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Article

Stochastic Inoculum, Biotic Filtering and Species Specific Transmission Shape the Rare Microbiome of Plants

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Simple Summary: Microbes living on and inside plants can significantly affect agricultural yield and quality. Because uncommon microbes are often ignored in plant microbiome studies, this report focuses on rare seed or soil transmitted bacteria and fungi in the 17 most important species of plant. Up to half of fungal cells observed in plants were uncommon, while less than 11% of bacterial cells could be considered rare. About 21% of uncommon fungal species were transmitted via seeds to the plant, whereas about 25% of uncommon bacteria were, implying the rest must've come from soil. Shoots were usually more heavily colonized by rare microbes than roots, while root associated soil contained the least. By focusing on these uncommon microbes, some significant associations were observed such as seed transmission of the biocontrol fungal endophyte *Sarocladium zeae* into maize, *Penicillium* (which aids in phosphorus absorption) into pea and *Phaseolus*, and *Curvularia* (known to help plants resist heat stress) into sugarcane. When grown on cassava farm soil, robust bacterial colonization of roots occurred with growth promoting *Leptolyngbya* going into *Arabidopsis* and *Panicum*, and biocontrol *Streptomyces* going into cassava. Becoming aware of these uncommon associations may allow scientists to optimize them to help plant agriculture, while breeders and seed companies should try to conserve them.

Abstract: A plant's health and productivity is influenced by its associated microbes. Although the common microbiome is often thought to be the most influential, significant numbers of rare or uncommon microbes (eg. specialized endosymbionts) may also play an important role in the health and productivity of certain plants in certain environments. To help identify rare/specialized bacteria and fungi in the most important angiosperm plants, we contrasted microbiomes of the shoots, roots and rhizospheres of *Arabidopsis*, *Brachypodium*, maize, wheat, sugarcane, rice, tomato, coffee, common bean, cassava, soybean, switchgrass, sunflower, *Brachiaria*, barley, sorghum, and pea. Plants were grown inside sealed jars on sterile sand or field soil. About 95% and 86% of fungal and bacterial diversity inside plants was uncommon, however judging by read abundance, up to half of the mycobiome consists of uncommon fungal cells, while less than 11% of bacterial endophytes are rare. Uncommon seed transmitted microbiomes consisted mostly of Proteobacteria, Firmicutes, Bacteriodetes, Ascomycetes and Basidiomycetes that most heavily colonized shoots, to a lesser extent roots and least of all rhizospheres. Soil served as a more diverse source of rare microbes than seeds, replacing or excluding the majority of the uncommon seed transmitted microbiome. With the rarest microbes, their colonization pattern could either be the result of stringent biotic filtering by most plants, or uneven/stochastic inoculum distribution in seeds or soil. Several strong plant-microbe associations were observed such as seed transmission to shoots, roots and/or rhizospheres of *Sarocladium zeae* (maize), *Penicillium* (pea and *Phaseolus*), and *Curvularia* (sugarcane), while robust bacterial colonization from cassava field soil occurred with the cyanobacteria *Leptolyngbya* into Arabidopsis and *Panicum* roots, and *Streptomyces* into cassava roots. Some abundant microbes such as *Sakaguchia* in rice shoots or *Vermispora* in *Arabidopsis* roots appeared in no other samples, suggesting they were infrequent, stochastically deposited propagules from either soil or seed (impossible to know based on the available data). Future experiments with culturing and cross inoculation of these microbes between plants may help us better understand host preferences and their role in plant

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productivity, perhaps leading to their use in crop microbiome engineering and enhancement of agricultural production.

Keywords: rhizosphere; phyllosphere; endophyte; plant microbiome; plant mycobiome; rare microbiome; fungi; bacteria; microbes; soil microbiology; inoculum; microbial ecology

1. Introduction

Modern plants are considered holobionts; an amalgam of different microbes that have co-evolved with the host to better survive and cope with numerous biotic and abiotic stresses [1]. Amongst the numerous beneficial plant-associated microbes, the most famous are arbuscular mycorrhizal fungi which extend through the soil to increase the absorptive area of the root (90% of plant species have this), aiding in nutrient acquisition, and nitrogen fixing bacteria colonizing the roots of leguminous plants [2]. Other classical examples of beneficial plant microbe interactions include the stress resistance conferred to grasses by seed transmitted *Epichloë* fungi [3] and biocontrol of take-all disease in wheat rhizospheres by antibiotic producing strains of *Pseudomonas* bacteria [4]. With the extensive technological advances realized in DNA sequencing in the past few decades, an immense diversity of additional plant associated "difficult-to-culture" microbes have begun to be observed, raising the uncomfortable realization that we do not understand how most of these microbes contribute to the life cycle of the plant, where they came from or the rules of microbial community structuring. Agricultural science's aspirations to optimize microbiomes, improving crop resilience and productivity will only be realized if we understand more about the structure, function and provenance of plant microbiomes [5-7].

The makeup of microbial populations occupying plants may vary by host genotype, plant age, geographic location, sampling date, and by tissue type sampled [8-10]. The biomes of most interest when studying plants are its rhizosphere or the soil immediately surrounding the roots, the endosphere inside of the plant including within and between cells, and the phyllosphere which includes all the above-ground surfaces of the stem and leaves. Less studied, seeds have also begun to be understood as important microbial habitats containing diverse bacteria and fungi that can contribute to the microbiome of the next generation of plants [11]. Besides a few examples of vertically transmitted endophytes, traditionally most plant inhabiting microbes have been believed to derive from soil, passing through the spermosphere and rhizosphere before colonizing the seed, phyllosphere and/or endosphere in much the same way that mycorrhizae and rhizobia do [6,12]. Horizontal transfer of some microbes is also believed to occur as plant surfaces come into contact with insects [13], dust, rain, and other plants [14]. Having landed on a plant surface, microbes either have to gain symbiotic access or enter the endosphere through cracks, wounds or stomata [15].

With an interest in bioprospecting for agriculturally useful plant associated microbes, we have attempted to ascertain how and where it is best to search. While soil is clearly an important source of the most well-known beneficial plant associated microbes [2] we have previously found that maize seeds are also an important source bacterial endophytes [16] which go on to dominate root endospheres [17] and rhizospheres [18]. Many other publications have likewise been discovering seeds to be rich sources of non-pathogenic bacteria and fungi [11,19,20] which can go on to form significant parts of the microbiomes of tomato, maize, rice, wheat, and *Arabidopsis thaliana* [11,21]. It makes intuitive sense that after millions of years of holobiont co-evolution [6], plants would ensure that important symbionts are transmitted through their seed to the next generation rather than gambling that these microbes would happen to be present in the soil at the germination site [11]. Some of the important functions these seed transmitted microbes might provide is aiding in germination, protecting against pathogens, aiding in nutrient acquisition and increasing the seedling vigor [11,21,22].

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Containing multitudes of different microbes, it can be difficult to know where to begin the study of a plant's microbiome. A common approach to find ecologically important species is to search for a positive correlation between its occupancy and its abundance [23], which has been implemented in microbial ecology to help identify core microbiomes [24]. Such microbes, must have developed a robust transmission strategy, consistently colonizing the plant each generation, contributing to the host specie's growth, survival and/or reproduction [25]. Because of the theoretical importance for agriculture, attempts to find plant core microbiomes have occurred in *Arabidopsis* [26,27], grape [28], potato [29], rice [30], sugarcane [31], switchgrass [32], tomato [33] and wheat [34] to name a few. Theoretically, the common ancestor of angiosperms had a core microbiome before it diverged into monocots and dicots about 150 MYA [35], and this core microbiome may have been transmitted along with all its attendant ecological functions to modern plants. Such multi-species core microbiomes have been observed amongst the axenically sprouted seedlings of 28 different crop species [36], amongst roots of various Brassicaceae [37], amongst rhizospheres and roots from 30 species of crop plant grown in soil from surface sterilized seed [38] and in the roots of 31 plant species buried in the sand dunes of a national park in Australia [39]. In a recent publication studying the microbiomes of a 17 distinct species of plant raised inside sealed jars, we also found evidence of a core bacterial seed transmitted microbiome, as well as many bacteria and some fungi that were common across all plants [20].

While core and common members of microbiomes might perform physiological functions that are conserved across plant species, rare or uncommon microbes may also be important to the health and survival of specific plant hosts. This is the outcome of co-evolution between plants, their endophytes and pathogens - specialized processes of signaling, recognition or defense evasion [40] which restrict the microbes to a narrow range of hosts. Legumes for example enjoy a symbiosis with specific soil transmitted rhizobacterial endosymbionts which use sophisticated molecular communication with the plant to trigger the formation of symbiotic organs in the root where they fix atmospheric nitrogen in exchange for carbon [41]. Beneficial seed transmitted *Epichloë* fungi are restricted in their symbiosis to certain types of grasses [3] and similarly specific is the symbiosis between orchids and their rhizoctonia-like endosymbionts, without which their seeds will neither germinate nor survive [42]. Grasses (*Dichanthelium lanuginosum*) growing in geothermally heated soils at over 60°C are able to do so thanks to a fungal endophyte (*Curvularia protuberate*) and its beneficial virus, which if transferred out of their host can also increase the drought and heat tolerance of other plants such as tomato [43]. These host specific mutualisms/symbiosis are well known because they produce very strong or ecologically obvious plant phenotypes, however it is easy to imagine that plant-microbe interactions resulting in more subtle plant phenotypes would be harder to identify. Modern plant microbiome research generates very large amounts of data which may contain evidence of these subtle and host-plant restricted plant-microbe associations, but for practical reasons analysis often ends up focusing only on the most common or core microbes.

This paper aims to document the uncommon (appearing in less than 53% of samples) microbes transferred by seeds or soil and inhabiting rhizospheres, roots and shoots of 17 of the most important angiosperms. These plants include the model plants *Arabidopsis thaliana* (Columbia-0) and *Brachypodium distachyon* (Bd21); the monocot crops rice (*Oryza sativa* ssp. japonica Nipponbare), wheat (*Triticum aestivum*), switchgrass (*Panicum virgatum* Alamo), maize (*Zea mays* ssp. mays B73), sorghum (*Sorghum bicolor* ssp. bicolor)*Brachiaria decumbens*, barley (*Hordeum vulgare* ssp. vulgare) and sugarcane (*Saccharum officinarum*); the dicot crops common bean (*Phaseolus vulgaris* G19833), tomato (*Solanum lycopersicum* Heinz 1706), cassava (*Manihot esculenta*), soybean (*Glycine max*), coffee (*Coffea arabica* Geisha), sunflower (*Helianthus annuus*) and pea (*Pisum sativum*). Plants were grown for up to 2 months inside sealed jars filled with either field soil or sterile sand, then harvested for DNA extraction from rhizospheres, root endospheres and phyllospheres. Microbiomes of plants raised in soil or sterile sand, were bioinformatically contrasted by the PCR amplified sequencing of their fungal ITS and bacterial 16S. Uncommon microbes transmitted by

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seeds to particular plant species and able to maintain robust populations under agricultural conditions may represent unnoticed but important ecological relationships which could contribute to our ability to fine tune plant microbiomes for agricultural benefits in the future.

2. Materials and Methods

2.1. Sources of seed

Seeds for this experiment were obtained from different sources in different countries. *Manihot esculenta* var. 19 (DI-2015), *Phaseolus vulgaris* var. G19833 and *Brachiaria decumbens* var. Basilisk (CIAT606) were donated by the CIAT Genebank (Palmira, Valle del Cauca, Colombia). *Solanum lycopersicum* var. Heinz 1706 (LA4345) was supplied by the C.M. Rick Tomato Genetics Resource Center (Davis, CA, USA). *Brachypodium distachyon* var Bd21 and *Arabidopsis thaliana* var. Columbia-0 were given to us by the Hazen lab at the University of Massachusetts (Amherst, MA, USA). The U.S. National Plant Germplasm System of the U.S. Department of Agriculture supplied us with (accession numbers in brackets): *Hordeum vulgare* ssp. vulgare var. Beaver (CIho 1915), *Helianthus annus* var. Arrowhead (PI 650649), *Panicum virgatum* var. Alamo (PI 422006 01 SD), *Oryza sativa* ssp japonica var. Nipponbare (GSOR 100), *Sorghum bicolor* ssp. bicolor var. BTx623 (PI 564163 02 SD), *Triticum aestivum* var. Prospect (PI 491568 TR04ID), *Pisum sativum* var. Aa134 (PI 269818), *Zea mays* ssp mays var. B73 (PI 550473). Cenicaña (Florida, Valle del Cauca, Colombia) gave us sedes of *Saccharum officinarum* var. CS#725 (CC93-4112 x CC91-1987), while *Glycine max* var. Paramo 29 was purchased from Semillas del Pacifico (Cartago, Valle del Cauca, Colombia). We bought seeds of *Coffea arabica* var. Geisha from Agro Ingenio (El Chantatduro, Valle del Cauca, Colombia.

2.2. Sources of substrate

River sand was bought from a hardware store in Palmira, Colombia and manually sieved using a 500 micron metal sieve. Sterilization was realized using autoclaving twice for 20 minutes at 121°C, followed by transfer to glass jars, and yet another autoclaving for 20 minutes at 121°C.

The soil (a mollisol) used in this experiment was excavated from a cassava field at CIAT near Palmira, Colombia at GPS coordinates 3.498434, -76.354959. Clods were disrupted with crushing, which were then sieved to a uniform consistency using a 500 micron metal sieve.

Autoclaved glass jars that were 7 cm wide and 13 cm tall were filled with 100 mL of sterile sand (then autoclaved again) or with 100 mL of 1:1 soil:sterile sand, then 10 mL of sterile distilled water poured in and the sealed with a plastic lid.

2.3. Experimental setup and plant growth conditions

Of each plant species, either 0.5 g of small seeds or 20 large seeds were immersed in sterile, distilled water within 2 mL or 15 mL tubes, then soaked for 6 hours. 50% of the soaked seed were then transferred to a sterile Whatman #1 filter paper (GE HealthCare: USA) inside a sterile Petri dish, at which point they received 3 mL of sterile water, while the other 50% received 1 g of field soil resuspended in 3 mL of sterile water. Plates were sealed and together with seeds, were incubated at 32°C for several days in the dark until seeds germinated.

Once germinated, seedlings were transplanted 2 at a time to a glass jar filled either with sterile sand or a blend of soil and sand. Jars were incubated in a Panasonic MLR-352H Plant Growth Chamber set at 28°C for 12 hours with 5 lumens of fluorescent light, and for 12 hours at 22°C of dark. Plants were allowed to grow from 2 weeks to 2 months, until they achieved a significant size or until they hit the lid of the jar. Before plants were harvested, lids were detached inside a laminar flow hood, and plants permitted to dry off for 24 hours (Figure 1A, 1B). There were 6 unplanted control jars that were watered with

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10 mL of sterile water and incubated in the growth cabinet for 14 days: 3 filled with sterile sand, and 3 filled with a mix of sand and field soil.

2.4. Harvesting rhizospheres, phyllospheres, spermospheres, root and seed endospheres

The harvesting of spermospheres and seed endospheres involved either 2 (maize, phaseolus, sunflower), 5 or 0.1 g (Arabidopsis, Brachiaria, sugarcane) of seeds of each species. These were positioned inside a 15 mL conical tube along with 5 mL sterile, distilled water, then incubated in darkness for 48 hours at 32°C. Tubes were shaken by hand to extricate microbes from the seed surfaces, supernatant transferred off into sterile tubes as spermospheres and these were then frozen at -80°C. The remaining seeds were then surface sterilized/cleaned of DNA by with 30 minutes of incubation in full strength bleach (6% Na_2HPO_4), then rinsed 3 times in sterile, distilled water and frozen at -80°C. Two reps of spermosphere and seed endosphere of each species were harvested (68 samples).

For each plant species, 3 repetitions per substrate were sampled by pooling plants inside each jar and then separating them into rhizosphere, root and shoot (306 samples). Using sterile forceps and scissors, phyllospheres were harvested by clipping each shoot just above where it emerged from the substrate, any remaining seed coat was removed, the shoot relocated to a clean 50 mL tube, cut into smaller pieces within the tube, and then frozen at -80°C. Rhizospheres were collected from unwashed roots that had been exhumed, that were then shaken free of loosely attached soil, and subsequently relocated into 50 mL conical tubes. To each tube there was then 10 mL of sterile, distilled water added, followed by vigorous shaking by hand, with the resulting "muddy water" decanted off into a separate 15 mL tubes and labelled as rhizosphere which was then frozen at -80°C. After removal of rhizosphere, the roots were washed many more times with sterile, distilled water until both the root surfaces and wash water were clean and clear. Using sterile scissors, clean roots were then cut into pieces within the tube, relocated to a fresh 50 mL conical tube, then frozen at -80°C.

2.5. Sample Preparation and DNA extraction

Frozen liquid samples (spermospheres and rhizospheres) were centrifuged at 15,000 g for 15 min, to concentrate microbial cells as a pellet. Supernatant was taken off and the procedure repeated until 3 mL of sample had been concentrated. The resulting microbial pellet was re-suspended in an additional 1 mL of unfrozen rhizosphere or spermosphere. On the other hand, after unfreezing, to shoots, roots and seeds in tubes was added 1 mL of sterile, distilled water and five 6.35 mm carbon steel ball bearings, followed by hand shaking until the liquid took on the consistency of thick soup.

From these slurries was taken 400 uL and transferred to 2 mL microcentrifuge tubes containing five 2.3 mm zirconia/silica beads (Cat#11079125z, Biospec Products, USA) and RNAse A, Phenolics Blocker, and Solution SL 500 uL of Qiagen Powerbead solution (Qiagen, USA). For 20 minutes these samples were shaken using a Harbil 5G-HD 5 Gallon Shaker (Part#32940, Fluid Management, USA), followed by centrifugation for 2 minutes at 13,000 RCF. 700 µL of the supernatant was transferred to a fresh tube. The rest of the protocol was followed as per Qiagen instructions with the DNeasy PowerPlant Pro HTP 96 Kit (Qiagen, USA).

2.6. Metagenomic Sequencing Library Preparation

16S and ITS amplicons were prepared for sequencing on the Illumina MiSeq platform using a 2-step PCR strategy. First was amplification of all 384 DNA samples using bacterial 16S primers and fungal ITS primers (768 PCR reactions) followed by dual labelling with 6 bp indexes and flow cell adapters. The first PCR was performed using an equimolar mix of staggered, universal fungal ITS (ITS1F and ITS2R [44]) or bacterial 16S (515FB and 806RB [45]) primers containing 19 or 20 bp 5′ tail sequences complementary to Illumina MiSeq indexing primers (Table S1). Peptide nucleic acid (PNA) blockers against chloroplasts (5′-GGCTCAACCCTGGACAG-3′) and mitochondria (5′-

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GGCAAGTGTTCTTCGGA-3′) were added to the bacterial 16S PCR reactions to reduce amplification of mitochondria and chloroplast [45]. Reactions were setup with a total volume of 25 uL including 18.3 µL of nuclease free water, 4 µL of 5X Phusion® HF buffer, 0.4 µl of each PNA blocker, 0.4 µl of each forward and reverse primer at 10 mM, 0.4 µl of 10 mM dNTPs, 0.2 µl of BSA, 0.1 µl of Phusion® enzyme (NEB, USA) and 0.5 uL of template DNA. The reaction program used was 35X(denaturation at 98°C for 10 sec, PNA annealing at 81°C for 10 sec, primer annealing at 50°C for 10 sec, elongation at 72°C for 20 sec), then a final elongation at 72°C for 5 min and a cooldown to 4 °C.

Without evaluating for reaction success, 0.5 uL of PCR product from each of the first 768 PCR reactions was used as template in a second PCR with the purpose of adding dual indexes and flow cell adapter sequences. The 768 distinct labelling reactions were realized using 24 diverse forward primers (TruSeq_F) containing unique 6 basepair long indexes, and 32 diverse reverse primers (TruSeq_R) containing unique 6 basepair long indexes (Table S1). Labelling reactions (step 2) were setup in a total volume of 25 uL including 19.2 µL of nuclease free water, 4 µL of 5X Phusion® HF buffer, 0.4 µl of each TSf and TSr primer at 10 mM, 0.4 µl of 10 mM dNTPs, 0.1 µl of Phusion® enzyme (NEB, USA), and 0.5 uL of unpurified PCR product from step 1. Reactions began with 98°C for 30 sec, 15x(denaturization at 98°C for 10 sec, primer annealing and elongation at 72°C for 20 sec), final elongation at 72°C for 5 min and then a cooldown to 4 °C.

Amplicons of these 768 labelling reactions were inspected visually for success (bacterial 16S of 428 bp and fungal ITS of 470-525 bp) on 1% agarose gels and estimates of quantity made with ImageJ [46]. Based on these estimates of amplicon quantity equimolar amounts of each PCR product within a 96 well plate was mixed into 8 molecular pools. (note: except for negative controls, reactions were repeated until there was sufficient PCR product for mixing of equimolar amounts of all samples in a 96 plate). Pooled PCR products were then concentrated using ethanol precipitation and resuspended in 10% their volume of pure water. Target amplicons were purified by loading and running 200 uL of each concentrated pool (8 different pools) on a 2% agarose gel, the appropriate bands excised using a scalpel, and gel blocks extracted employing an Omega Bio-Tek E.Z.N.A.® gel extraction kit (Norcross, Georgia, USA). The 8 purified pools were again checked visually for purity on an agarose gel, quantified using the Picogreen® dsDNA quantitation assay (ThermoFisher Scientific, USA) and sent for super-pooling and sequencing on a single 2 × 300 bp paired-end run on the Illumina MiSeq platform at a commercial sequencing facility (GENEWIZ, NJ, USA).

2.7. Bioinformatics

Data was demultiplexed by the sequencing service provider and received as FastQ files (one per sample). Additional processing involved USEARCH 11 and recommended paramters (www.drive5.com). Briefly, pair end reads were aligned, then merged, forming full length sequences known as “Uniques”, which were then quality filtered to remove unmatched and poor quality reads. Next, the software collected full length reads together at a similarity threshold of 97%, then created a representative reference sequence for each collection which is known as an operational taxonomic unit (OTU). OTUs represented by only one raw read were discarded from further analysis. To taxonomically annotate bacterial 16S OTUs, USearch was trained on the RDP training set v16 (13,000 sequences), while fungal ITS OTUs were identified by RDP Classifier [47] trained on the RDP Warcup training set v2 (18,000 sequences). OTU annotations and read counts were exported to Excel (Microsoft, USA) for analysis and visualization. Statistics were conducted by XLStat (Addinsoft, France) and PAST 4 (https://folk.uio.no/ohammer/past/). Based on taxonomic annotation, we manually removed OTUs representing mitrochondria, chloroplasts, plant ribosomes, or other non-target sequences. OTU sequences and annotations are supplied as table S2 (fungal ITS OTU counts/taxonomy) and table S3 (bacterial 16S OTU counts/taxonomy). Summed and averaged bacterial 16S and fungal ITS OTU counts are supplied as tables S4 and S5 respectively.

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3. Results

3.1. Plant growth

Sand and soil were collected and seived, seeds were soaked in water to prime for germination and revive microbial populations, then germination was conducted within Petri dishes, seedlings were transferred to sealed glass jars these were left to grow for up to two months until they were harvested for DNA extraction. Cassava plants are shown growing in jars containing sterile sand and field soil (Figure 1A) while sorgum is shown in Figure 1B.

3.2. Sequencing Summary

After removal of chimeras, followed by length, singleton and quality filtering, there were only 2,573,506 high-quality fungal ITS reads to be clustered at 97% sequence identity into 680 OTUs (Table S2). Manual checking of OTU taxonomy turned up 127 non-target OTUs that had been annotated as plant, protist or bacterial ribosome DNA, so these were also removed, leaving 1,826,066 reads. Of the 306 fungal ITS samples, 304 returned high quality data, with read counts ranging from 1 for barley sand root #1, to 44,304 for *Panicum* soil grown rhizosphere #3. Fungal ITS read counts averaged 5,967 per sample.

Bacterial 16S read data was also quality filtered, resulting in 4,119,547 high-quality reads that (at 97% sequence identity) were clustered into 1,178 OTUs (Table S3). Hand checking of bacterial OTU annotation revealed that despite our use of PNA blockers, 102 non-target OTUs were nevertheless annotated as plant/fungi mitochondria or chloroplast, thus these were eliminated, leaving 3,904,600 bacterial 16S reads. All bacterial 16S samples contained read count data, ranging from 12 for Maize soil shoot #2, to 80,970 for *Brachypodium* spermosphere #2. Bacterial 16S read counts averaged about 12,760 per sample.

To compare diversity across different samples as heatmaps, we summed reads of each OTU from all 3 repetitions, then transformed the reads into relative proportion for fungi (Table S4) and for bacteria (Table S5). The microbial sequence data generated in this study using MiSeq have been deposited and are available in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA731997 and are also provided as Supplemental Materials (annotated sequences and OTU counts).

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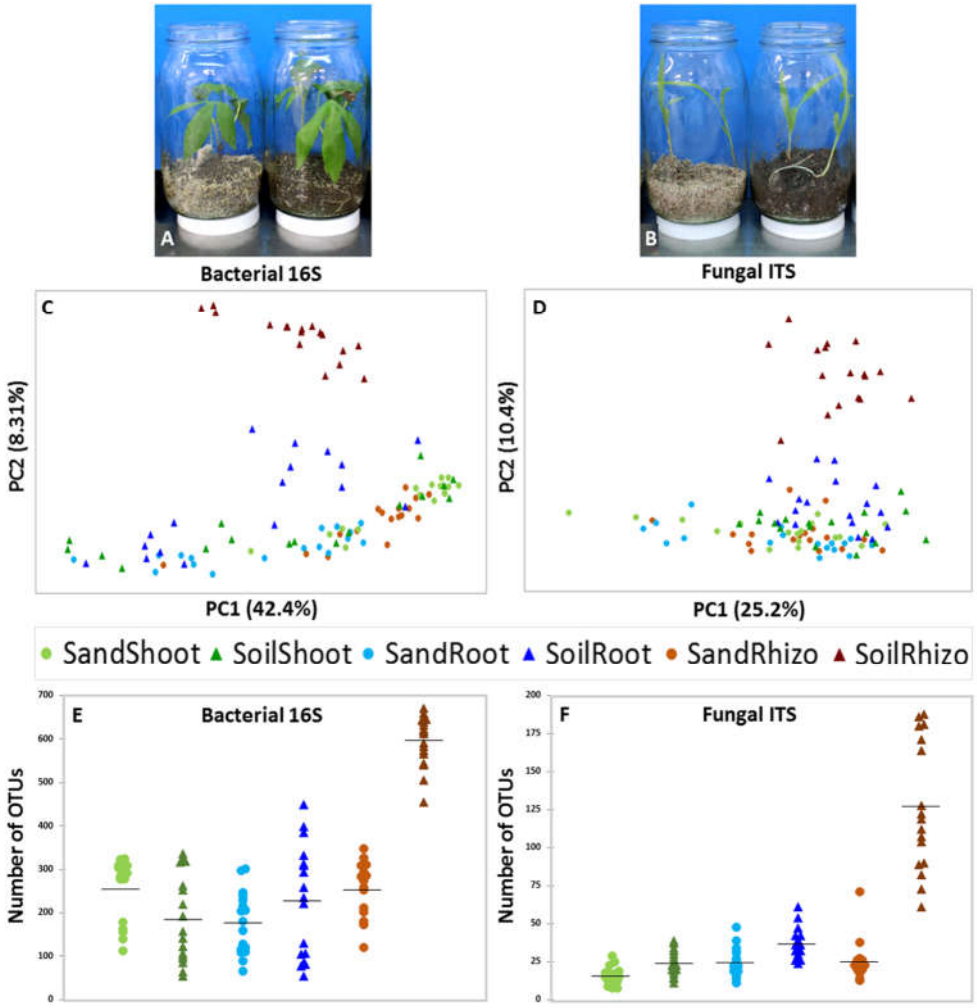

Figure 1. Statistical analysis of microbial diversity in 17 different plant species grown on sterile sand or field soil. Juvenile cassava (A) and sorghum (B) plants in sterile sand on the left, field soil mixed with sand on the right. PCA of binary transformed bacterial 16S (C) and fungal ITS (D) OTU counts. Scatterplots of the number of different bacterial 16S (E) and fungal ITS (F) OTUs observed in each sample Sand grown samples are displayed as circles, while soil grown samples are displayed as triangles. Mean values of OTUs observed are indicated by horizontal black bars.

3.3. Differences in microbial diversity between sample types

To relate the microbial diversity in different tissue types without weighting by abundance, OTU counts (summed and averaged across reps) were transformed into presence-absence data, then ordinated by principle component analysis of covariance (n-1) and visualized as scatterplots grouped by tissue type and substrate (Figure 1C, D) or the number of different OTUs per sample was plotted by substrate and tissue type, allowing visualization of differences in diversity amongst bacterial (Figure 1E) and fungal (Figure 1F) populations. The most obvious separation between groups is evident for both bacterial and fungal populations in rhizospheres of soil grown samples, which are clearly distinct from sterile sand ground samples. About half of the root bacterial and fungal samples grown on soil were also observed to shift away from sterile sand root samples. There wasn't a clear difference in the PCA of either bacterial or fungal diversity consistently varying between shoot tissues grown on sterile sand or soil.

The strong shift in rhizosphere microbial diversity is easy to see when plotting numbers of bacterial (Figure 1E) and fungal (Figure 1F) OTUs next to each other where the mean values in sand went up from 252 and 25 respectively, to 594 and 127 when grown on soil. There were only modest average increases in the number of OTUs that are gained

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by bacterial or fungal populations in roots when grown on soil relative to sterile sand, going from 177 and 24 on sand, to 225 and 37 on soil. While the average number of fungal OTUs in shoots increased slightly when plants were grown on soil instead of sand (from 16 to 24), the average number of bacterial OTUs in shoots instead went down when plants were grown on soil rather than sand (from 255 to 185).

To get a taxonomic summary of all uncommon OTUs, all reps of each treatment (eg. bacteria in soil grown maize roots) were summed together, normalized to 100%, identified as uncommon if present in less than 10/17 plant species, then averaged across tissue and soil type (eg. all uncommon fungi in sand grown shoots), categorized taxonomically, and counted or summed. Figure 2 shows the total number of uncommon fungal OTUs (Figure 2A) or uncommon bacterial OTUs (Figure 2C), as well as the average number of uncommon reads for fungi (Figure 2B) and bacteria (Figure3D). First of all, it is important to note that all plants that were grown on either field soil or sterile sand inside hermetically sealed jars nevertheless developed microbiomes containing uncommon microbes in their shoots, roots and rhizospheres. The fungal populations in sterile grown shoots contained the lowest number of OTUs with only 92 (96% of total) observed across all 17 plant species, increasing to 140 (96% of total) in roots and 155 (95% of total) in rhizospheres. Judging by read abundance, these uncommon, apparently seed transmitted microbes made up a very large proportion of the fungal population, totaling 51, 52 and 52% of the reads observed in sterile sand grown shoots, roots and rhizospheres (Figure 2B). When plants were grown on soil, the total number of uncommon fungal OTUs observed in shoots, roots and rhizospheres went up to 126 (95% of total), 203 (95% of total) and 399 (87% of total) respectively (Figure 2A). Although the total number of uncommon fungal OTUs went up when grown on soil, their average abundance went down making up only 30, 42 and 26% of the total reads in shoots, roots and rhizospheres (Figure 2B). The majority of fungal OTUs and reads observed in all plants and sample types were Ascomycetes followed by Basidiomycetes. It was interesting to note the presence of Glomeromycota (mycorrhizae) and Chrytidiomycota (root pathogens) in soil grown roots and/or rhizospheres, although they were not represented by a large number of reads.

Table 1. Sorenson similarity index comparing uncommon microbial OTUs in soil vs sand grown plants.

	Fungal Similarity between Plants			Bacterial Similarity between Plants		
	Rhizosphere	Roots	Shoots	Rhizosphere	Roots	Shoots
Arabidopsis	0.05	0.18	0.08	0.17	0.36	0.50
Barley	0.11	0.18	0.30	0.04	0.11	0.30
Brachiaria	0.08	0.15	0.11	0.21	0.07	0.07
Brachypodium	0.05	0.11	0.00	0.18	0.32	0.47
Cassava	0.06	0.13	0.16	0.14	0.30	0.41
Coffee	0.07	0.17	0.26	0.13	0.31	0.41
Maize	0.09	0.40	0.32	0.12	0.08	0.05
Panicum	0.06	0.07	0.19	0.28	0.33	0.11
Pea	0.03	0.15	0.31	0.06	0.17	0.49
Phaseolus	0.02	0.24	0.24	0.25	0.17	0.21
Rice	0.34	0.50	0.12	0.10	0.25	0.17
Sorghum	0.10	0.22	0.10	0.13	0.23	0.14
Soy	0.01	0.51	0.48	0.10	0.16	0.11
SugarCane	0.18	0.33	0.31	0.23	0.39	0.17
Sunflower	0.01	0.13	0.06	0.11	0.30	0.25
Tomato	0.09	0.17	0.38	0.27	0.36	0.46
Wheat	0.02	0.09	0.14	0.10	0.11	0.15
Average	0.08	0.22	0.21	0.15	0.24	0.26

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There was a substantial number of uncommon bacteria (478 OTUs representing 72% of the total) in sterile grown shoots that must've been transferred there through seeds, and growth in soil did not dramatically increase this number (525 OTUs representing 84% of the total). Similarly, a total of 499 (82% of total) uncommon OTUs were observed in sterile sand grown roots, which increased to 647 (88% of total) in soil grown plants (Figure 2C). Surprisingly, growth on soil reduced the number of uncommon OTUs from 486 (73% of total) in sterile sand grown plants, to 351 (38% of total). Because numbers of uncommon bacterial OTUs were usually about 3 times higher than those of uncommon fungal OTUs, it was surprising to see that these represented very little (less than 10%) of the average read abundance in a sample (Figure 2D). Uncommon bacterial OTUs in sterile sand grown shoots represented only 2% of the total, while in roots they were 6% and in rhizospheres 3%. Growth on soil vs sterile sand approximately doubled the proportion of uncommon bacterial reads in shoots, going on average from about 2% to 5% and from about 6% to 11% in roots, while in rhizospheres, soil caused a drop to only 1%. The most abundant, uncommon bacteria were Proteobacteria and Firmicutes, but in soil grown roots it was Cyanobacteria that dominated, representing 5% of the total reads.

In order to observe the ability of seeds to robustly transmit the uncommon microbiome and of growth on soil to alter it, we calculated the Sørenson similarity index by comparing the occurrence of every uncommon OTU between field soil and sterile sand grown plants (Table 1). For example, FungalOTU32 was found in maize roots grown on both sterile sand and soil, thus it was considered seed transmitted and raised the corresponding index. By this metric, seed transmission appears to have been responsible for only a small amount of the uncommon diversity observed in soil grown plants, contributing on average only 8 and 15% of fungal and bacterial diversity in rhizospheres, 22 and 24% of fungal and bacterial diversity inside roots, and 21 and 26% of fungal and bacterial diversity in shoots. Because soil grown plants had both relatively few seed transmitted OTUs and a higher number of uncommon microbes than did sterile sand grown plants, this potentially means that propagules of uncommon seed inhabiting microbes are stochastically distributed amongst seed and/or that the majority of uncommon seed microbes which are transmitted to plants are quickly displaced by soil transmitted ones (ie. most uncommon plant microbiome inhabitants are soil transmitted). It is also worth noting that there is substantial variation in plant microbiomes depending on what substrate they were grown on, with for example 0% of uncommon fungi in *Brachypodium* shoots being observed on both substrates, while there was a similarity of 51% between uncommon fungi in soy roots. Other samples with relatively high similarity between substrates were fungi in maize/rice roots and maize/soy/tomato shoots, bacteria in *Arabidopsis*/sugarcane/tomato roots and *Arabidopsis*/*Brachypodium*/pea/tomato shoots. There was much more similarity between roots and shoots (0.21-0.26) grown on different substrates, than there was for rhizospheres (0.08-0.15).

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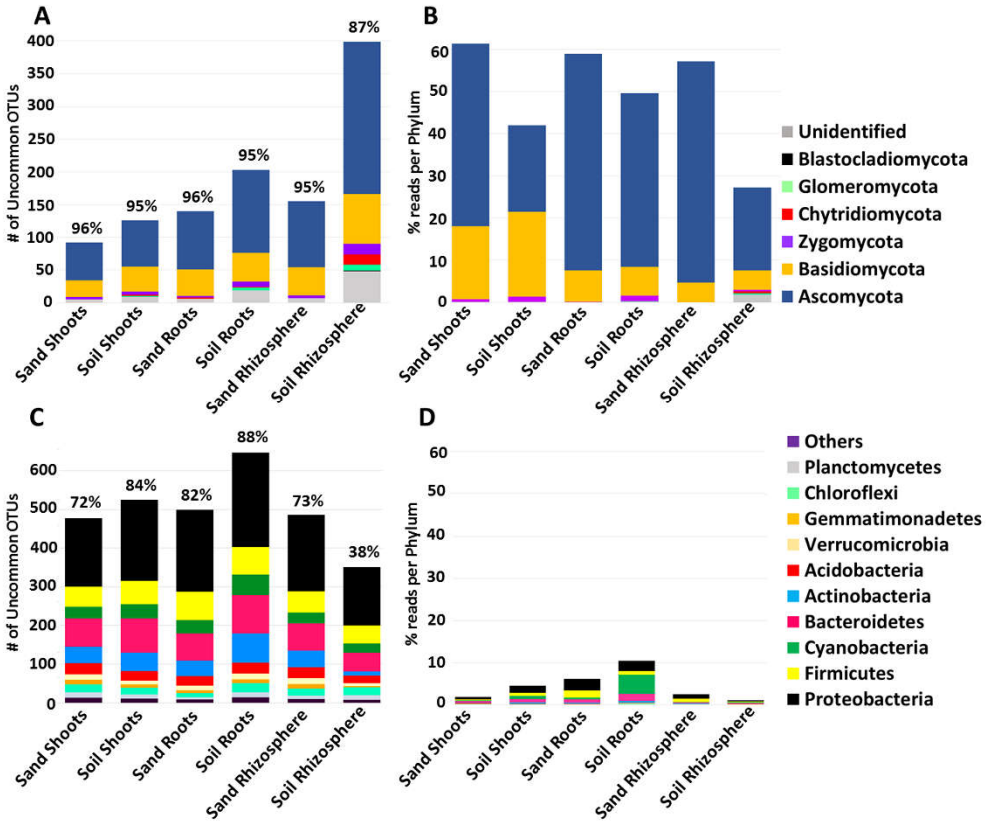

Figure 2. Phylum level classification of uncommon OTU diversity and read abundance for fungi (A and B) and bacteria (C and D) in shoots, roots and rhizospheres. Uncommon OTUs were those that were found in 9 or fewer of the 17 plant species and were calculated by addition across all plant species per sample type. Percentage of uncommon versus total OTUs in each sample type is indicated above each bar. Read proportion per phylum was the result of averaging the number of reads in uncommon OTUs across all plant species. OTU and read taxonomy is indicated by coloring according to the legends at the right.

3.4. Patterns in microbial abundance data

In an effort to visualize patterns in the abundance and distribution of uncommon microbes (appearing in less than 10/17 of samples) in different tissues of each plant species grown on either sterile sand or soil, all reads in each repetition of each sample were added together across OTUs and normalized to proportional read count %, then the top 40 most abundant but uncommon OTUs were displayed as heat maps sorted by Bray Curtis dissimilarity with hierarchical trees shown above (Figure 3). Groupings by plant are indicated with a violet bar across the top.

Focusing on the dark red squares (greater than 5% read abundance) there are few present in bacterial heatmaps with 0 in shoots, 3 in roots, and only 1 in coffee rhizospheres. Three other interesting patterns are evident depending on clustering of vertical dark blue lines where a sample type isn't registering almost any of the OTUs which are present in other plants. Nine soil grown samples including *Phaseolus*, soy, rice, maize and wheat, showed a striking absence of uncommon OTUs in shoots, suggesting that exposure to bacteria in the substrate somehow reduced diversity in above ground tissues. Conversely (and more expectedly) roots of 8 different sand grown plants including rice, soy, and wheat were low in their diversity of uncommon bacterial OTUs suggesting that a lack of inoculum resulted in a lack of bacterial endophytes. Most expectedly of all, rhizosphere populations of uncommon bacteria appear to be strongly influenced by inoculation with soil, with all 17 samples clustering together by substrate. Clustering by plant rather than by soil might indicate seed transmission of microbes, and the greatest number of instances of this phenomenon were observed in shoots where *Bracharia*, coffee and pea grouped

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together, while in roots there was only 2 groupings (*Brachypodium* and soy) and in rhizospheres only 1 (maize).

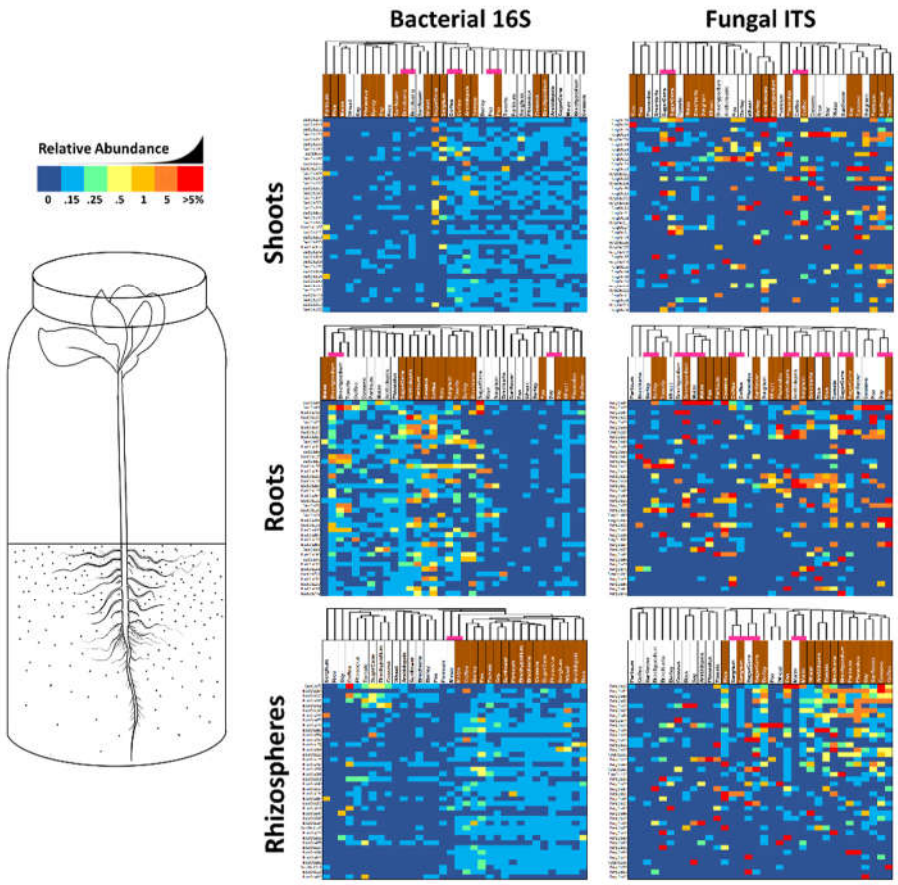

Figure 3. The 40 most abundant bacterial fungal ITS and bacterial 16S OTUs which occurred in less than 53% of Miseq samples obtained by analysis of shoots, roots and rhizospheres of 17 different plant species grown in sealed jars on either field soil or sterile sand. Reads were summed across repetitions and then transformed into relative percentages. Sand grown plants are labelled in white, while soil grown samples have a brown label. Samples were organized by Bray-Curtis dissimilarity and those that grouped by plant species are highlighted by a pink block atop the column. Square shading is by percentage value with dark blue being 0%, up to 0.1% being light blue, between 0.1-0.25% being green, 0.25-0.5% being light yellow, 0.5-1% being dark yellow, 1-5% being orange and greater than 5% being red.

Fungal heatmaps also show the importance of soil in populating rhizospheres with uncommon microbes such as FungOTUs32, 52 and 88, with most samples clustering by substrate type, exceptions being sorghum, maize and sugar cane which grouped by plant species instead. Neither root or shoot populations of uncommon fungi appeared to be strongly influenced by substrate microbiology, however there were 8 groupings of root by plant (almost half) suggesting that seed transmission to roots of uncommon fungi is significant in many species. There are two other interesting patterns in the fungal heatmaps: red fungal OTU squares quite often occur once per row and are unaccompanied by their matching plant species sample, for example FungOTU58 occurs at >5% in sand grown *Bracharia* shoots but not at all in soil grown shoots, while FungOTU76 occurs at >5% in soil grown *Arabidopsis* roots but not at all in any other root sample, suggesting that either seeds or soil (or both) are serving as a source of stochastic inoculum, and/or that very specific colonization potential exists between plants and fungi under particular growing conditions. Another interesting pattern is the horizontal doublet where two red or colored squares side by side show that a fungus is being seed transmitted to the shoots

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or roots under both sets of growing conditions. Examples include FungOTU32 in coffee/maize roots; FungOTU16 in *Arabidopsis*/coffee roots; FungOTU166 in soy roots, FungOTU338 in sugarcane shoots; and FungOTU170 in coffee shoots.

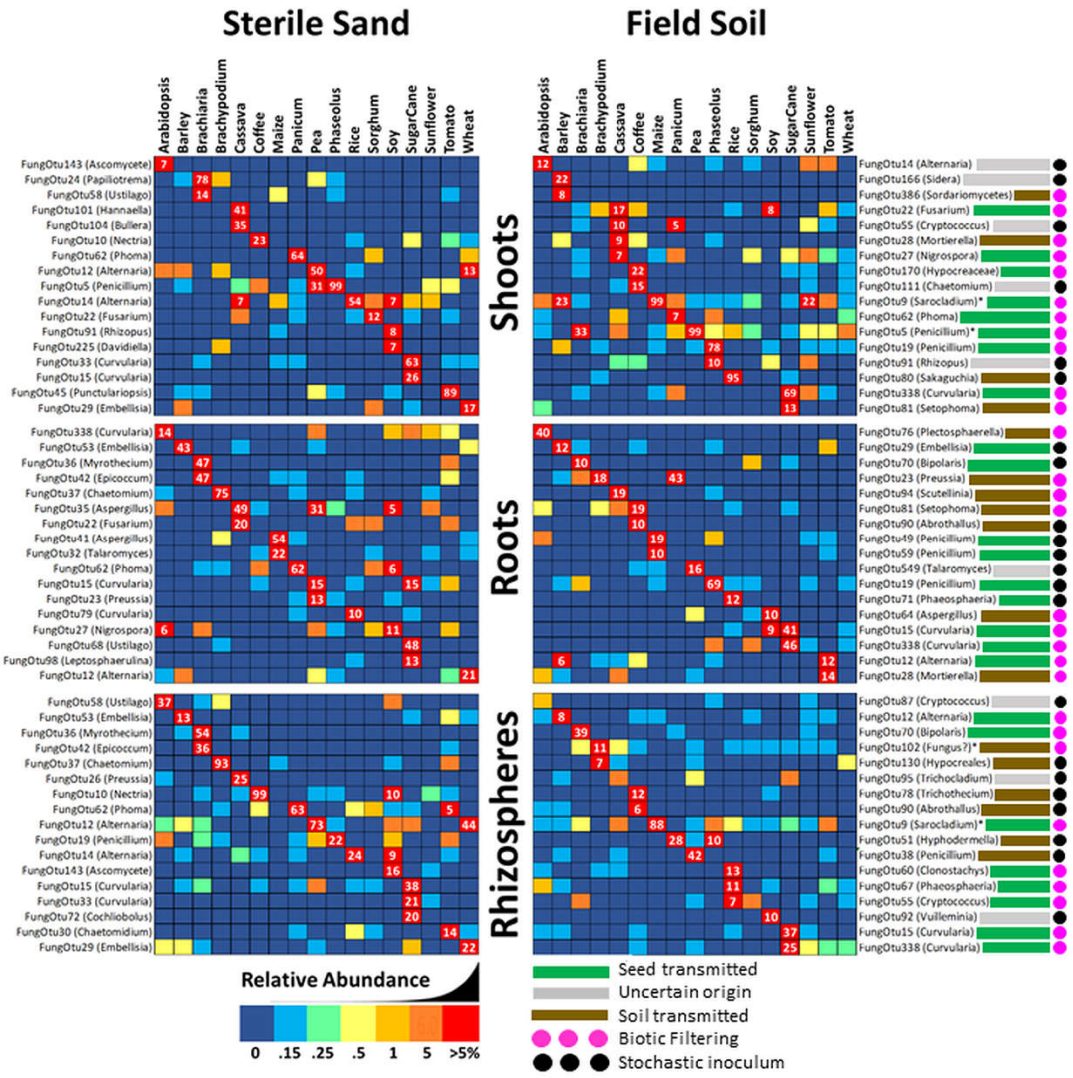

Figure 4. List of the 17 most abundant but uncommon fungal ITS OTUs occurring in less than 53% of Miseq samples of shoots, roots and rhizospheres from 17 different plant species grown in sealed jars on sterile sand or farm soil. Reads from soil grown samples were added across repetitions, transformed into relative percentages, and the most abundant OTU for each plant sample was shown, provided it represented a number higher than 5%. Rows with an asterisk were represented in more than 53% of samples, however appeared to show a large increase of abundance in one specific plant. Cells are shaded by percentage value with dark blue being 0%, up to 0.15% being light blue, between 0.1-0.25% being green, 0.25-0.5% being light yellow, 0.5-1% being dark yellow, 1-5% being orange and greater than 5% being red with white numbers inside. Predicted inoculum source in soil grown plants is shown as gray (unknown), green (seed) or brown (soil) colored bars, while pink dots indicate plants selectively filtering for the fungus and black dots indicate a stochastic supply of inoculum.

3.5. Taxonomy of Uncommon but Abundant Microbes

In order to better define which uncommon fungi (Figure 4) and bacteria (Figure 5) which might be helping to distinguish one plant microbiome from another, we again selected OTUs present in 9 or fewer of the 17 sterile sand or soil grown samples and show the most abundant in each sample type. Because almost no uncommon bacteria occurred at an abundance above 5%, the threshold for considering a bacterial OTU present was

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increased to 0.15%, thus a bacterial OTU present at less than 0.15% relative abundance was considered absent.

The majority of shoot mycobiomes were represented by a few dominant OTUs with limited representation amongst samples; a pattern that remained similar whether plants were grown on sterile sand or soil. For example 75.9% of the fungal reads in cassava shoots were contributed by FungOTUs 101 (*Hanaella*) and 104 (*Bullera*), which occurred in no other plant under any condition (suggesting stochastic seed borne inoculum), however when grown on soil the uncommon cassava shoot mycobiome was 43% FungOTU22, 27, 28 and 55 instead. Sterile sand grown tomato shoots were dominated (89.4%) by the fungus *Punctulariopsis* (FungOTU45), 99.8% of reads in *Phaseolus* belonged to FungOTU5 (*Penicillium*), and 77.5% of fungal reads in *Brachiaria* were from FungOTU24 (*Papiliotrema*) – when grown on soil only *Phaseolus* shoots maintained an uncommon fungus at a level of above 5% (78% for FungOTU19 and 10% for FungOTU91). In sterile sand, the most abundant, uncommon fungus in rice was FungOTU14 (*Alternaria*) but on soil it was replaced by FungOTU80 (*Sakaguchia*) at 95%. Although they weren't technically uncommon, it's worth noting that on soil, the dominant members (making up more than 99% of reads) in maize and pea shoot mycobiomes were seed transmitted FungOTU9 (*Sarocladium*) and FungOTU5 (*Penicillium*) respectively. The magnitude of OTU abundance in roots was generally less than that of shoots, with only six examples near or above 50% in sterile sand and one in soil, and these included 49% of reads in cassava roots belonging to FungOTU35 (*Aspergillus*), 75% of reads in *Brachypodium* coming from FungOTU37 (*Chaetomium*), 54% of reads in maize coming from FungOTU41 (*Aspergillus*), 62% of reads in *Panicum* roots coming from FungOTU62 (*Phoma*), 48% of reads in sugarcane roots growing in sterile sand coming from FungOTU68 (*Ustilago*), and between FungOTUs 36 (*Myrothecium*) and 42 (*Epicoccum*), 93.9% of reads in *Brachiaria* roots were accounted for. Although most soil grown roots contained one or two abundant and uncommon fungal OTUs, only FungOTU19 (*Penicillium*) in *Panicum* was recorded at an abundance above 50% and it appeared to have been stochastically transmitted through seed. Some OTUs such as #28 (*Mortierella*) and #76 (*Plectosphaerella*) appear to have been soil transmitted (they were detected in all soil grown rhizospheres) however they appeared in few roots, suggesting most plants were able to block them from colonizing inside their roots. Rhizospheres of plants grown in sterile sand had the same pattern of a few hyperabundant fungi dominating the mycobiomes and correlating with some of the same OTUs that were abundant in roots; examples with abundance of over 50% included 99% of the reads in coffee coming from FungOTU10 (*Nectria*), 73% of the reads in pea coming from FungOTU12 (*Alternaria*), 54% of the reads in *Bracharia* coming from FungOTU36 (*Myrothecium*), 93% of the reads in *Brachypodium* coming from FungOTU37 (*Chaetomium*) and 62% of the reads in *Panicum* were from FungOTU 62 (*Phoma*). Almost all soil grown rhizospheres contained dominant uncommon fungi, but only the seed transmitted/biotically filtered FungOTU9 (*Sarocladium*) in maize occurred at a level of over 50%.

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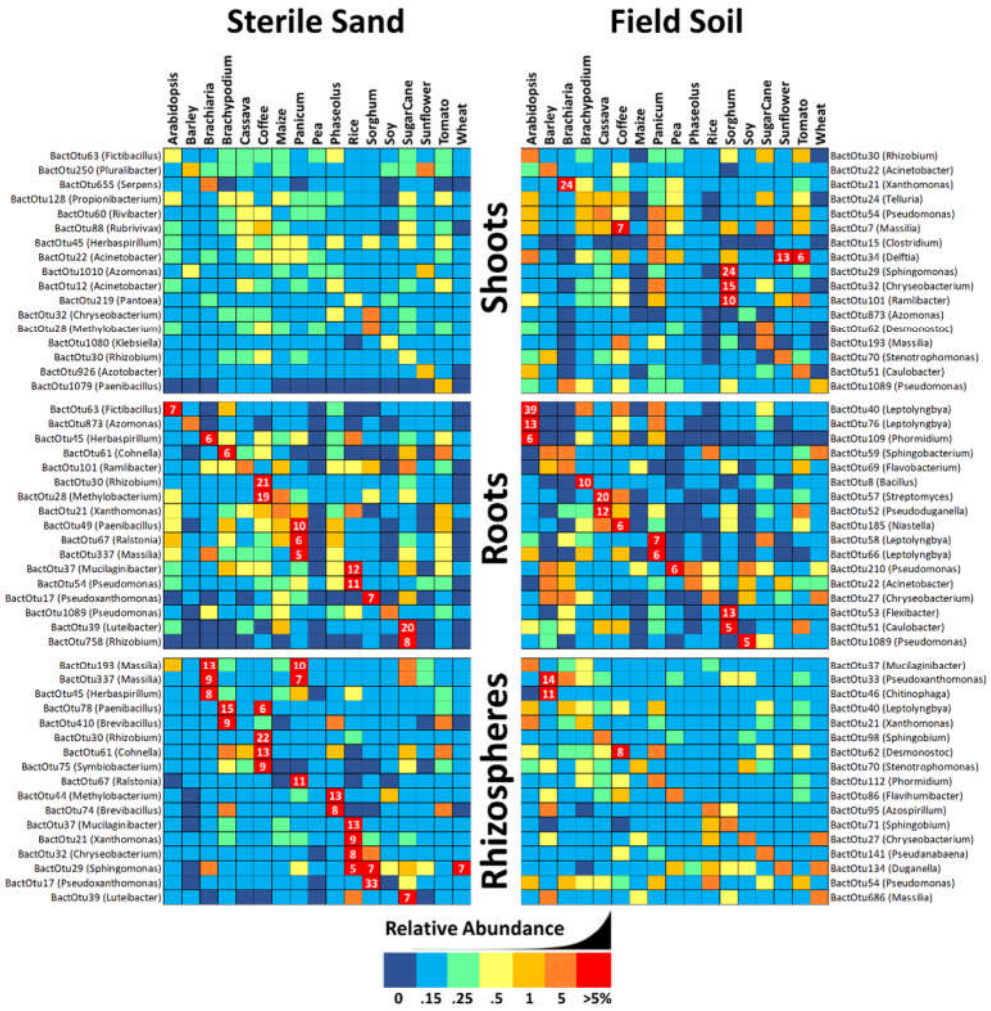

Figure 5. List of the 17 most abundant but uncommon bacterial 16S OTUs (occurring at a level of more than 0.15% in 9 or fewer different plant species) in shoots, roots and rhizospheres of 17 different plant species raised inside sealed jars on either farm soil or sterile sand. Reads from soil grown samples were added across repetitions, transformed into relative percentages, and the most abundant OTU for each plant sample was shown. Cells are shaded by percentage value with 0% being dark blue, up to 0.15% being light blue, between 0.1-0.25% being green, 0.25-0.5% being light yellow, 0.5-1% being dark yellow, 1-5% being orange and greater than 5% being red with white numbers inside.

Contrary to fungi, there were almost no both uncommon and abundant (> than 5% of the relative abundance) bacterial OTUs any of these samples, thus the threshold of counting an OTU as absent was raised from 0% to 0.15%, allowing for inclusion of a few additional and potentially interesting OTUs. Even with this elevated detection threshold, no bacterial OTU was observed to be present in sterile grown shoots with more than 5% of the total reads, although when grown on soil, shoots of *Brachiaria* did contain abundant BactOTU21 (*Xanthomonas*), shoots of coffee contained BactOTU7 (*Massilia*), shoots of sorghum contained abundant BactOTU29, 32, 101 and BactOTU34 (*Delftia*) was abundant in both sunflower and tomato. Eight plant species had roots with abundant bacteria in sterile sand and eight did in soil; in sterile sand these included coffee with *Rhizobium* (20% of BactOTU30) and *Methylobacterium* (19% BactOTU28) and sugarcane with *Luteibacter* (20% of BactOTU79) and *Rhizobium* (8% BactOTU758). While soy or pea roots grown in soil had been expected to accumulate large amounts of rhizobial bacteria, they instead accumulated abundant and uncommon OTUs of *Pseudomonas*. Interestingly, *Arabidopsis* and *Panicum* roots grown on soil did accumulate significant amounts of *Leptolyngbya* bacteria

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which they did not in sterile sand. Cassava roots grown in the cassava farm soil, also accumulated *Streptomyces* and *Pseudoduganella* that were not abundant in any other plant. Nine sand grown rhizospheres contained uncommon bacteria present at a level above 5% of the total reads, with the highest being BactOTU17 (*Pseudoxanthomonas*) at 33% in sorghum and BactOTU30 (*Rhizobium)* at 22% in coffee. In soil grown rhizospheres, only BactOTU33 (*Pseudoxanthomonas*) and BactOTU46 (*Chitinophaga*) in barley; BactOTU62 (*Desomontoc*) in coffee appeared at relative abundances above 5%.

4. Discussion

Nearly all angiosperms use seeds for their reproduction, and as such, it would make sense that they also serve as vectors for ecologically and evolutionarily important members of plant microbiomes. Having previously shown that seeds do indeed transmit many microbes that are common or core to angiosperms [20], we decided to re-evaluate the same dataset and focus on uncommon microbes which were ignored in the previous study. Once again, we studied the sterile sand and field soil grown rhizosphere, root and shoot microbiomes of many of the most important angiosperms plants including *Arabidopsis*, *Brachypodium*, wheat, coffee, rice and soybean. Are there uncommon seed transmitted bacteria or fungi which accumulate to high levels inside particular plant species, suggesting they might play an important role in the life cycle of a host? Do these uncommon seed transmitted microbes get displaced by uncommon soil transmitted microbes, and if so, by which ones? Using high throughput sequencing we identified and quantified bacteria and fungi found in these samples, focusing only on abundant (greater than 5% of the total reads in a sample) and "uncommon" OTUs which were found in 9 or less of the 17 plant species. We decided to focus on relatively abundant OTUs because of the macro-ecological mass–ratio hypothesis which predicts that the effect of a species on ecosystem function is proportional to its relative abundance [48,49], while understanding that scarce microbes could be important as well [50].

Uncommon but abundant microbes in rhizospheres

The layer of soil immediately around the root is the rhizosphere, where large populations of up to 10^{11} microbial cells / gram conduct ecological functions beneficial for controlling disease and aiding in nutrient absorption of the plant [51]. Roots manipulate the rhizosphere microbiome by attracting and feeding microbes via a plethora of released sugars, phytosiderophores, organic acids, amino acids, vitamins, mucilage and nucleosides [14]. Because plants invest so much energy in attracting and feeding soil microbes, it has traditionally been assumed that the entire rhizosphere microbiome "is recruited from the main reservoir of microorganisms present in soil" [12]. Publications about rhizosphere microbiomes echo this idea, suggesting that most or all rhizosphere microbes in *Arabidopsis* [26], maize [52], coffee [53], rice [30], common bean [54] and soy [55] are soil derived. Seeds are increasingly being shown to contribute microbes to the rhizosphere as well. Endophytic bacteria from seeds can travel systematically through the plant, exit roots and colonize the rhizosphere [16] perhaps depending on root hair expulsion [56] or by being sloughed off inside root cap border cells [57]. We've recently shown that the most abundant bacteria and fungi in juvenile plant rhizospheres are in fact, seed transmitted, while soil does transmit a large diversity of common microbes to this niche as well [20]. In the current study, uncommon fungal OTUs which were transmitted by seeds to the rhizosphere of sterile sand grown plants made up 50% of the mycobiome by abundance (Figure 2), but on average only 5% of these uncommon fungal OTUs went on to establish themselves in soil grown rhizospheres (Table 1). While soil grown plants had a higher number of uncommon microbes in their rhizospheres, the average total abundance of uncommon fungi went down to only 27% - this might be because soil also added many abundant, common fungi to rhizospheres. There were hundreds of seed transmitted, uncommon bacteria that were observed in sand grown rhizospheres making up a very small part of the population by abundance, but growth on soil only allowed about 10% of these to

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colonize the rhizosphere while further reducing uncommon bacteria in number and abundance – again this might be because soil also added many abundant, common bacteria to rhizospheres.

With the exception of maize, sorghum and sunflower (which were dominated by common fungi) all plants grown in sterile sand developed rhizospheres with abundant, uncommon fungi. In many cases, these uncommon fungal OTUs totally dominated the rhizosphere population, for example 93% of the reads in *Brachypodium* coming from FungOTU37 (*Chaetomium*) and 99% of the reads in coffee coming from FungOTU10 (*Nectria*). Because rhizospheres of sterile sand grown plants were not very diverse, containing on average only 25 OTUs, these fungi could represent stochastically selected "island" colonists or founders that rose to dominance through lack of competition [58]. Indeed, FungOTU10 and FungOTU37 were absent from soil grown rhizospheres of these plants, suggesting either they were not also present on the particular seeds planted in soil, or these fungi were outcompeted by more aggressive soil rhizosphere colonists. A microbe abundant in both sterile sand and soil grown rhizospheres might be an example of a specially adapted commensal, seed transmitted microbe and there were 3 of these: FungOTU70 (*Bipolaris*) appeared at high abundance (6-39%) in both sterile sand and soil grown rhizospheres of *Brachiaria*, while in sugar cane, FungOTU15 and FungOTU338 (both *Curvularia*) appeared at high abundance in both sterile sand and soil grown rhizospheres. Considered pathogens that should be eliminated from crop plants, *Bipolaris, Chaetomium, Curvularia, Penicillium* and *Phoma* (as well as *Alternaria, Aspergillus, Cladosporium, Colletotrichum, Fusarium* and *Rhizopus*) are seed transmitted fungi colonizing *Brachiaria* and *Panicum* seedlings [59] so perhaps that explains our observing them in *Brachiaria* and sugarcane rhizospheres. *Curvularia* is also known as a beneficial endophyte of tropical panic grasses, where its presence protects the host against extreme temperatures [43]. While technically not uncommon by our definition, FungOTU9 (*Sarocladium zeae*) in maize was found at high abundance in both sterile sand (12%) and soil grown rhizospheres (88%), suggesting a special relationship between the seed transmitted fungus and plant. *Sarocladium zeae* (also known as *Acremonium zeae*) is a seed-transmitted endophyte of maize that produces a range of insecticidal and antibacterial compounds with which it is thought to use to help protect its plant host [60]. As an endophyte with biocontrol potential, perhaps *Sarocladium zeae* is also able to protect maize against herbivory and infections while living in the rhizosphere.

A distinct pattern of OTU distribution was abundant fungi that were only observed in soil grown rhizospheres, for example FungOTU38 (*Penicillium*) found in pea, or FungOTU51 (*Hyphodermella*) found only in soil grown *Phaseolus* and *Panicum* rhizospheres. *Hyphodermella* is a white rot fungus that associates with decaying plant material [61] and has been isolated as an endophyte in wheat [62], but it is difficult to understand why it accumulated to high levels in some rhizospheres and not others. *Penicillium* has been reported often in rhizospheres where it can solubilize phosphate and increase its uptake by pea [63] and promote millet defense and growth [64]. Rather than soil containing high densities of evenly distributed fungal inocula with a good chance of colonizing their preferred hosts, it is possible that many fungal propagules are scarce in soil, resulting in random distribution from jar to jar and colonization of only certain rhizospheres. For context, arbuscular mycorrhizal spores in Indian sugarcane fields have been recorded at a density of as little as 1.19 per gram of soil [65], Fusarium wilt of spinach has been observed to require at least 10 propagules per gram of soil [66] and microsclerotia of *Verticillium* have been found to vary from 0 to over 400 per gram of soil within a single field [67]. It is thus easy to imagine that if fungal inocula were present at lower densities, say 0.5 spores per gram, our filling of different jars with 50 mL of soil could have resulted in stochastic fungal distribution and unequal inoculation of rhizospheres. OTUs such as FungOTU51, 78 and 92 were only present in one or two soil grown rhizospheres and are examples of soil transmitted fungi with low inoculum levels that were unevenly distributed into different soil filled jars. Conversely *Penicillium, Chaetomium* and *Curvularia* (as well as *Alternaria, Aureobasidium, Cladosporium, Fusarium, Acremonium*) have been shown to be cosmopolitan to

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soils around the world [68] so these genera might have been expected to occur in all soil rhizospheres (as we observed for *Fusarium* and *Alternaria* in our previous publication) rather than only one or two. Fungi that were observed in all soil, but not sterile sand grown rhizospheres, would arguably be present at high enough propagule densities to serve as a uniform inoculum for plants growing in all jars. Of all the uncommon and abundant rhizosphere fungi, FungOtu52 (*Alternaria*), 66 (*Tomentella*), 85 (*Xylogone*), 86 (*Mortierella*) and 88 (*Saitozyma*) appeared in either 16 or 17 of soil grown rhizospheres but nearly no sterile sand grown rhizospheres.

Because there were almost no "uncommon and abundant" bacteria in any sample type, it was necessary to lift the minimum threshold of detection to ignore any OTU with less than 0.15% of the reads in a sample. With this relaxed threshold, 9 sterile sand grown rhizospheres had abundant, uncommon bacteria, while only 2 plants grown in soil did, which we think was caused by soil supplying mostly common bacteria at high densities to rhizospheres, reducing the stochastic effects of segregating soil into different jars. Of the 3 uncommon and abundant OTUs in soil grown rhizospheres, none were abundant in sand grown plants, suggesting that they could have been specially selected from the soil and cultivated by the plant. In general however, under the conditions in our experiment, uncommon bacteria appear to be numerically insignificant in plant rhizospheres, being reduced in both diversity and abundance when challenged with soil microbes. While this appears to suggest there is little or no specialized co-evolution between any of these crop plants and rhizobacteria, it also re-enforces our previous observation that plant rhizospheres are dominated by common, abundant seed transmitted bacteria [20].

Uncommon but abundant microbes in roots

Roots anchor the plant in the substrate where they also absorb water and nutrients while secreting biochemicals to manipulate and cooperate with soil microbes, which have typically been assumed to invade and populate the root as endophytes [6]. Root microbiome variation between plant genotypes has been often observed [37-39,69-72] and is usually explained as uneveness in the plant's filtration abilities which restrict or promote soil microbe entry. Assuming a soil origin for most of the root microbiome, many recent studies still sterilize their seeds and leave out sterile substrates as negative controls, and nevertheless continue to conclude that endophytes come from the soil. For example, growing all plants in microbe filled soil without a sterile substrate for comparison, the recruitment of *Brassica napus* seedling microbiota was deemed to derive almost entirely from soil or other unknown sources rather than seeds [73]. To the contrary, we show that seeds of all plants tested transmit microbes to offspring and most of these microbes go on to dominate the microbiome of juvenile plants [20]. That being said, these seed transmitted microbiomes may fluctuate in makeup over time, increasing in diversity during germination [36] and later decreasing as plants age [74]. Ordination of microbiome sequence data in this study suggested that more than half of samples received most of their bacterial diversity from seeds, while the same was true for about two thirds of fungal populations in roots (Figure 1C,D). Focusing exclusively on uncommon microbes, fungal abundance was about 50% in sterile sand grown roots and went down to 42% when plants are grown in soil (Figure 2). Uncommon bacterial abundance was about 7% in sterile sand grown roots and went up to 11% when plants when grown in soil (Figure 2) although seed transmitted bacteria only account for 23% of diversity (Table 1). There were only 4 uncommon and abundant bacterial OTUs in roots, but these were all observed in sterile sand grown plants and thus seed transmitted (Figure 3). Conversely, there were instances of abundant and uncommon fungal OTUs found in nearly every root whether grown in sterile sand or soil, suggesting both seed and soil transmission of different abundant/uncommon fungi. Levels of fungal propagules in seeds and soils seemed to vary greatly, making it difficult to know whether detection inside a root was a product of biotic filtering by the plant or chance inoculation.

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Root endophytes (bacteria) are most Actinobacteria, Bacteroidetes and Proteobacteria [14] and mostly include the genus *Acidovorax, Agrobacterium, Arthrobacter, Bacillus, Curtobacterium, Enterobacter, Erwinia, Methylobacterium, Micrococcus, Phyllobacterium, Pantoea, Pseudomonas, Rhizobium, Serratia, Stenotrophomonas, Streptomyces* and *Xanthomonas* [75]. The only uncommon and abundant bacteria in roots were BactOTUs 39 (*Luteibacter*), 61 (*Cohnella*), 109 (*Phormidium*), and 758 (*Rhizobium*), which all appeared to be seed transmitted, appearing in roots of both sand and soil grown plants. After raising the threshold of detection to include more candidate OTUs for analysis, there were 8 sterile sand grown roots (*Arabidopsis*, *Brachiaria*, *Brachypodium*, coffee, rice, sorghum, switchgrass and sugarcane) where we observed uncommon/abundant strains of *Methylobacterium*, *Fictibacillus, Cohnella, Herbaspirillum, Luteibacter, Pseudomonas, Paenibacillus, Ralstonia, Mucilaginibacter* and/or *Rhizobium* (in coffee and sugarcane). While soy or pea roots grown in soil had been expected to accumulate large amounts of rhizobial bacteria, surprisingly they did not. Relative to sterile sand, an equal number of soil-grown roots accumulated uncommon but abundant bacteria, however with the exception of *Pseudomonas*, none of these were the same that were observed in sterile sand. Some seed transmitted bacteria did however increase in abundance on soil: BactOTU63 in coffee, BactOTU39 in *Phaseolus*, BactOTU78 and BactOTU410 in rice, BactOTU185 in sunflower. Of these apparently soil derived bacteria, switchgrass and especially *Arabidopsis (*52% of its root microbiome*)* accumulated significant amounts of *Leptolyngbya* which they did not in sterile sand. *Leptolyngbya* are filamentous cyanobacteria colonizing rhizospheres where they produce a variety of metabolites such as auxins which are beneficial in pea, rice [76] and wheat [77] agriculture. Cassava roots in field soil accumulated 20% of their bacteria reads as BactOTU57 of the genus *Streptomyces*, bacteria that are known to be beneficial to tomato and maize growth and stress resistance [78,79]. Because the soil we used came from a field used continuously to grow cassava for many years, it is possible that this abundant *Streptomyces* represents a cassava specific strain that has been built up over the years in much the same way that protective strains of *Pseudomonas* build up year after year of planting wheat, until they can suppress take-all disease [4]. Plant specific microbiological inoculation/enrichment of agricultural soil is especially well known for soybeans: there weren't any compatible symbionts in American soils when soybeans first arrived in the early 1800s, necessitating first the practice of infected soil transplants from farm to farm and later through soil inoculation with pure bacterial strains [80].

In nature, root endospheres are generally dominated by Ascomycetes (Pezizomycetes, Dothideomycetes, Sordariomycetes, Eurotiomycetes and Leotiomycetes), Basidiomycota (Polyporales, Russulales and Agaricales) and Zygomycota [81], although in grasslands monocot roots are dominated by Dothideomycetes (ie. *Fusarium* and *Alternaria*) while in forests it is Leotiomycetes that predominate [82,83]. The soil dwelling Chrytidiomycete *Olpidium* can also make up a large portion of root mycobiomes in tomato [84], *Arabidopsis* [85], melon [86] and lettuce [87]. In our experiment, uncommon and very abundant genera in roots of seed transmitted fungi included *Aspergillus, Chaetomium*, *Embellisia, Epicoccum, Myrothecium* and *Ustilago*, however when grown on soil the most abundant fungi changed to *Curvularia, Penicillium, Plectosphaerella* and *Preussia*. The most famous seed transmitted endophytes are *Epichloë, Acremonium (Sarocladium)* or *Neotyphodium* in grasses [3] but there was evidence for other uncommon/abundant seed transmitted root fungi in many plants here as well, such as FungOTU16 (*Waitea*) in *Arabidopsis*, FungOTU32 (*Talaromyces*) in maize, FungOTU166 (*Sidera*) in soy, FungOTU15 and 338 (*Curvularia*) in sugarcane together representing 87% of reads, FungOTU37 (*Chaetomium*) in *Brachypodium* and FungOTU19 (*Penicillium*) in *Phaseolus*. Although assumed to derive from the soil rather than from seed as we observed, *Waitea* occurs at high levels inside *Arabidopsis* flavonoid mutant roots [88] and *Chaetomium* has been observed to be the most abundant fungus in Australian grown rhizospheres of *Brachypodium* [89]. *Talaromyces* has previously been isolated from maize seed [90]. The occurrence of other uncommon fungi was harder to explain, perhaps because inoculum on seeds was uneven, making it difficult to know if root colonization was specifically encouraged by that plant or whether it was the result of

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a chance inoculation by a wayward propagule. An even more extreme example of a rare, stochastically distributed propagule was FungOTU283, a nematophagous fungus of the genus *Vermispora* which was abundant in sterile sand grown *Arabidopsis* roots, but was observed nowhere else in any sample type.

Seeing abundant microbes in soil, but not sand grown roots, was evidence of a soil origin. Fungal endophytes of roots are thought to have a soil provenance and thus are likewise believed to be very sensitive to the biogeography of soil [71,83,85] although we controlled for this by using soil from only one location. Despite thoroughly homogenizing the farm soil, we were unable to achieve an even distribution of rare fungal propagules into each soil filled jar. Nevertheless, by studying fungal abundance in rhizospheres we were able to characterize fungi as soil transmitted, while also noticing that not all fungi were evenly distributed into different jars or present at high densities in soil. FungOTU23 (*Preussia*) appeared in 4 soil grown roots including at a level of 44% inside *Panicum*, while being detected in 12 soil grown rhizospheres. Another great example of a fungus that made it past plant biotic filters to colonize roots, FungOTU76 (*Plectosphaerella*) made up 40% of the *Arabidopsis* root mycobiome while not being detected in any other root, despite appearing in 12 of the 17 soil grown rhizospheres. *Plectosphaerella cucumerina* is a soil fungus that can enter soybean stems as an endophyte [91] and that can infect *Arabidopsis* leaves as a necrotroph [92]. Conversely FungOTU90 which represents an obligately lichenicolous microbe of the genus *Abrothallus [93]*, was only observed inside coffee roots and rhizospheres, apparently being so sparsely distributed in the soil that it only made it into one of jars where coffee was planted, leaving us to wonder if it could've colonized any of the other 14 plant species had it been given a chance?

Uncommon but abundant microbes in shoots

Microbes inside the aerial parts of the plant can influence source/sink relationships, the flux of sugars and nutrients and even fix nitrogen [94], while in the phylloplane they could affect the harvesting of light and gas exchange or help prevent the establishment of pathogens. Despite having an incomplete sense of where shoot inhabiting microbes come from [14], they are usually believed to be horizontally acquired from rain, dust, soil, and contact with other organisms, followed by biotic filtering that shapes the resulting microbiomes over time [95-99]. Speaking to the high diversity of endophytic fungi in coffee shoots, many of these environmentally transmitted microbes have been described as "accidental tourists" playing no direct role in the life cycle of the plant [100]. Some evidence suggests transmission from seeds to shoots can be significant: maize seeds transmit fungi to the leaves [101], rice seeds populate their shoots with bacteria [102], oak embryos within the seed are heavily colonized by microbes [103] and we've previously shown that juvenile shoot microbiomes are dominated by core seed transmitted bacteria and fungi [20]. We again show by ordination of total microbiome sequence data that soil did not greatly affect the diversity of either bacteria or fungi in any shoot sample, implying that most microbes in shoots came from seeds (Figure 1). Focusing exclusively on uncommon microbe abundance in soil grown plants (Figure 2), bacteria made up only a small amount (5%) of the shoot endosphere, whereas uncommon fungi were about 30% of the shoot mycobiome. More so than in rhizospheres or roots, seeds appear to supply a substantial number of microbes to (soil grown) shoots, representing 35% of the bacterial OTUs and 19% of fungi (Table 1). Shoot microbiomes had a lower number of uncommon microbes than roots or rhizospheres, suggesting that perhaps plants were able to impose tighter controls on microbial invasion of stems and leaves. The ability of shoots to effectively filter out undesirables is exemplified by maize or *Bromus tectorum* which are able to maintain an unvarying leaf mycobiome despite being grown on different soils that are known to contain distinct endophytic fungal communities [72,101]. In addition to filtering, shoots may be able to become saturated by core bacterial endophytes [20] which could serve to outcompete and block less common microbes from invading the niche.

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Plant shoots are usually reported to contain Proteobacteria, Firmicutes, Actinobacteria and Bacteriodetes with such genera as *Pantoea, Pseudomonas, Bacillus, Sphingomonas, Erwinia, Acinetobacter, Gluconobacter* and *Xanthomonas [104].* We observed most of these bacteria plus others such as *Klebsiella* and *Massilia*, as part of the common or core population of shoots of our experiment [20], but strictly speaking, there were no bacteria that were both uncommon and abundant in any shoots. Even with our relaxed detection threshold for bacteria (Figure 5) there were still no uncommon and abundant bacteria in sand grown shoots and only 7 of the genera *Xanthomonas, Massilia, Delftia, Sphingomonas, Chryseobacterium* and *Ramlibacter* were abundant inside of 5 different soil grown shoots. In sorghum the 3 uncommon and abundant OTUs total 49% of the total reads, while BactOTU21 is 24% of the shoot microbiome of soil grown *Brachiaria*, however it otherwise seemed that shoot microbiomes were mostly made up of common bacteria.

Common fungal genera reported in the literature to occur in shoots include *Alternaria, Acremonium, Penicillium, Cladosporium, Mucor, Sporobolomyces, Rhodotorula, Cryptococcus* and *Aspergillus* [104]. We previously observed *Fusarium, Alternaria, Pseudozyma, Sarocladium, Phoma* and *Penicillium* as common fungi in angiosperm shoots [20], but the list of uncommon and abundant shoot fungi is much larger, also including the genera *Waitea, Sakaguchia, Punctulariopsis, Curvularia, Papiliotrema, Phoma, Hannaella, Nectria, Bullera, Cryptococcus, Sidera, Embellisia, Rhizopus, Ustilago, Chaetomium, Nigrospora, Setophoma, Mortierella, Davidiella, Occultifur, Clonostachys* and *Aspergillus*. Perhaps because fungal diversity in shoots was so low, with an average of only 16 OTUs in sand grown plants and 24 OTUs in soil grown plants, it was easy for one or two OTUs to appear very abundant. In our experiment, uncommon and very abundant (over 50%) genera of sterile sand grown fungi in shoots included *Alternaria, Papiliotrema, Penicillium, Phoma, Curvularia* and *Punctulariopsis,* however when grown on soil the most abundant fungi changed to *Sarocladium* (FungOTU9) in maize with 99% of the reads, *Penicillium* (FungOTU5) in pea with 99% of the reads, *Penicillium* (FungOTU5) in pea with 99% of the reads, *Penicillium* (FungOTU19) in *Phaseolus* with 78% of the reads, *Sakaguchia* (FungOTU80) in rice with 95% of the reads, and *Curvularia* (FungOTU338) in sunflower with 69% of the reads. FungOTU5, 9 and 338 were seed transmitted since they occurred in sterile sand grown shoots too, but FungOTU19 and 80 seem like they may have been deposited there by chance as infrequent inoculum in either seeds or soil. *Sakaguchia* is a Basidiomycete yeast that has been previously been observed in the phyllosphere of turfgrasses [105] and rice [106].

5. Conclusions

As a complement to a previous study searching for core microbiomes [20], this experiment aimed to document the seed and soil transmitted diversity of uncommon bacteria and fungi associated with a panel of 17 important, juvenile plant species grown inside hermetically sealed jars. We observed that about 95% and 86% of fungal and bacterial diversity inside plants was uncommon, however by abundance these fungi represent only up to about half of the mycobiome, while less than 11% of bacterial endophytes are rare. When grown on sterile sand, all plants developed microbiomes with uncommon Proteobacteria, Firmicutes, Bacteriodetes, Ascomycetes and Basidiomycetes, proving that seeds can transmit uncommon and varying communities of microbes to the resulting seedlings. A minority of these uncommon vertically transmitted microbes were robust colonizers of soil grown plants, and although rhizospheres were poor niches for these seed transmitted microbes, roots hosted relatively more of them and shoots a greater number still. Except for bacteria inhabiting rhizospheres, soil served as a more diverse source of uncommon microbes than seeds, replacing or excluding the majority of the seed transmitted microbiome. It was difficult to know whether a microbe was absent from a particular sample because of biotic filtering from the plant, or because of uneven/stochastic inoculum distribution in seeds or soil. By focusing on uncommon microbes rather than searching for a core microbiome, a few interesting plant-microbe associations were observed such as seed transmission and robust endophytic colonization of shoots, roots and/or rhizospheres by

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the beneficial maize endophyte *Sarocladium zeae*, the phosphate solubilizing and growth promoting *Penicillium* in pea and *Phaseolus*, and the stress resistance enhancing endophyte *Curvularia* in sugarcane. There was evidence for robust soil transmission into *Arabidopsis* and *Panicum* roots of the phytohormone producing cyanobacteria *Leptolyngbya*, while the colonization of cassava roots by cassava field soil dwelling *Streptomyces* invites speculation that this was a specialized endosymbiont allowed in by its preferred plant host. Some abundant microbes such as *Sakaguchia* in rice shoots or *Vermispora* in *Arabidopsis* roots appeared in no other samples, suggesting they were stochastically deposited propagules from either soil or seed, making it impossible to know what their relationship may be with other plants. Future experiments which succeed in culturing some of these uncommon microbes, would allow for cross inoculation to better understand their host specificity without worrying about stochastically uneven inoculum and may help explain their role in plant health and productivity, perhaps leading to their future implementation in crop microbiome engineering and agricultural production enhancement.

Supplementary Materials: Table S1:Primer Sequences, Table S2: Fung ITS seq and taxonomy, Table S3:Bact 16S seq and taxonomy, Table S4:Fungal Shoot Root and Rhizosphere OTU Counts, Table S5: Bacterial Shoot Root and Rhizosphere OTU Counts.

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Data Availability Statement: The bacterial and fungal sequence data generated in this study using MiSeq have been deposited and are available in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA731997 and are also provided as Supplemental Materials (annotated sequences and OTU counts) in this publication.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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