.[83,84] Negative control seedlings were all free of EcM colonization.[37] Seedlings were exposed to four experimental watering treatments – low, medium, or high-water regimes or medium watering followed by acute drought, which mimics the natural variation that Bishop pines encounter along their natural species distributions (details can be found in Erlandson et al.[37]). Likewise, since water availability impacts all biological systems, the microbial activity-function relationships that we observe in our RNA-seq data point to common and widespread features of plant-microbe interactions. At the end of the experiment, roots and soils were collected for RNA extractions and sequencing, and plant biomass and fungal colonization were measured. The amount of root tissue used in extractions was the same across treatments and thus independent of plant biomass or fungal colonization. The MoBio RNA PowerSoil Kit was used to extract RNA from soils (Qiagen, Germantown MD, USA). The Qiagen RNeasy Plant and Fungi Mini Kit was used to extract RNA from root tissue (Qiagen, Germantown MD, USA). After RNA extraction, all samples were treated with DNase I enzyme using MoBio DNaseMax kit to remove genomic DNA, then cleaned with Zymo Research RNA Clean & Concentrator-5 (Zymo Research,Irvine CA, USA). To select for mRNA, we used the NEBNext Poly(A) mRNA Magnetic Isolation Kit. cDNA libraries were prepared from poly(A) mRNA using the NEBNext Ultra RNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina Dual Index Primers Set 1 (New England Biolabs, Ipswich MA, USA). RNA and cDNA quality and quantity were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA, USA) or a Qubit Fluorometer (ThermoFisher, Waltham MA, USA). High-quality cDNA libraries were pooled and sequenced on an Illumina HiSeq 4000 (2 x 150 bp) at the Stanford Functional Genomics Facility. A total of 228 × 106 reads were obtained from 24 root and 31 soil samples with five controls for both root and soil samples.[37] All sequences files and meta-data are available through the National Center for Biotechnology (NCBI) Short Read Archive (SRA: PRJNA771376). Seedling biomass and colonization data are available through Dryad (https://doi.org/10.5061/dryad.qnk98sfht).

We used three approaches to examine the nature of tripartite interactions in these microcosms. First, we used MG-RAST to quantify bacterial taxa in root and soil samples, which uses BLAT, SILVA, GenBank, SEED, IMG, UniProt, KEGG, and eggNOGs databases to match and align input sequences (clustering at 97% identity).[85] An average of 18,439 bacterial reads per sample were recovered from our root samples (average reads aligned = 1.4%). An average of 372,731 bacterial reads per sample were recovered from our soil samples (average reads aligned = 12%). The low number of bacterial reads obtained and aligned is a product of poly-A selection. Thus, most of reads aligned to either the *Suillus pungens* reference transcriptome (average reads aligned = 21%) or *Pinus* genomes (average reads aligned = 15%), and most of the bacterial reads aligned to rRNA genes (~60%). Second, we downloaded genomes from GenBank for key bacterial taxa (i.e., those that were (1) among the top 50 bacterial genera, (2) abundant in the endosphere across regions (i.e., HC, PRNS, CINP; see Figure 1B), and (3) shown to affect model predictions of EcM fungal relative sequence abundance; see Figures 2 and S4) and then used kallisto[69] using default parameters and 100 bootstraps to map transcripts in each sample back to reference genomes. The reference genomes were selected based on availability and closest sequence matches based on 16S rRNA gene data. The accession numbers for each bacterial genus used are GenBank: NC_008578.1 (*Acidothermus cellulolyticus* 11B), GenBank: NC_004463.1 (*Bradyrhizobium diazoefficiens* USDA 110), GenBank: NZ_CM000774.1 (*Burkholderia pseudomallei* 1106b, Chromosome 1), GenBank: NZ_CM000775.1 (*Burkholderia pseudomallei* 1106b, Chromosome 2), GenBank: NZ_AP022569.1 (*Mycobacterium cookii* strain JCM 12404), GenBank: BMJC00000000 (*Puia dinghuensis* CGMCC 1.15448), and GenBank: NZ_CP090059.1 (*Sphingomonas* sp. DM2-R-LB4). Lastly, we mapped expressed genes to metabolic pathways using the PATRIC database version 3.26.4.[40] The genus *Puia* was not used for pathway mappings because mapped transcripts totaled less than 0.05% per sample. Data tables were merged and analyzed in R version 4.2.1.[86]

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QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed in R studio version 4.2.1.[86] The *phyloseq* package was used to derive alpha and beta diversity estimates.[75] Observed alpha diversity estimates are reported in Figure S1. The *microbiome* package was used to transform our abundance data and derive relative abundance measures for different regions and soil compartments.[87] We tested for differences in bacterial (n=218) and fungal (n=213) community composition between soil compartments and across geographic regions using permutational analyses of variance (PERMANOVA[88]) as implemented in the adonis2 function from the *vegan* package.[89] To account for regional differences, we tested for differences in microbial communities across compartments by restricting permutations to within geographic region (strata = region). The betadisper function was used to test for homogeneity of variances. The *DESeq2* package was used to determine differential abundance(s) of microbial genera between regions and soil compartments.[79]

To investigate interkingdom interactions in the field, we used linear regression models to examine correlations between the abundance of bacterial genera and EcM fungi from our amplicon datasets. Our models were generated with the *stats* R package using the Benjamini-Hochberg p-value correction method.[86] For each bacterial genus that was among the 20 most abundant in the endosphere and was also enriched in the endosphere (using DESeq2), soil chemistry, soil compartment, and geographical region were included as co-correlates to examine the effects of bacteria on EcM fungi independent of environment. The R-squared and proportional reduction in error were then compared between models to determine the impact of bacterial abundance (predictor variable) on EcM abundance (response variable).

To investigate interkingdom interactions in the lab, we constructed linear regression models that predicted ectomycorrhizal colonization based on bacterial transcript abundance. In total, our RNA-seq dataset included 24 root and 31 soil samples. Key covariates known to affect colonization (i.e., experimental watering treatment; Table S3) were included in our regression models. Because the amount of mycorrhizal tissue collected for RNA extraction was the same across experimental treatments, bacterial abundance estimates from RNA is not simply a function of increased fungal biomass when the percentage of EcM colonization is high or when plant biomass is large. When we analyzed the effects of bacteria on pine biomass, we also adjusted for the effects of EcM colonization since EcM colonization tends to increase plant biomass (Table S3). After controlling for these variables, our models should then indicate the direct effects of target bacterial taxa on fungal and plant growth. All models were constructed in R Studio version 4.2.1,[86] and all figures were created with the R package *ggplot2*.[90]