SEED MICROBIOME RESEARCH

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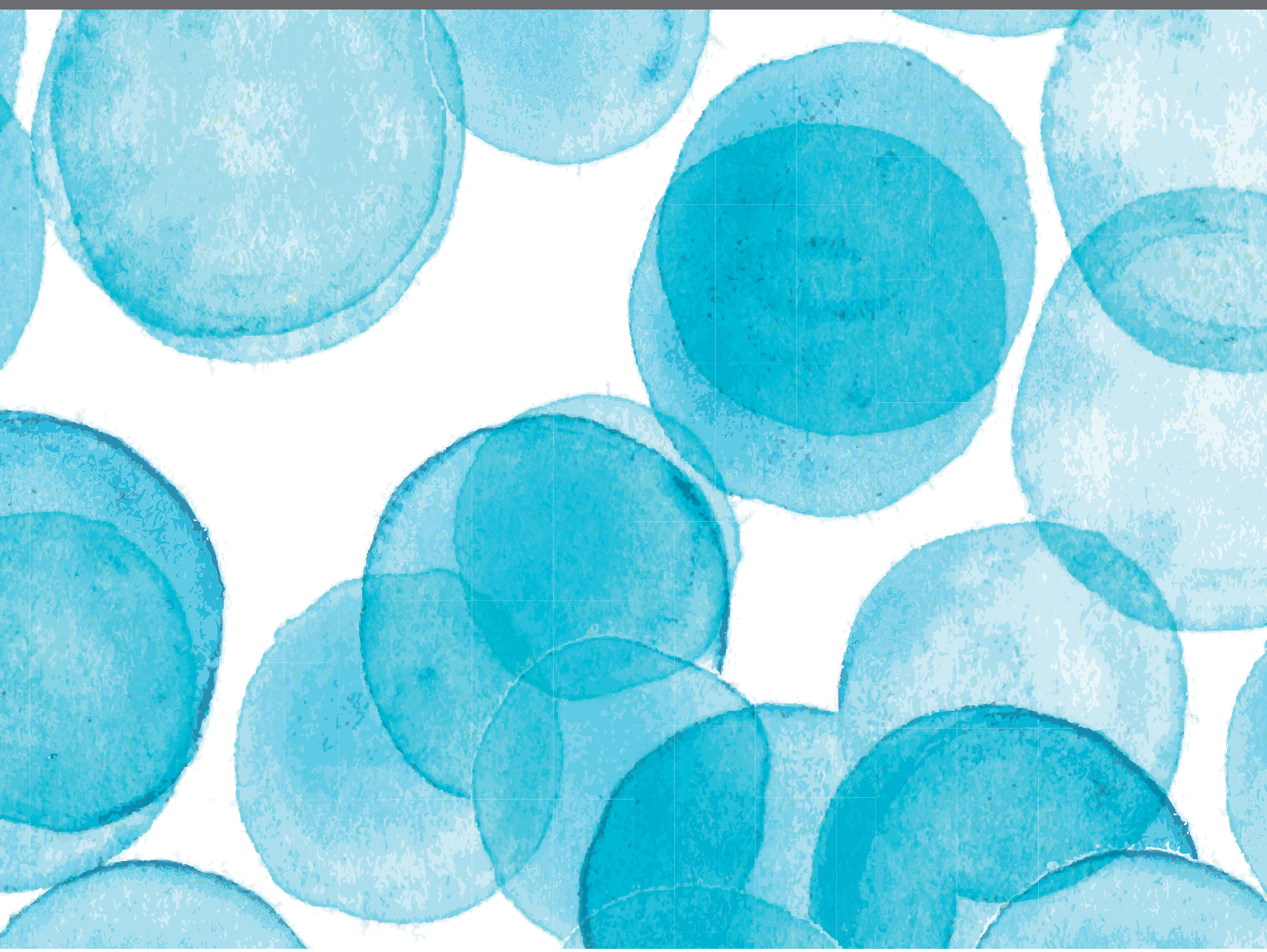

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SEED MICROBIOME RESEARCH

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EDITORIAL
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Editorial: Seed Microbiome Research

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Editorial on the Research Topic

Seed Microbiome Research

With sequencing technologies, we are able to detect viruses, bacteria, and fungi that are outside (ectosphere) or inside (endosphere) of plants. Accordingly, there is a boom in articles exploring factors that control the plant associated microbiome by specifically looking at different plant organs/tissues across species, and different biotic and abiotic environmental conditions. Viewing the plant together with the associated microorganisms as a supra-organism (i.e., holobiont) presumes that symbionts play functional roles in determining host phenotype (Zilber-Rosenberg and Rosenberg, 2008; Vandenkoornhuyse et al., 2015). However, while there are plant-microorganism systems in which the functional roles of symbionts are well-established (Rodriguez et al., 2009; White et al., 2019), the fact is that most of the studies today are descriptive, predominantly determining which microbes are present.

Members of the plant microbiome can be taken from the soil (Walsh et al., 2021), or can come within—or attached to—the seed (Truyens et al., 2015; Shade et al., 2017; Berg and Raaijmakers, 2018). Plant-microorganism symbiosis can present different degrees of coevolution and so, stability, and degree of integration (Ellers et al., 2012). Some symbiotic systems have evolved to a point that the microorganism/s is/are conserved across maternal linages by means of vertical transmission (i.e., microorganisms pass from mother plant to seeds) (Gundel et al., 2011; Abdelfattah et al., 2021). There are conditions that lead symbiosis with free-living microorganisms to evolve into stable mutualistic interactions (see Thompson, 2005; Ellers et al., 2012). Vertical transmission of microorganisms may result in evolution of mutualisms because fitness of both partners is tightly aligned (Ewald, 1987; Herre et al., 1999; Gundel et al., 2008). For instance, the association of certain grasses (Family: Poaceae) with vertically transmitted *Epichloë* fungal endophytes is labeled as a defensive mutualism since fungal alkaloids confer resistance against herbivory (Rodriguez et al., 2009; White and Torres, 2009; Panaccione et al., 2014). Therefore, besides identifying the variables controlling the plant microbiome, we want to understand the functions these microorganisms play in the evolution of plant phenotype (Gundel et al., 2017; White et al., 2019).

Manipulation of the symbiotic states of plants is a breeding strategy in agriculture (Gundel et al., 2013; Wei and Jousset, 2017). In some programs, different fungal endophyte strains are inoculated to improve forage cultivars and turf grasses, a strategy that relies on the persistence in—and transmission through, the seed (Johnson et al., 2013). Spraying plants with beneficial microorganisms at flowering has been proposed as a technical strategy for manipulating the seed microbiome and add agricultural desirable traits in crops (Mitter et al., 2017). However, the flowering stage is also critical for pathogen infection, which once in the seed, will affect the next generation of plants. It is interesting then, to understand the effect of the seed microbiome on the infection rates and transmission of phytopathogenic microorganisms (Barret et al., 2016).

In this special topic, we focused on seed microbiomes because: (i) they may be critical for seed germination and seedling establishment, (ii) they may affect the ecology and evolutionary dynamics of plant symbiosis by connecting the maternal environment with that of offspring,

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(iii) they may be critical pathways for pathogenic diseases to spread, and (iv) seed microbiomes represent a way to manipulate beneficial symbionts in agriculture. One paper (Chandel et al.) highlights the storage strategy implications on seed microbiomes in seed vaults and another two (Thomas and Saha; Thomas et al.) reveal the presence of "cytobacts" (bacteria that enter into plant cells) in multiple plant species. Three papers (von Cräutlein et al.; Bastías et al.; Laihonen et al.) deal with fungal endophytes in the genus *Epichloë*, which associate with grasses forming a systemic and asymptomatic symbiosis. Three other articles (Kim and Lee; Johnston-Monje et al.; Kumar et al.) involve the seed microbiome and microbial endophytes for seedling growth, development, and nutrient acquisition. Two other articles (Parmar et al.; Redman et al.) focus on the association of plant health regarding abiotic and biotic stress and associated microbes.

Fungal endophytes in the genus *Epichloë*, associate with grasses forming a systemic and asymptomatic symbiosis. These endophytes grow in the apoplast of host aerial tissues producing alkaloids that protect plants against herbivores. While most of these endophytes are interspecific hybrids that reproduce asexually by growing hyphae in developing seeds (vertical transmission), some haploid species conserve the capacity of reproducing sexually and transmit both vertically and horizontally. An important volume of research shows that these endophytes alter the ecology and evolution of grass populations. In this special issue, von Cräutlein et al. were able to infer underlying processes to the population structure of *Epichloë festucae* Leuchtm., Schardl & M.R. Siegel (a haploid endophyte) in the host species *Festuca rubra* L. (Red Fescue) combining different molecular approaches. Their results suggest that sexual reproduction is important in southern European populations while asexual reproduction and vertical transmission seem to prevail in northern populations (von Cräutlein et al.). There was also a great variation in alkaloid genes but, interestingly, the variation within populations in this variable was lower in northern populations (von Cräutlein et al.). In common garden experiments, Laihonen et al. showed that the seed transmitted *E. festucae* also affected the ecological interaction of *F. rubra* plants with pathogens and herbivores. Interestingly, they found that plants associated with *E. festucae* were more susceptible to be infected by the biotropic pathogen *Claviceps purpurea* (Fr.) Tul.; but the presence of the pathogen was associated with a lower incidence of aphids (*Sitobion* sp.) due to a higher content of ergot alkaloids (Laihonen et al.). Bastías et al. tested the hypothesis that as a persistent and vertically transmitted endophyte, *E. occultans* (C.D.Moon, B.Scott & M.J.Chr.) Schardl controls the seed microbiome of *Lolium multiflorum* plants. In their experiment, *L. multiflorum* plants with and without endophyte were challenged by the aphid *Rhopalosiphum padi* and the microbiomes of the produced seeds were characterized by sequencing the bacterial 16S ribosomal RNA (rRNA) gene. *Epichloë* endophytes increased the bacterial diversity and affected the bacterial communities which were more equitable (all bacterial groups were equally abundant) than that of endophyte-free seeds (Bastías et al.). These three works illustrate how a vertically transmitted microorganism affects the ecology of host grasses by modulating the interaction with other microorganisms.

Several of the articles in this collection deal with seed-vectored bacteria or fungi. In a genomic study, Johnston-Monje et al. show that bacteria present on seeds colonize seedlings to dominate the seedling microbiome. This article and other studies underline the importance of the seed microbiome and microbial endophytes for seedling growth, development and nutrient acquisition (White et al., 2019). Using microscopy and metagenome profiling in a diverse set of plants, different cultivation-recalcitrant endophytic bacteria living intracellularly were found in many plant parts and deep in the seeds and embryos of watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai] and grapevine [*Vitis vinifera* L.] (Thomas et al.). Many of these bacteria belong to the phylum Proteobacteria. This paper sheds a new light on intracellular endophytic bacteria as ubiquitous entities in vascular plants that can be transmitted vertically to the next generation. These authors refer to such intracellular bacteria as "cytobacs," in reference to their presence in plant cell cytoplasm (see also White et al., 2019 for another discussion of these intracellular microbes). Thomas and Sahu examined bacteria transmitted within watermelon seeds and showed that bacteria transmitted in seeds could be seen within plant cells after seedlings form. An article by Kim and Lee examines diversity and distribution of both bacteria and fungi in seedlings and mature plants of rice. Redman et al. takes an ecological approach and shows that plant microbes in symbiosis with plants may drive expansion of coastal plants in the San Juan Archipelago of Washington State. Collectively, these papers suggest the importance of seed-vectored symbiotic microbes in facilitating plant growth, development, and ecological success.

Plants recruit diverse communities of microorganism from their surroundings and absorb them into their tissues as endophytes. These plant-associated microbes, or plant microbiota, play important roles in modulation of plant development (Berg and Raaijmakers, 2018; White et al., 2019). Although microbes have been isolated and reported from all parts of plants, occurrence of microbes in seeds is advantageous to plants because seed associated microbes may colonize the emerging seedlings to promote plant health (Verma et al., 2019; White et al., 2019). Composition and diversity of seed microbiota may be influenced by genotype, storage, and environmental conditions. Studies on influence of storage conditions on seed microbiota conservation have not been explored adequately. Chandel et al. showed that in soybean (*Glycine max* (L.) Merr.) initial seed drying before storage reduced the microbial composition. Further study suggested that storage of seeds at −20°C is standard for conservations of seed microbiota. Being an early colonizer of seedlings, seed endophytic microbes may provide stress tolerance against salt, drought, and heavy metals, and also may protect seedlings from soil pathogens during early stages of seedling development (Verma and White, 2018). Parmar et al. reported that a seed endophytic fungus *Epicoccum nigrum* improved Cd tolerance in *Dysphania ambrosioides* (L.) at all stages of its development. In another study of seed endophytic bacteria of pearl millet (*Pennisetum glaucum* L.), Kumar et al. found that seed endophytic bacteria are important during seedling development. They reported that removal of seed endophytic bacteria compromised the seedlings growth and increased susceptibility against *Fusarium* sp., however, when the

same bacteria were re-inoculated, the seedlings restored their growth with reduced susceptibility against pathogens (Kumar et al.).

We hope this special issue sparks interest in seed microbiome research. We are in an unprecedented time in which we may study microbiomes and their effects on plant development and how interactions between microbes and plants are regulated. Seeds of plants are genetic resources that ensure plant propagation but also contain a plethora of microbes that may be used to secure healthy plants and agricultural soils.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Spatiotemporal Assembly of Bacterial and Fungal Communities of Seed-Seedling-Adult in Rice

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Seeds harbor not only genetic information about plants but also microbial communities affecting plants' vigor. Knowledge on the movement and formation of seed microbial communities during plant development remains insufficient. Here, we address this knowledge gap by investigating endophytic bacterial and fungal communities of seeds, seedlings, and adult rice plants. We found that seed coats act as microbial niches for seed bacterial and fungal communities. The presence or absence of the seed coat affected taxonomic composition and diversity of bacterial and fungal communities associated with seeds and seedlings. Ordination analysis showed that niche differentiation between above- and belowground compartments leads to compositional differences in endophytic bacterial and fungal communities originating from seeds. Longitudinal tracking of the composition of microbial communities from field-grown rice revealed that bacterial and fungal communities originating from seeds persist in the leaf, stem, and root endospheres throughout the life cycle. Our study provides ecological insights into the assembly of the initial endophytic microbial communities of plants from seeds.

Keywords: endophytic microbiota, niche differentiation, rice, vertical transmission, seedling growth

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INTRODUCTION

The relationship between a plant host and its microbiota begins in the seed, which has its own microbial community. The composition and diversity of the seed microbial community affects the vigor of seedlings and fitness of adult plants. For example, variations in seed microbiota contribute to the rate (Nelson, 2018; Rodríguez et al., 2020) and speed (Rochefort et al., 2019) of germination of plant seeds. The seed microbial communities also drive the successional assembly of environment-derived microbial communities in the root (Ridout et al., 2019). These effects of the seed microbial community on plant fitness provide opportunities for improving plant health. Previous studies have reported that compositional variations of the seed microbial community are affected by the geographical location where seeds are harvested (Barret et al., 2015), as well as by the harvesting year (Rochefort et al., 2019), seed compartment (Eyre et al., 2019), and plant domestication

(Kim et al., 2020). Therefore, it is important to understand the variations in microbial communities associated with seeds.

Microbial communities associated with seeds were known to be distributed in the outer and inner space of seed coat, surface of grain, embryo, and endosperm (Eyre et al., 2019; Wang et al., 2020; Abdelfattah et al., 2021). A culture-dependent approach also reported that bacterial and fungal isolates are primarily retrieved from the seed coats of rice, barley, and alfalfa (Maude, 1996; Singh and Mathur, 2004). A previous study in rice reported that the seed coat (or husk) possesses higher richness and diversity of bacterial and fungal communities than grain does (Eyre et al., 2019). In particular, 85% of bacteria and 81% of fungi were found in the seed coat and the surface of the grain (Eyre et al., 2019). These findings indicate that the seed coat plays crucial roles in protecting genetic information of plants and microbes potentially associated with plants. Understanding on the effects of the seed coat on the assembly of microbial communities in developing seedlings will provide ecological importance of the seed coat in the plant–microbiome relationship.

Germination and seedling development are crucial steps in the life cycles of plants. The metabolic environment changes dynamically throughout seed germination and seedling growth (Bewley, 1997; He and Yang, 2013). Seed germination begins with imbibition (rehydration of seeds), which is a process of diffusion wherein water is absorbed by solid particles such as colloids (Bewley and Black, 1978). Upon imbibition, substrate, and energy starvation activate the embryo, and phytohormones (primarily gibberellic acid) are then produced. Gibberellic acid diffuses into the aleurone layer, leading to the synthesis of hydrolytic enzymes including α-amylases. These hydrolytic enzymes degrade storage compounds in the endosperm to support seedling establishment (He and Yang, 2013). Proteomic analysis showed sequential expression of protein sets involved in cell wall biosynthesis, as well as the assembly of mitochondria, biosynthesis of amino acids and starch, and aerobic respiration (He et al., 2011). Postgerminative growth of seedlings is primarily driven by cell expansion along the embryonic axis (shoot and root meristem) (Wolny et al., 2018). Transcriptional and hormonal regulation processes are involved in the early developmental stages of seedlings (Hoffmann-Benning and Kende, 1992; Magneschi and Perata, 2009; Pucciariello, 2020). Thus, the physiological changes that occur in germinating seeds and growing seedlings have been thoroughly examined from a biochemical perspective. However, little attention has been paid to the associated microbial communities.

One unsolved question regarding plant microbiomes is the origin of the microbial communities residing in seeds. Three putative transmission pathways, i.e., internal (via non-vascular or xylem tissues in maternal plants), floral (via the stigma of maternal plants), and external (transmitted from the external environment including air and soil), have been proposed (Maude, 1996; Nelson, 2018; Shahzad et al., 2018). Studies have reported the assembly of progeny seed microbial communities in this context. In bean and radish, bacterial communities could be transmitted via the floral pathway, as revealed in investigation of bacterial communities in reproductive organs (buds, flowers, and fruits) and seeds (Chesneau et al., 2020). In oilseed rape, the pollination activity of honeybee (*Apis mellifera*) affects progeny seed microbial communities by introducing insect-associated bacteria into flowers (Prado et al., 2020). Although the assembly of seed microbial communities has been examined, how these communities are transferred within internal tissues during plant development and colonize progeny seeds remains largely unknown.

In the present study, we describe the endophytic bacterial and fungal communities of the leaves, stems, and roots of rice plants grown in axenic culture and field conditions. The objectives of the study were: (1) to examine the temporal dynamics of seed bacterial and fungal communities during the germination and postgerminative growth of seedlings in axenic culture, (2) to identify vertical transmission of seed bacterial and fungal communities to progeny seeds, and (3) to reveal the distribution of vertically transmitted bacteria and fungi in rice endophytic tissues of field-grown rice.

MATERIALS AND METHODS

Rice Cultivation in Axenic Cultures

To investigate the movement of seed-borne bacterial and fungal communities in rice seedlings grown under axenic conditions, surface-sterilized seeds were planted in 60 ml test tubes with 15 ml of Murashige and Skoog (MS) medium (4.4 g L^{-1} MS powder and 9 g L^{-1} agar powder; autoclaved at 121°C for 20 min). The test tubes were sealed with autoclaved silicone rubber stoppers and Parafilm to prevent entry of exogenous microbes. Rice seedlings were grown at 28°C and 80% humidity with 16-h light/8-h dark cycle in a growth chamber. Rice seedling samples were collected at 0, 1, 4, 7, and 14 days after planting. Three seedlings were collected at each sampling point. To exclude epiphytic fractions in the seedling samples, surface sterilization was performed. After surface sterilization, 14-day-old seedlings were divided into leaves, stems, and roots. Collected samples were stored in −80°C until DNA extraction. To compare the effects of seed coats on the bacterial and fungal communities of seedlings, hulled seeds (seeds without seed coats) were prepared using autoclaved forceps. Sample preparation of seedlings grown from hulled seeds was identical to of the process used for the intact seeds.

Collection of Rice Samples Grown Under Field Conditions

To track the temporal changes in rice-associated microbial communities under field conditions parallelly with axenic cultures, rice plants were grown in a field located at the university farm of Seoul National University (37°16′06.7″N 126°59′24.5″E). For rice cultivation, a total of 9 kg nitrogen, 4.5 kg phosphate, and 5.7 kg potassium per 1,000 m^2 was applied three times during the growing season. Sampling began 48 days after transplanting (tillering stage) into the field. Additional rice samples were collected at 90 (heading stage) and 141 (harvest) days after transplanting. The collected plant samples were divided into leaf, stem, and root portions. Roots were obtained using the

method described by Edwards et al. (2015). The divided samples were surface-sterilized to remove epiphytic communities. The sterilized plant compartments were ground using sterilized mortars and pestles. 0.5 g of each ground sample was transferred to the Lysing Matrix E tube provided in the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, United States). Seed samples were collected from the heading stage to harvest. The collected seeds were surface-sterilized and transferred to Lysing Matrix S tubes (MP Biomedicals, Solon, OH, United States). All samples were kept at −80°C until DNA extraction.

Sample Preparation and DNA Extraction

DNA from all samples was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, United States). Plant tissues were prepared and pulverized using a bead beater (Biospec Products, Bartlesville, OK, United States) at 4,000 rpm for 2 min. This step was repeated after cooling in ice for 1 min. Plant DNA was extracted following the instructions of the manufacturer. The concentration of DNA samples was quantified using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The extracted DNA was stored at −20°C until amplicons generation.

Polymerase Chain Reaction Amplification and Sequencing

16S ribosomal RNA (rRNA) and internal transcribed spacer (ITS) amplicons were generated via a two-step polymerase chain reaction (PCR) amplification protocol. The V4 region of bacterial 16S rRNA gene was amplified with the universal PCR primers 515F and 806R (Caporaso et al., 2011). To reduce plant mitochondrial and plastid DNA contamination, peptide nucleic acid (PNA) PCR blockers were added during the first PCR step (Lundberg et al., 2013). The fungal ITS2 region of the 18S rRNA gene was amplified using ITS3 and ITS4 PCR primers (White et al., 1990). Each sample was amplified in triplicate in a 25-μl reaction tube containing 12.5 μl of 2 × PCR i-StarTaq™ Master mix solution (iNtRON Biotechnology, Seongnam, South Korea), 0.4 μM each forward and reverse primers, 0.8 μM diluted DNA template and PNA clamps for chloroplast (pPNA) and mitochondria (mPNA), at 0.75 μM each. For generation of the ITS libraries, the conditions were the same except that PNA clamps were not included. PCR was performed using the following program: initial denaturing at 98°C for 3 min, followed by 32 cycles of denaturing at 98°C for 10 s, PNA annealing at 78°C for 10 s, primer annealing at 55°C for 30 s and extension at 72°C for 60 s. For ITS PCR amplification, the same program was used, but without the PNA annealing step. Each library was accompanied by negative PCR controls to ensure that the reagents were free of contaminant DNA. Amplicon replicates were pooled and then purified using the MEGAquick-spin Plus DNA Purification Kit (iNtRON Biotechnology, Seongnam, South Korea) with an additional ethanol clean-up step to remove unused PCR reagents and resulting primer dimers. Next, PCR was conducted with the Nextera XT Index Kit (Illumina, San Diego, CA, United States). DNA templates were diluted to equal concentrations after measurement with the Infinite 200 Pro (Tecan, Männedorf, Switzerland). The libraries were then pooled at equal concentrations into a single library and concentrated using AMPure beads (Beckman Coulter, Brea, CA, United States). The pooled library was then subjected to a final gel purification step to remove any remaining unwanted PCR products. Pooled libraries were sequenced using the Illumina MiSeq platform with 2 × 300 base pair read length. Sequencing was conducted at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University, South Korea.

Processing of Microbial Reads, and Statistical Analysis of Microbial Communities

The sequenced reads were processed with the QIIME2 (version 2018.6) pipeline (Callahan et al., 2016). After demultiplexing, the resulting sequences were merged using PEAR (Zhang et al., 2014) and then quality filtered with the DADA2 plugin in the QIIME2 (version 2018.6) pipeline (Callahan et al., 2016). High-quality sequences were clustered into operational taxonomic units (OTUs) using the open reference vsearch algorithm (vsearch cluster-features-open-reference) (Rognes et al., 2016) against the Silva 99% OTU representative sequence database (v132, April 2018) (Quast et al., 2012), and then assembled into an OTU table. Bacterial OTUs were filtered for chimeras using the vsearch uchime-*de novo* algorithm (Edgar et al., 2011). Fungal OTUs were checked for chimeric sequences using the Uchime-ref algorithm against the dedicated chimera detection ITS2 database (June 2017 version) (Nilsson et al., 2015). The taxonomy of non-chimeric OTUs was assigned using the Naïve Bayes algorithm implemented in the q2-feature-classifier prefitted to the Silva database for the V4 region of 16S rRNA genes (Bokulich et al., 2018). For the ITS2 region, taxonomic assignment was conducted with the q2-feature-classifier prefitted to the UNITE database (UNITE_ver7_dynamic of January 2017) (Nilsson et al., 2019). Bacterial sequences over 300 bp in length and fungal sequences shorter than 100 bp were discarded. The OTU table was imported into R software by the phyloseq package (McMurdie and Holmes, 2013) for further analysis. Sequences from host DNA and OTUs that were unassigned at the kingdom-level were removed (bacterial OTUs: orders "*Chloroplast*" and "*Rickettsiales*"; fungal OTUs: kingdoms "*Unassigned,*" "*Chromista,*" and "*Plantae*").

Statistical Analysis and Visualization

Unless otherwise noted, all statistical analyses were performed using R software (version 3.5.2) (R Core Team, 2013) and statistical significance was determined at $\alpha = 0.05$; where appropriate, the statistical significance was corrected for multiple hypothesis testing using the false discovery rate (FDR) method. The OTU table was normalized through cumulative-sum scaling (CSS) and log-transformed with the cumNorm function from the R package metagenomeSeq (v3.8) (Paulson et al., 2013). Rarefaction was assessed when calculating alpha diversity (McMurdie and Holmes, 2014). The Shannon and Simpson indices were calculated using the alpha function in the R package microbiome (v1.9.13) (Lahti and Shetty, 2019). The Kruskal–Wallis test and Dunn's test were also performed in R. Taxa

with relative abundances greater than 0.5% were visualized with the R package ggplot2 (v3.2.1) (Wickham, 2016) for taxonomic composition analysis. A Bray–Curtis dissimilarity matrix was constructed for principal coordinate analyses (PCoA) to compare community structure among examined samples based on both abundances and profiles of OTUs. Permutational multivariate analysis of variance (PERMANOVA) was conducted using the adonis function in the vegan package (Oksanen et al., 2018).

Assessment of Movement of Seed Microbial Communities to Seedlings and Adult Plants

To assess the movement of microbial communities from seeds to seedlings, the community composition of germinating seeds and seedling compartments was compared to seed bacterial and fungal communities before planting. In this assessment, we assumed that community membership between seeds and other tissues is similar if seed OTUs are moved to the shoot, leaves, stems, and roots of seedlings and adult plants in the presence and absence concept. Since Jaccard dissimilarity is calculated by dividing the number of the shared OTUs between two communities by total numbers of OTUs (not consider OTU abundances), community dissimilarity was estimated using the Jaccard dissimilarity index using the R package vegan. OTU profiles of seeds and seedlings were compared using a Venn diagram to identify OTUs co-occurring among seeds prior to planting in MS agar medium and 7- and 14-day-old seedlings. For this analysis, Venn diagrams were constructed using InteractiVenn[1] (Heberle et al., 2015). To investigate the movement of seed microbial communities to adult plants under field conditions, we first identified OTUs present in both seeds and rice compartments, including the leaf, stem, root, and progeny seeds. These OTUs were defined as seed-borne OTUs. Then, the proportion of seed-borne OTUs was calculated by dividing the number of seed-borne OTUs by the total number of OTUs in each compartment. The taxonomic composition of seed-borne OTUs was visualized using bar plots showing cumulative relative abundances.

RESULTS

Composition of Bacterial and Fungal Communities in Rice Seedlings Grown in Axenic Cultures

We investigated the bacterial and fungal communities of rice seedlings grown in axenic culture (MS agar medium) to verify the microbial assembly present during the early growth of rice seedlings. Rice seedlings were grown from seeds with (unhulled condition) or without (hulled condition) seed coats. As the seedlings were grown in a germ-free medium, which prevents the input of microbes from the surrounding environment, the leaf, stem, and root endospheres of seedlings may harbor microbial communities originating solely from seeds. To completely exclude epiphytic microbes, additional surface sterilization was performed. Based on the sampling procedures, the collected samples were labeled using presence and absence of seed coats (H, hulled condition; U, unhulled condition), sampling point (0, prior to planting; 1, 1 day after planting; 4, 4 days after planting; 7, 7 days after planting; and 14, 14 days after planting), and plant compartment (Sh, shoot; L, leaf; S, stem; and R, root). As a result, 67,211 bacterial and 64,909 fungal reads were acquired in the hulled condition, and greater number of reads were obtained in the unhulled condition (251,968 bacterial and 300,500 fungal reads) (**Supplementary Table 1**).

The taxonomic composition of bacterial and fungal communities also differed between unhulled and hulled seeds (**Figure 1**). In the bacterial community, *Alphaproteobacteria* was dominant in the shoot (58.8%) and leaf endospheres (64.1%) of seedlings grown from hulled seeds, whereas *Gammaproteobacteria* was the most abundant class in unhulled seeds (**Figure 1A** and **Supplementary Table 2**; U0, 77.4%; U1, 71.1%; and U4, 90.1%) and their seedlings (U7Sh, 57.7%; U7R, 98.1%; U14L, 79.3%; U14S, 86.9%; and U14R, 91.5%). A similar pattern was observed in the fungal community (**Figure 1B**). In unhulled seeds, *Dothideomycetes* dominated the seed fungal community (**Supplementary Table 2**; U0, 97.6%; U1, 99.2%; and U4, 79.8%). Meanwhile, *Sordariomycetes* (H0, 36.4%; H1, 37.0%; and H4, 16.4%), *Leotiomycetes* (H0, 7.27%; H1, 15.0%; and H4, 36.2%), and *Agaricomycetes* (H0, 9.5%; H1, 16.1%; and H4, 4.7%) were dominant in hulled seeds. *Dothideomycetes* (U7Sh, 13.3%; U7R, 49.6%; U14L, 53.8%; U14S, 65.9%; and U14R, 1.8%) and *Sordariomycetes* (U7Sh, 67.7%; U7R, 31.8%; U14L, 40.4%; U14S, 32.3%; and U14R, 95.9%) were abundant in seedlings developed from unhulled seeds, whereas *Sordariomycetes* dominated the endospheric community of seedlings grown from hulled seeds (**Supplementary Table 2**; H7Sh, 55.1%; H7R, 65.7%; H14L, 88.7%; H14S, 89.8%; and H14R, 95.4%). These results indicate that seed coats provide ecological niches for the microbial communities. The compositional difference in seedling microbial communities in the presence vs. absence of seed coats suggests that the microbial pools present in seeds affect the endophytic microbial community composition of seedlings grown under axenic conditions.

Effects of Seed Coat Presence, Compartment, and Age on Bacterial and Fungal Community Variations

Next, we investigated the factors that contribute to compositional variations in bacterial and fungal communities during the early growth of rice seedlings. For this assessment, we employed ordination analysis (**Figure 2**). PCoA results showed that the bacterial communities cluster into two groups ("Hulled" and "Unhulled") more clearly than fungal communities based on the presence or absence of the seed coat (**Figure 2A**). Bacterial and fungal communities were also grouped by compartment and age (**Figures 2B,C**). The effects of these factors on bacterial and fungal compositional variations were quantified using PERMANOVA. We found that the bacterial community (R^2 = 0.26385, P = 0.0001) is more strongly

[1]http://www.interactivenn.net/

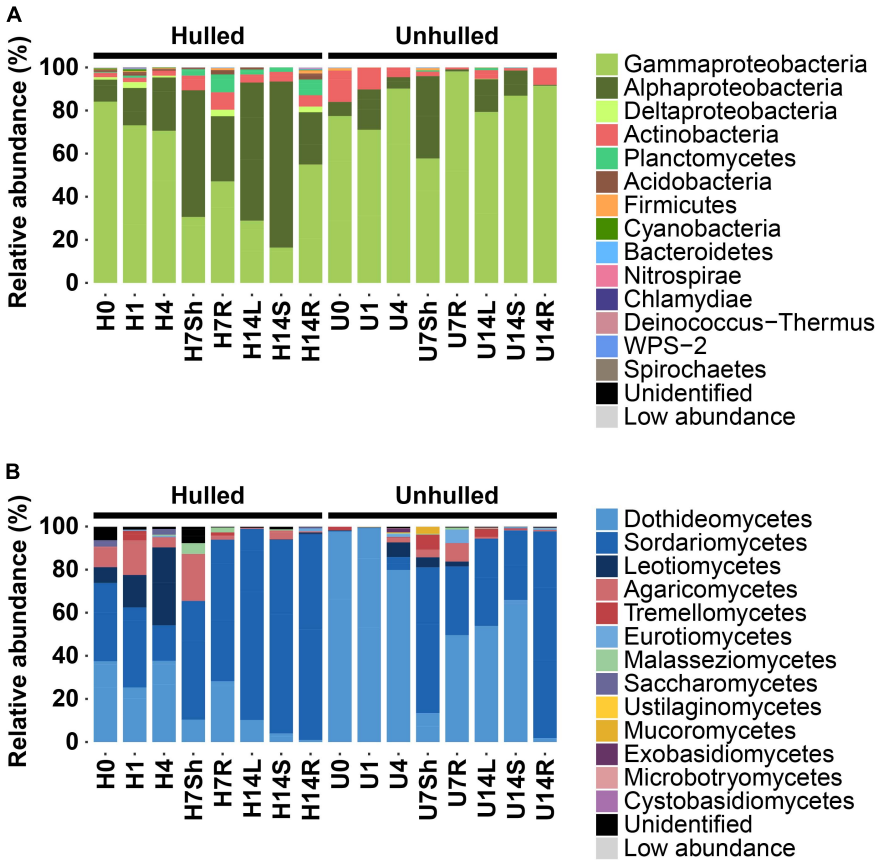

FIGURE 1 | Abundance patterns of bacterial and fungal communities in seeds and seedlings. **(A)** Taxonomic composition of bacterial communities associated with seeds and seedlings grown from hulled and unhulled seeds. **(B)** Taxonomic composition of fungal communities associated with seeds and seedlings grown from hulled and unhulled seeds. In panels **(A,B)**, each bar indicates the taxonomic composition of a given sample. Colors indicate different phyla and classes in the bacterial and fungal communities. Low abundance refers to the group of phyla or classes with relative abundances lower than 0.05%. H and U indicate the absence and presence of seed coats, respectively. The numbers 0, 1, 4, 7, and 14 indicate the day(s) after planting on Murashige and Skoog (MS) agar medium. Sh, L, S, and R represent the endospheric regions of the shoots, leaves, stems, and roots, respectively. The exact numbers of sequence reads and relative abundances of samples are available in **Supplementary Tables 1**, **2**.

affected by the presence of seed coats than the fungal community (R^2 = 0.05366, P = 0.001) (**Supplementary Table 3**). Meanwhile, rice compartments contributed more to fungal compositional variations (R^2 = 0.22301, P = 0.0001) than bacterial variations (R^2 = 0.17518, P = 0.0001). On the other hand, age did not significantly affect bacterial compositional variations (R^2 = 0.05187, P = 0.1869), while fungal community variations were significantly influenced by age (R^2 = 0.08878, P = 0.0078).

Alpha diversity indices of bacterial and fungal communities also showed significant differences with compartment and age [**Supplementary Figure 1**; Kruskal–Wallis test, P = 0.00035 (bacterial richness); P = 0.01449 (fungal richness); P = 0.00137 (bacterial diversity); and P = 0.00103 (fungal diversity)]. In general, bacterial and fungal richness and diversity decreased under hulled conditions (**Supplementary Figure 1**). However, the bacterial richness and diversity of roots under hulled conditions decreased at 7 days after planting and increased thereafter. Similar patterns were observed for bacterial richness and diversity under unhulled conditions (**Supplementary Figure 1**). On the other hand, fungal richness and diversity increased in 4-day-old germinating seeds and decreased at 14 days after planting. These results suggest that microbial community composition and diversity are affected by the development of seedlings.

Movement of Seed Microbial Communities Into Compartments of Seedlings

We examined whether seed-borne OTUs are transmitted to the endosphere of the resulting seedlings. For this assessment, OTU profiles were compared between the microbial communities of seeds at the sowing stage (G0) and compartments of seedlings at 7 and 14 days after planting. We assumed that bacterial and fungal communities of seeds and seedlings consist of similar microbial members if seed-borne OTUs are moved to the compartments of developing seedlings. Dissimilarity of community membership was quantified using the Jaccard

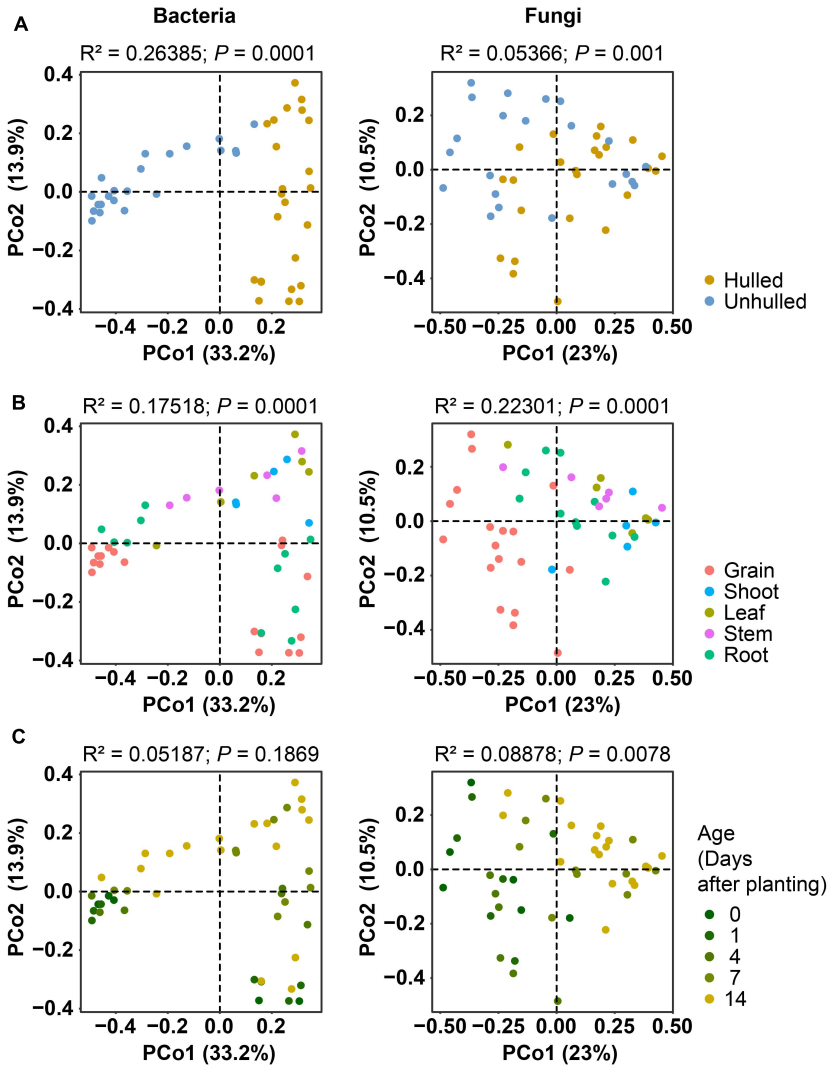

FIGURE 2 | Ordination analyses of bacterial and fungal community compositions showing the effects of seed coat presence, plant compartment, and age. **(A)** Contribution of seed coats to compositional variations. The presence of seed coats ("Unhulled") is indicated with blue dots, and the absence of seed coats ("Hulled") is indicated with yellow dots. **(B)** Influence of rice compartments on compositional variations. Dots are colored according to compartment. **(C)** Effect of age on compositional variations. More vivid yellow dots denote older rice plants. In panels **(A–C)**, the ordination results for bacterial communities are displayed in the left panel, and those for fungal communities are shown in the right panel. Bacterial and fungal community distances were estimated using Bray–Curtis distances with cumulative sum scaling (CSS)-normalized and log-transformed operational taxonomic unit (OTU) abundances. Results on the permutational multivariate analysis of variance (PERMANOVA) are indicated on the top of each plot.

distance (D = 1−Jaccard similarity). D values near 0 indicate that the two microbial communities share a large number of OTUs. In bacterial communities, the Jaccard distances between seeds and germinating seeds or seedlings ranged from 0.5623 to 0.9627 for the hulled condition and from 0.4401 to 0.9367 for the unhulled condition (**Supplementary Table 4**). Meanwhile, the Jaccard distances of the fungal communities ranged from 0.6227 to 0.9492 for the hulled condition and from 0.5829 to 1 for the unhulled condition (**Supplementary Table 4**). The bacterial community composition of seeds at 1 day (Hulled, D = 0.6683 ± 0.078; Unhulled, D = 0.5463 ± 0.059) and 4 days (Hulled, D = 0.7943 ± 0.054; Unhulled, D = 0.5887 ± 0.057) after planting differed less from that of seeds at the sowing stage under both hulled and unhulled conditions compared to other compartments (**Figure 3**; **Supplementary Table 4**). The compositional difference increased with time and the differentiation of plant parts. For example, the endophytic bacterial communities of 7-day-old shoots (Hulled, D = 0.8702 ± 0.005; Unhulled, D = 0.9032 ± 0.023) and 14-day-old leaves (Hulled, D = 0.8992 ± 0.029; Unhulled, D = 0.8564 ± 0.022) showed large differences compared to the seeds at the sowing stage (**Figure 3** and **Supplementary Table 4**). This tendency was also observed for fungal communities, although the differences were less significant than those

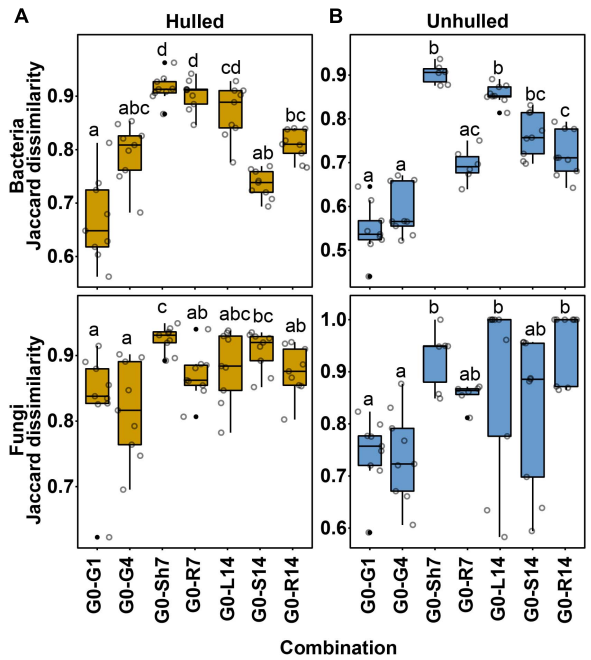

FIGURE 3 | Community dissimilarity between microbial communities before and after planting. **(A)** Community dissimilarity in bacterial and fungal communities between samples taken before and after planting under hulled conditions. **(B)** Community dissimilarity in bacterial and fungal communities between samples taken before and after planting under unhulled conditions. Community dissimilarity was calculated using the Jaccard dissimilarity index. In panels **(A,B)**, the upper and lower panels show bacterial and fungal communities, respectively. Dots on each box plot indicate dissimilarity values estimated through pairwise comparison among biological replicates. Letters indicate the statistical significance, estimated using the Kruskal–Wallis test followed by Dunn's test. The results of the statistical analysis are available in **Supplementary Table 4**. G0, seed microbial community before planting; G1, seed microbial community at 1 day after planting; G4, seed microbial community at 4 days after planting; Sh7, microbial community of the shoot endosphere at 7 days after planting; R7, microbial community of the root endosphere at 7 days after planting; L14, microbial community of the leaf endosphere at 14 days after planting; S14, microbial community of the stem endosphere at 14 days after planting; and R14, microbial community of the root endosphere at 14 days after planting.

observed for bacterial communities. This finding suggests that the differentiation and growth of plant tissues may lead to differentiation of the endophytic microbial communities.

Next, we examined the distribution of OTUs in seeds at the sowing stage and each compartment of 7- and 14-day-old seedlings under hulled and unhulled conditions. We found that limited fractions of the seed microbial communities could be moved to both above- and belowground compartments. For example, 2–6 bacterial OTUs and 1–3 fungal OTUs co-occurred in the seeds and aboveground compartments of 7- and 14-day-old seedlings (**Figure 4**). These OTUs belonged to the bacterial genera *Burkholderia*, *Ralstonia*, *Sphingomonas*, and *Bradyrhizobium* and the fungal genus *Pyricularia* in hulled seeds and their seedlings (**Supplementary Table 5**). On the other hand, in unhulled seeds and their seedlings, co-occurring OTUs were assigned to the bacterial genera *Pantoea* and *Sphingomonas*, the fungal genus *Cladosporium*, and fungal family *Didymellaceae*. Greater numbers of seed OTUs were shared with root endosphere of seedlings than with other compartments. Those shared OTUs belonged to the bacterial genera *Burkholeria*, *Sphingomonas*, *Leifsonia*, *Aquabacterium* (in the hulled condition), *Pseudomonas*, *Pantoea*, *Methylobacterium*, *Curtobacterium* (in the unhulled condition) and the fungal genera *Pyricularia*, *Cladosporium*, and *Alternaria*. Generally, bacterial OTUs co-occurring between seeds and the stem endosphere of 14-day-old seedlings (Hulled, 64.7%; Unhulled, 45.4%) were more shared than the leaf endosphere of seedlings of the same age (Hulled, 60%; Unhulled, 28.5%). In contrast, in the fungal community, more OTUs were shared between seeds and the leaf endosphere (Hulled, 45.4%; Unhulled, 56.2%) than stem endosphere (Hulled, 33.3%; Unhulled, 50%).

Movement of Seed Bacterial and Fungal Communities Under Field Conditions

Since the movement of seed microbial communities from the seed to seedling endosphere had been observed under *in vitro* conditions, we investigated whether seed microbial communities can be moved to and colonize the rice leaf, stem, root, and progeny seeds during rice growth under field conditions. For this test, the distributions of seed-borne microbial communities in leaves, stems, roots, and progeny seeds collected from field-grown rice plants were examined. First, we identified seed-borne OTUs present at the sowing stage (0 days after transplanting) and in other compartments at the tillering, heading, and harvest stages. Almost half of bacterial (51.4%) and fungal (47.3%) OTUs co-occurred in seeds at the sowing and harvest stages (**Figure 5** and **Table 1**). The proportions of seed-borne bacterial and fungal OTUs were lowest at the tillering stage, and increased throughout rice growth in the leaf, stem, and root endospheres (**Figure 5** and **Table 1**). The proportion of seed-borne OTUs was small but accounted for the vast majority of relative abundances of bacterial and fungal communities. In progeny seeds, seed-borne OTUs accounted for 98.6 and 96.1% of relative abundances of the bacterial and fungal communities, respectively (**Figure 5**, **Table 1**, and **Supplementary Table 6**). Meanwhile, aboveground (leaf and stem) and belowground compartments exhibited differing patterns of seed-borne OTU abundances. Among the aboveground compartments, seed-borne OTUs in the leaf endosphere accounted for 25.7% (bacteria) and 40% (fungi) of relative abundances of microbial communities at the tillering stage. In the stem, 28.9% (bacteria) and 36.4% (fungi) of relative abundances were seed-borne OTUs at the tillering stage. During rice development, the cumulative relative abundances of seed-borne OTUs increased to 83.2% (leaf bacterial community), 56.5% (stem bacterial community), 82.5% (leaf fungal community), and 55.9% (stem fungal community) (**Figure 5**, **Table 1**, and **Supplementary Table 6**). On the other hand, seed-borne OTUs in the root endosphere were less dominant in the bacterial (tillering, 0.4%; heading, 1.3%; and harvest, 6.3%) and

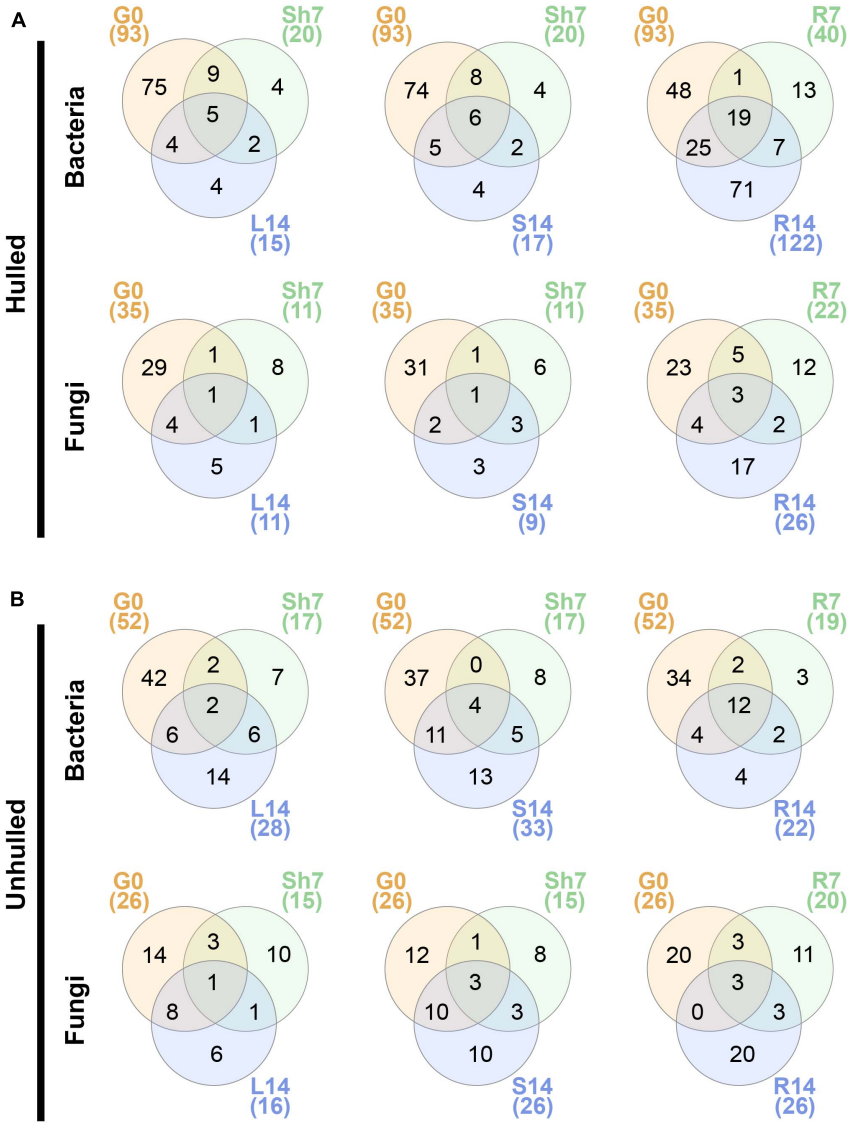

FIGURE 4 | Distribution of bacterial and fungal OTUs in seeds and seedling compartments. **(A)** Distribution of bacterial and fungal OTUs in hulled seeds and their seedlings. **(B)** Distribution of bacterial and fungal OTUs in unhulled seeds and their seedlings. Venn diagrams were constructed using InteractiVenn (http://www.interactivenn.net/). Each circle in the Venn diagrams represents a given sample. The numbers in parentheses indicate the total numbers of OTUs detected in each sample. The numbers on the Venn diagrams indicate the numbers of OTUs uniquely detected or co-occurring across different samples. Taxonomic information for OTUs co-occurring in seeds, 7-day-old seedlings, and 14-day-old seedlings is available in **Supplementary Table 5**. G0, seed microbial community before planting; Sh7, microbial community of the shoot endosphere at 7 days after planting; R7, microbial community of the root endosphere at 7 days after planting; L14, microbial community of the leaf endosphere at 14 days after planting; S14, microbial community of the stem endosphere at 14 days after planting; and R14, microbial community of the root endosphere at 14 days after planting.

fungal (tillering, 17.8%; heading, 23.7%; and harvest, 9.8%) communities compared to aboveground compartments at the same developmental stages. This suggests that seed-borne OTUs are dominant in aboveground compartments, whereas the majority of root microbial communities originate from the soil environment under field conditions.

When focusing on the taxonomic composition of seed-borne OTUs, *Pantoea*, *Sphingomonas*, *Methylobacterium*, *Curtobacterium*, and *Pseudomonas* were dominant in the bacterial communities of aboveground compartments (**Figure 5** and **Supplementary Table 7**). Meanwhile, in fungal communities, *Nigrospora*, *Alternaria*, *Sarocladium*, *Cladosporium*, and *Moesziomyces* were identified as seed-borne OTUs under field conditions (**Figure 5** and **Supplementary Table 7**). The bacterial genera *Pantoea*, *Ralstonia*, *Methylobacterium*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Burkholderia-Caballeronia-Paraburkholderia*, *Herbaspirillum*, and the fungal genera *Chaetomium*, *Alternaria*, *Cladosporium*, *Pyrenochaetopsis*, and *Setophoma* were identified as seed-borne taxa present in root

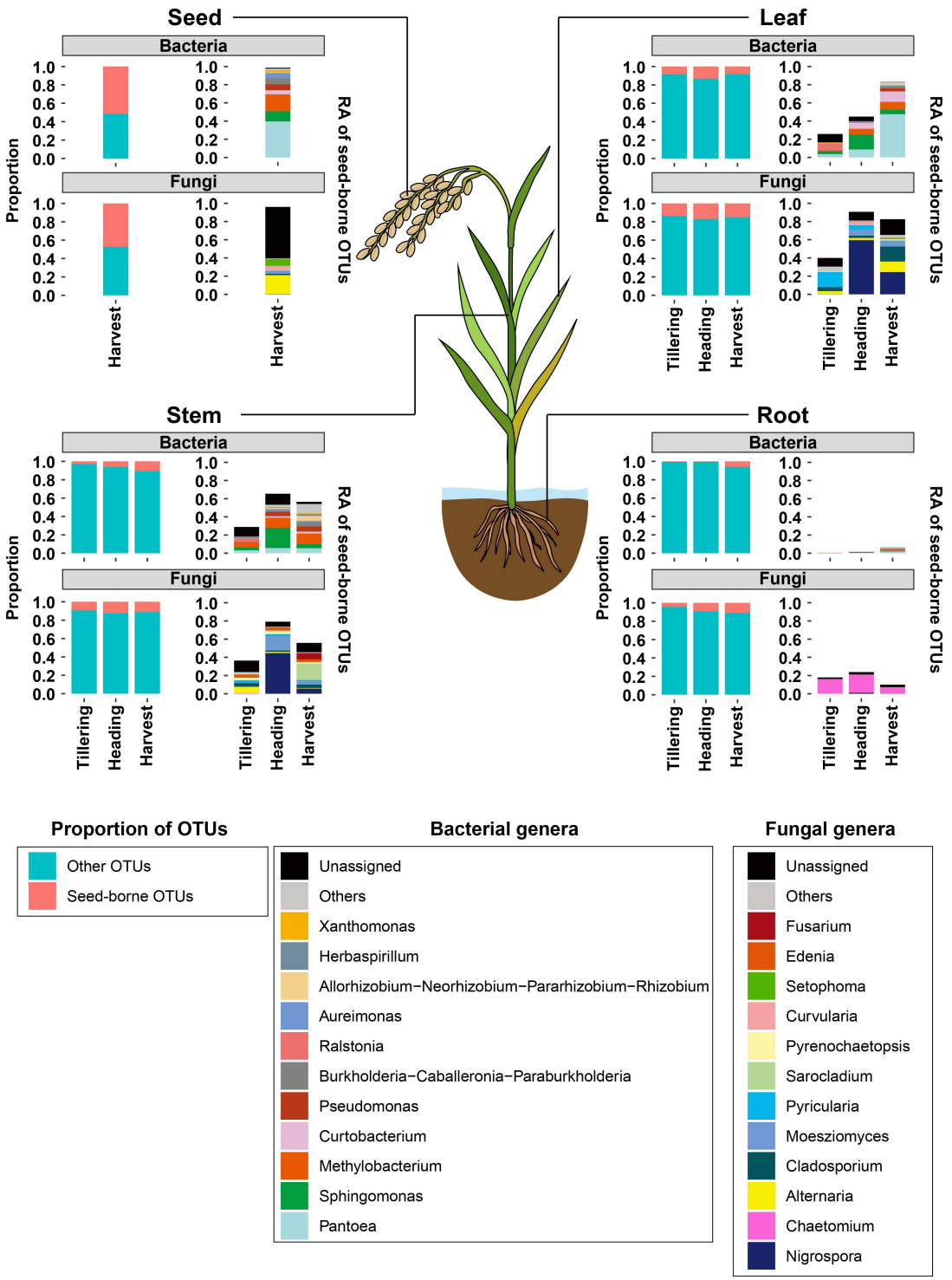

FIGURE 5 | Distribution of seed-borne bacterial and fungal communities in compartments of field-grown rice plants. The distributions of seed-borne bacterial and fungal OTUs were investigated at three developmental stages: tillering, heading, and harvest. At each developmental stage, the proportion of seed-borne OTUs in the leaf, stem, root, and seed endospheres was calculated as the number of seed-borne OTUs divided by the total numbers of OTUs in that compartment. The proportions of seed-borne OTUs are indicated with magenta-colored bars. The remaining portions are indicated with cyan-colored bars. Taxonomic composition of seed-borne OTUs in plant compartments is shown at the genus level. Each color on the bar plots represents a bacterial or fungal genus. Genera with relative abundance lower than 0.03 are grouped into "Others." The raw data on the relative abundances of bacterial and fungal genera are available in **Supplementary Table 6**. RA, relative abundance.

TABLE 1 | Seed-borne bacterial and fungal operational taxonomic units (OTUs) in the leaf, stem, root, and progeny seeds.

Kingdom	Compartment	Stage	Number of seed-borne OTUs	Total number of OTUs	Proportion of seed-borne OTUs	Cumulative abundance of seed-borne OTUs
Bacteria	Seed	Harvest	36	70	0.5143	0.986
		Tillering	11	127	0.0866	0.257
	Leaf	Heading	22	166	0.1325	0.449
		Harvest	49	588	0.0833	0.832
		Tillering	25	855	0.0292	0.289
	Stem	Heading	51	843	0.0605	0.654
		Harvest	65	601	0.1082	0.565
		Tillering	7	1,116	0.0063	0.00427
	Root	Heading	6	1,356	0.0044	0.0136
		Harvest	27	436	0.0619	0.0633
Fungi	Seed	Harvest	63	133	0.4737	0.961
		Tillering	39	285	0.1368	0.4
	Leaf	Heading	54	317	0.1703	0.907
		Harvest	83	552	0.1504	0.825
		Tillering	58	613	0.0946	0.364
	Stem	Heading	81	653	0.1240	0.792
		Harvest	84	754	0.1114	0.559
		Tillering	10	233	0.0429	0.178
	Root	Heading	18	195	0.0923	0.237
		Harvest	19	171	0.1111	0.0984

microbial communities (**Figure 5** and **Supplementary Table 7**). Among these taxa, we aimed to identify the specific bacterial and fungal taxa that could move to seedling compartments under axenic conditions and were also present under field conditions. *Pantoea*, *Methylobacterium*, and *Sphingomonas* were identified in the bacterial communities of both the leaf and stem endospheres under axenic and field conditions (**Supplementary Tables 5**, **7**). In the fungal community, *Nigrospora*, *Alternaria*, *Malassezia*, Unidentified genus belonging to *Didymellaceae*, and *Pyricularia* were seed-borne OTUs in aboveground compartments under both axenic and field conditions (**Supplementary Tables 5**, **7**). These common OTUs suggest that seed-borne OTUs may outcompete environmentally transmitted microbial communities.

DISCUSSION

The seed coat is a protective layer, as well as a channel for transmitting environmental cues to the interior of the seed (Radchuk and Borisjuk, 2014). The seed coat could also act as an ecological niche to support microbial communities. We found that the compositions of the microbial communities associated with seeds and seedlings differed according to the presence of the seed coats (**Figures 1**, **2**). This is corroborated by the study reporting that seed compartments (outer husk, husk, outer grain, and grain) harbor different bacterial and fungal community compositions (Eyre et al., 2019). Compositional differences between hulled and unhulled seeds were also detected in seedlings grown under axenic conditions. This finding suggests that endophytic microbial communities in emerging tissues are shaped by endogenous seed microbial pools under axenic conditions.

In the present study, we investigated movement of seed-borne OTUs to seedlings and adult plants under axenic and field conditions. We found that bacterial OTUs belonging to *Pantoea*, *Sphingomonas*, and *Methylobacterium* and fungal OTUs belonging to *Alternaria*, *Cladosporium*, and *Pycularia* were commonly found as seed-borne taxa able to colonize in leaf and stem endosphere under axenic and field conditions (**Figure 5** and **Supplementary Table 5**). However, the distribution patterns of seed-borne OTUs showed differences between axenic and field conditions in root endosphere communities. In the axenic culture, about 50% of bacterial and fungal OTUs co-occurred in the root endosphere and seeds (**Figure 4**), whereas the proportion and cumulative relative abundance of seed-borne OTUs were lower in the root endospheres of field-grown rice (**Figure 5**). These findings suggest that root bacterial and fungal communities originating from seeds might be depleted through the competition with invaders from the soil. Meanwhile, at the late stage of life cycle of rice under field condition, these seed-borne taxa dominated the endophytic communities of leaf and stem, suggesting that seed-borne microbial communities colonizing in the endosphere of the aboveground compartments could stand competition with external microbes.

Ordination and community dissimilarity analyses revealed that both bacterial and fungal compositions differed by compartment (above- and belowground tissues) and age (**Figures 2**, **3**). In particular, compartment was the significant

factor shaping bacterial and fungal community compositions. This relationship could be driven by a combination of niche differentiation due to temporal changes in plant physiological conditions, plant regulatory cues limiting microbial transmission, and microbial traits (Abdelfattah et al., 2021). Previous studies reported physiological and metabolic differences between the leaves and roots of wheat (Kang et al., 2019), rice (Baldoni et al., 2016), and barley (Klem et al., 2019). Morphological differences, photosynthesis and chlorophyll metabolism, and differential distributions of metabolites (including amino acids and carbohydrates) lead to niche differentiation between above- and belowground compartments during plant development. Parallel and longitudinal investigation of the transcriptome, proteome, metabolome, and microbiome in plant tissues will reveal the molecular mechanisms underlying niche differentiation and their relationships with plant microbiomes.

The diversity of bacterial and fungal communities was affected by the development and growth of seedlings (**Supplementary Figure 1**). A decrease in microbial diversity during seedling growth was reported for 28 plant genotypes belonging to Brassicaceae (Barret et al., 2015). During the germination and development of seeds and seedlings, the chemical properties of the seeds and surrounding environment change dramatically due to exudation (Bewley, 1997; Higashinakasu et al., 2004). Seed exudates contain carbohydrates, organic acids, fatty acids, amino acids, proteins, and other secondary metabolites (Schiltz et al., 2015). This finding indicates that seed exudates could cause the surrounding environment to become copiotrophic. The exudates of germinating seeds may affect distribution of oligotrophs and copiotrophs and may reduce microbial diversity similar to the effects of nutrient addition on soil microbial communities (Leff et al., 2015; Zeng et al., 2016). After germination, the changes in microbial richness and diversity differed according to the presence of seed coats and the kingdom (**Supplementary Figure 1**). An investigation of viable seed microbial communities and their correlations with seed exudates could provide more information about the effects of plant physiology on seed-borne microbial communities.

We identified not only OTUs shared among seeds and compartments of developing seedlings but also OTUs uniquely distributed in seeds or developing seedlings under axenic conditions (**Figure 4**). The compositional differences between microbial communities of seeds and compartments of seedlings might be related to intrinsic variability in seed microbial communities and seed rare taxa which abundances are below detection level in seeds but can proliferate in developing seedlings. The variability of microbial communities within seed populations have been reported in maize and common beans (Rosenblueth et al., 2012) and wheat (Özkurt et al., 2020). This seed-to-seed variability of microbial communities may affect compositional variability of developing seedlings. The similar results were also reported in acorn (Abdelfattah et al., 2021). Experimentally, test tubes where seedlings were grown were capped by a silicone rubber stopper that is widely used for microbial cultures. We also used a MS agar medium on which both plants and microbes can grow. We did not observe visible bacterial colonies and fungal mycelia which can be considered as contamination on the MS agar media during the seedling growth. Based on this observation, we considered that external contaminations are blocked during the experiment. Although we could not specify the reasons for the existence of unique OTUs in the developing seedlings, our findings suggest that seed bacterial and fungal communities could move from seed to developing seedlings partially.

We revealed that seed-borne OTUs in both bacterial and fungal communities are the main colonizers of the endospheres of leaves and stems (**Figure 5**). Movement of these OTUs occurred during growth of seedlings (**Figure 4**). Previous research reported that 45% of the bacterial community of first generation are vertically transmitted to seeds of second generation (Hardoim et al., 2012). We also found that 51.4% of bacterial OTUs and 47.3% of fungal OTUs were inherited by second-generation seeds. *Pantoea*, *Methylobacterium*, *Pseudomonas*, and *Sphingomonas* were commonly identified among the inherited taxa. These taxa were also identified in the seeds of wild species and their relatives in the genus *Oryza* (Kim et al., 2020). Seeds of radish (Rezki et al., 2018), bean (Chesneau et al., 2020), and maize (Johnston-Monje and Raizada, 2011) also harbor *Pantoea* and *Pseudomonas*, suggesting that these taxa may be preserved in the seeds of many plant species. These taxa may persist across generations in radish via the floral pathway, in which microbes are transmitted via reproductive organs (Chesneau et al., 2020). This finding implies that *Pantoea* and *Pseudomonas* may be vertically transmitted through both internal and floral pathways.

We found that fungal communities in rice seeds and seedlings are dominated by *Nigrospora*, *Pyricularia*, *Alternaria*, and *Cladosporium.* While seed fungal communities are less studied than bacterial communities, there have been a few reports that *Alternaria*, *Cladosporium*, and *Pleosporaceae* are abundant in the seed fungal communities in rice cultivars and wild rice species (Eyre et al., 2019; Kim et al., 2020), as well as in plants belonging to *Brassicaceae* (Barret et al., 2015), suggesting that ecological niches in seeds might be suitable for these fungal genera regardless of plant species. Similar to bacterial communities, we observed that fungal communities could be vertically transmitted through the seed. OTUs belonging to *Nigrospora*, *Alternaria*, *Cladosporium*, and *Moesziomyces* could persist throughout the growing season (**Figure 5** and **Supplementary Table 7**). These OTUs were distributed in the leaf and stem compartments (**Figure 5** and **Supplementary Table 7**). In forbs, which are herbaceous dicot plants, *Alternaria* and *Cladosporium* can be vertically transmitted and occur in seeds, cotyledons, and true leaves (Hodgson et al., 2014). Fungal taxa abundant in seeds were less common in the roots and rhizosphere of the common sunflower (*Helianthus annuus*) (Leff et al., 2017). The findings of the present study and previous reports suggest that fungi capable of vertical transmission might proliferate in ecological niches of the endosphere of aboveground compartments. Future works similar to the previous study on *Epichloë*, which demonstrated systemic colonization of in stem endosphere by the fungus during the growth of grasses (Kayano et al., 2018), will provide the evidence for vertical transmission of fungal community members via aboveground tissues.

CONCLUSION

The present study provides novel insights into the dynamics of seed microbial communities during early developmental stages and seed maturation. We conducted temporal analyses of the bacterial and fungal communities associated with rice seeds and adult plants using amplicon-based community profiling. Through this approach, we found that seed bacterial and fungal communities could move from seeds to above- (shoots, leaves, and stems) and belowground compartments of seedlings. We also identified bacterial and fungal OTUs that were vertically transmitted and systemically distributed in aboveground compartments. The next step is to assess the functional properties of microbial communities and identify host factors, including genes, phytohormones, and metabolites, affecting their distribution in germinating seeds, seedlings, and adult plants over time. This approach will provide comprehensive insights into the temporal shift in ecological niches caused by host factors, as well as microbial functions that affect host physiology. This study provides an ecological basis for understanding the establishment of seed-borne plant microbiomes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA728672 and PRJNA733292. Raw input files, and all the codes used for statistical analyses in this study are available at https://github.com/hyunkim90/seed_to_seedling_movement.

AUTHOR CONTRIBUTIONS

HK and Y-HL conceived and designed the study, discussed and interpreted the results, and contributed to the writing of the manuscript. HK carried out all experiments and analyzed the data. Both authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Microbiology

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Seed-Transmitted Bacteria and Fungi Dominate Juvenile Plant Microbiomes

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Plant microbiomes play an important role in agricultural productivity, but there is still much to learn about their provenance, diversity, and organization. In order to study the role of vertical transmission in establishing the bacterial and fungal populations of juvenile plants, we used high-throughput sequencing to survey the microbiomes of seeds, spermospheres, rhizospheres, roots, and shoots of the monocot crops maize (B73), rice (Nipponbare), switchgrass (Alamo), *Brachiaria decumbens*, wheat, sugarcane, barley, and sorghum; the dicot crops tomato (Heinz 1706), coffee (Geisha), common bean (G19833), cassava, soybean, pea, and sunflower; and the model plants *Arabidopsis thaliana* (Columbia-0) and *Brachypodium distachyon* (Bd21). Unsterilized seeds were planted in either sterile sand or farm soil inside hermetically sealed jars, and after as much as 60 days of growth, DNA was extracted to allow for amplicon sequence-based profiling of the bacterial and fungal populations that developed. Seeds of most plants were dominated by Proteobacteria and Ascomycetes, with all containing operational taxonomic units (OTUs) belonging to *Pantoea* and *Enterobacter*. All spermospheres also contained DNA belonging to *Pseudomonas*, *Bacillus*, and *Fusarium*. Despite having only seeds as a source of inoculum, all plants grown on sterile sand in sealed jars nevertheless developed rhizospheres, endospheres, and phyllospheres dominated by shared Proteobacteria and diverse fungi. Compared to sterile sand-grown seedlings, growth on soil added new microbial diversity to the plant, especially to rhizospheres; however, all 63 seed-transmitted bacterial OTUs were still present, and the most abundant bacteria (*Pantoea*, *Enterobacter*, *Pseudomonas*, *Klebsiella*, and *Massilia*) were the same dominant seed-transmitted microbes observed in sterile sand-grown plants. While most plant mycobiome diversity was observed to come from soil, judging by read abundance, the dominant fungi (*Fusarium* and *Alternaria*) were also vertically transmitted. Seed-transmitted fungi and bacteria appear to make up the majority of juvenile crop plant microbial populations by abundance, and based on occupancy, there seems to be a pan-angiosperm seed-transmitted core bacterial microbiome. Further study of these seed-transmitted microbes will be important to understand their role in plant growth and health, as well as their fate during the plant life cycle and may lead to innovations for agricultural inoculant development.

Keywords: plant microbiome, plant mycobiome, spermosphere, rhizosphere, endophyte, phyllosphere, core microbiome, seed microbiome

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INTRODUCTION

Over hundreds of millions of years, angiosperms have coevolved with microbes that helped them acquire nutrients, resist stress, and combat pathogens. Today, plants are considered to be holobionts, a community of microbes cooperating and coevolving with their host to stimulate its anatomy, physiology, development, immunity, behavior, and genetic variation (Rosenberg and Zilber-Rosenberg, 2016). Agricultural science began to appreciate the importance of these plant–microbe interactions with the discovery of soil-inhabiting arbuscular mycorrhizal fungi, which colonize about 90% of land plant species and aid in nutrient acquisition, and also by the realization that nodules on the roots of leguminous plants are powered by nitrogen-fixing bacteria (Johnston-Monje et al., 2021). Besides mycorrhizae and rhizobia, agriculture in some parts of the world has for many decades appreciated that other microbes may also play important roles in plant growth and productivity; for example in the 1970s, stem-inhabiting bacterial endophytes were discovered in Brazil (principally coordinated by EMBRAPA Agrobiologia scientist Johanna Döbereiner) to be important in the nitrogen economy of graminaceous grasses (Baldani and Baldani, 2005). It was not until the advent of high-throughput sequencing technologies at the beginning of this new millennium, however, that the immense diversity of plant-associated microbes began to be understood by the broader scientific community, highlighting the potential to discover many new beneficial plant-associated bacteria and fungi. As this exciting frontier of agricultural science continues to unfold, rational microbiome engineering to improve crop resilience and productivity will only become possible if the rules of microbiome function, provenance, transmission, assembly, and inheritance are elucidated (Vandenkoornhuyse et al., 2015; Busby et al., 2017; Arif et al., 2020).

Plant-inhabiting microbial populations may vary between host species or cultivar; organs or tissues or surfaces; developmental stage; geographic location; plant health; and even year sampled (Berg et al., 2016; Müller et al., 2016; Compant et al., 2019). Microbes in the soil immediately around roots (rhizosphere) are studied for their importance in breaking down organic materials and producing nutrients for plant absorption, while microbes inside the plant (endosphere) influence plant physiology and help control pathogens. Microbes in aboveground parts of the plants inhabit what is known as the phyllosphere, where again they mostly help their host by influencing physiology and controlling pathogens. Less studied, seeds and the area around the germinating seed (spermospheres) are increasingly being appreciated as microbial habitats contributing microbiota that can protect seeds against rotting in the soil, aid in germination, and increase seedling vigor (Nelson, 2018). Probably because of the legacy effects relating to the agricultural importance of soil-transmitted mycorrhizae and rhizobia, even today, agricultural science still largely believes the majority of plant-inhabiting microbes in phyllospheres, endospheres, and rhizospheres are acquired by horizontal transmission from soil (Bakker et al., 2013; Vandenkoornhuyse et al., 2015). For example, a foundational study describing the core root microbiome of *Arabidopsis* concluded that all of the plant's rhizosphere and endosphere comes from the soil, although it should be noted that the seeds used were surface sterilized and no microbe-free substrate was included as a negative control (Lundberg et al., 2012). Besides soil, microbes are also believed to horizontally colonize plant surfaces and endospheres through contact with insects (Allard et al., 2018), dust, rain, and other plant surfaces (Bulgarelli et al., 2013). Unlike phyllospheres or rhizospheres, the inside of the host plant (endosphere) is a controlled habitat, requiring horizontally transmitted endophytes to find ways to enter through cracks, wounds, stomata, or complex signal-based mechanisms (Ibáñez et al., 2017).

In the last few decades, various publications began documenting the presence of non-pathogenic bacteria and fungi in and on seeds of many plant species (Truyens et al., 2015; Nelson, 2018). Evidence has also begun accumulating that vertical or seed transmission also significantly contributes to the plant microbiome (Li et al., 2019). As an example, our previous studies on the juvenile maize microbiome have found that bacterial seed endophytes can colonize other plant tissues, travel throughout the endosphere, and exit the roots to colonize the rhizosphere (Johnston-Monje and Raizada, 2011). We have also found that bacterial populations in maize seeds are a more important source of inoculum for juvenile root endobiomes, than is soil (Johnston-Monje et al., 2014), and that the most abundant bacteria in juvenile maize rhizospheres are vertically rather than horizontally transmitted (Johnston-Monje et al., 2016). A variety of other plant species, including rice, *Arabidopsis thaliana*, wheat, and tomato, have been shown to acquire at least some of their microbiome from their seeds (Nelson, 2018). If plants are truly holobionts that have survived and coevolved with microbes for hundreds of millions of years (Vandenkoornhuyse et al., 2015), it makes sense that their most important symbionts would be vertically transmitted through seed rather than gambling that all of the correct soil-dwelling microbes might be available at the germination site (Nelson, 2018). Vertical transmission may also give beneficial microbes the chance to establish founder populations and claim priority effects, helping define the microbiome of the plant from early on (Toju et al., 2018). Although much work needs to be done to better understand the importance of seed endophytes, it has been shown that they can aid in germination, provide protection from pathogens, and improve mineral nutrition and vigor of the seedling (Puente et al., 2009; Nelson, 2018; Li et al., 2019). It is troubling to think that because of the use of vegetative propagation in plants like cassava, potatoes, and strawberries, in addition to the phytosanitary standards requiring the physical and chemical disinfestation of botanical or vegetative seeds in order to have pathogen-free crops, the normal transmission of microbes from seeds to seedlings may have been interrupted by modern agriculture (Berg and Raaijmakers, 2018).

Regardless of provenance, with thousands of different species of microbe in the plant biome, how does one determine which are the most important to the plant's well-being and productivity? In ecology, a positive relationship between a specie's abundance and occupancy is considered a robust indication of its ecological importance (Gaston et al., 2000). These principles also function

well in the study of plant microbial ecology, where microbial abundance and occupancy are important for the identification of core microbiomes (Shade and Stopnisek, 2019). The ecological importance of abundance is intuitively easy to understand. Sequence the bacterial populations in soybean root nodules, and by far the most abundant members observed are *Bradyrhizobium*, which are the preferred nitrogen-fixing endosymbionts of those plants (Sharaf et al., 2019). Watermelon cultivars which are susceptible to fusarium wilt, will accumulate much higher levels of *Fusarium* in their roots than will resistant cultivars (Xu et al., 2020). Identify soils with high levels of plant pathogens (such as *Fusarium solani*, *Verticillium dahliae*, *Rhizoctonia solani*, and *Colletotrichum truncatum*), and it is possible to identify the part of the field the sickest strawberry plants will develop (Mirmajlessi et al., 2018).

When trying to identify a core microbiome, occupancy (how often a microbe is observed in a sample) is most often considered the defining characteristic. For example, one definition of a core microbiome are those bacteria and fungi that are closely associated with a particular species or genotype of plant (i.e., high occupancy), independent of environmental conditions (Toju et al., 2018). Core microbiomes are thought to contain key microbial taxa that have been important for plant survival and reproduction over evolutionary time (Shade and Handelsman, 2012; Lemanceau et al., 2017). Such microbes, must have over millions of years, developed a robust and efficient transmission strategy and retained the ability to colonize the plants and also to provide beneficial functions that contribute to plant growth, survival, and/or reproduction; traits which could be under positive selection in the holobiont (Wassermann et al., 2019a). Because of the theoretical importance for agriculture, searches for core microbiomes have been attempted in *Arabidopsis* (Lundberg et al., 2012; Brachi et al., 2017), potato (Pfeiffer et al., 2017), grape (Zarraonaindia et al., 2015), sugarcane (Hamonts et al., 2018), tomato (Lee et al., 2019), wheat (Schlatter et al., 2020), switchgrass (Bowsher et al., 2020), and rice (Edwards et al., 2015) among others. Beyond being important in the microbiome of a single plant species, microbes that are core to multiple plant species may be evidence of a larger pattern of transmission, environmental inoculation, or host evolution. It is interesting to speculate that the holobiont common ancestor of angiosperm plants, which split into monocots and dicots about 150 MYA (Chaw et al., 2004), would have also possessed a core microbiome that may have been passed on to all of its descendants. A few searches for core microbiomes across plant species have already been conducted, for example among the germinating seeds of 28 different species of agricultural plant including *A. thaliana*, *Solanum lycopersicum*, and *Phaseolus vulgaris* (Barret et al., 2015), among soil-grown roots of three *A. thaliana* ecotypes and wild relatives (Schlaeppi et al., 2014), among the roots and rhizospheres of 30 different crop plants grown from surface-sterilized seed in soil (Fitzpatrick et al., 2018), and inside the roots of 31 taxonomically diverse plant species growing on sand dunes in an Australian nature reserve (Yeoh et al., 2017). From a technological point of view, microbes with high occupancy and beneficial bioactivity across a spectrum of plant species are very attractive, theoretically allowing one strain to become an inoculant for diverse crop species. For example, *Burkholderia phytofirmans* is an endophytic bacteria that can colonize and promote the growth of a wide range of angiosperms including *A. thaliana*, grape, maize, potato, switchgrass, tomato, and wheat (Afzal et al., 2019). Another bacterial endophyte, *Gluconacetobacter diazotrophicus*, is claimed by the company Azotic Technologies to be able to both colonize and fix substantial amounts of nitrogen in a wide variety of plants including rice, wheat, maize, tomato, potato, tobacco, cotton, sunflower, lettuce, cassava, soybean, pea, beans, and even *A. thaliana* (Dent et al., 2017). The fungal endophyte *Piriformospora indica* has been tested on over 150 different species of plants, where it has consistently been shown to promote plant growth and enhance yield, increase seed germination and vigor, increase flowering/fruiting, augment nutrient uptake, and aid in abiotic and biotic stress resistance (Singhal et al., 2017).

Beneficial microbes are usually discovered in the lab and then screened in small-scale assays within labs or greenhouses. After isolation and screening, the strain then needs to be properly formulated for delivery into a farmer's field if it ever hopes to impact agriculture, and this bottleneck can dramatically reduce the number of candidates that find commercial success. The most efficient and practical method of agricultural microbe delivery is through the seed, where a relatively small amount of inoculum is needed (compared to soil) and microbes are well positioned to colonize the emerging seedling and potentially the whole plant for its entire life (O'Callaghan, 2016). *B. phytofirmans* for example, was originally isolated from onion roots (Sessitsch et al., 2005), and despite consistently being able to promote growth of a wide variety of plants under controlled conditions, without development of an effective, practical, and scalable way to coat it onto crop seeds, this bacteria has not been able to directly impact agriculture as a commercial product. Rather than attempting to develop inoculant formulations to help root, shoot, rhizosphere, or soil microbes survive on the seed surface, the study of seed microbiomes from a variety of crop species may yield insights into which microbes are already best suited/preadapted to be seed inoculants (von-Maltzahn et al., 2015, 2017). The ideal situation for a company would be to find core microbes, adapted to life on the dry surface of a seed and with the ability to survive there for weeks or months until germination in farm soil, whereupon it can colonize the developing roots and shoots of any crop plant and begin to influence plant growth and health in a beneficial manner.

Our experiment attempts to document the common (appearing in over 60% of samples) and core (appearing in 100% of samples) microbes inhabiting seed interiors and seed surfaces (spermospheres) of a panel of 17 academically and economically important plant species, many of which have had their genomes sequenced and serve as model organisms. These plants include the monocot crops maize (*Zea mays* ssp. mays var. B73), rice (*Oryza sativa* ssp. japonica var. Nipponbare), switchgrass or Panicum (*Panicum virgatum* var. Alamo), *Brachiaria decumbens*, wheat (*Triticum aestivum*), sugarcane (*Saccharum officinarum*), barley (*Hordeum vulgare* ssp. vulgare), and sorghum (*Sorghum bicolor* ssp. bicolor); the dicot crops tomato (*S. lycopersicum* Heinz 1706), coffee (*Coffea arabica* var. Geisha), common bean (*P. vulgaris* G19833), cassava (*Manihot*

esculenta), soybean (*Glycine max*), pea (*Pisum sativum*), and sunflower (*Helianthus annuus*); and the model plants *A. thaliana* (Columbia-0) and *Brachypodium distachyon* (Bd21). In order to try to see how much of the seed microbiome goes on to make up the microbial populations of developing plants, these were planted in sealed jars filled with sterile sand and water, then left to develop up to 2 months until harvesting their rhizospheres, root endospheres, and phyllospheres for DNA extraction. As soil is classically considered to be the most important source of a plant's microbiome, the sealed jar experiment was also carried out using soil from a cassava field at the International Tropical Agriculture Research Institute in Colombia. Microbiomes of all sample types of plant species growing on sterile or non-sterile soil were compared bioinformatically based on sequencing of the bacterial 16S and fungal internal transcribed spacer (ITS). The primary purpose of this study was to demonstrate the importance of seed transmission to the establishment of plant microbiomes, which have traditionally been assumed to acquire all their microbes from soil. Showing that seeds are dominant players in establishing plant microbiomes could lead to a paradigm shift in our understanding (and ability to manipulate) of plant microbiome assembly, which has been assumed to depend largely on soil. A secondary purpose of this study was to establish whether core seed-transmitted microbiomes might exist across these economically important plant species. Core seed-transmitted microbes are evidence of evolutionary conservation and may point to important physiological functions these microbes perform for angiosperm seeds, as well as suggesting the existence of microbes that could function as broad host range inoculants in agriculture.

MATERIALS AND METHODS

Sources of Seed

Seventeen different seed accessions were obtained for this experiment. From the U.S. National Plant Germplasm System of the U.S. Department of Agriculture were obtained (with accession numbers in brackets) the following: *H. annuus* var. Arrowhead (PI 650649), *H. vulgare* ssp. vulgare var. Beaver (CIho 1915), *O. sativa* ssp. japonica var. Nipponbare (GSOR 100), *P. virgatum* var. Alamo (PI 422006 01 SD), *P. sativum* var. Aa134 (PI 269818), *S. bicolor* ssp. bicolor var. BTx623 (PI 564163 02 SD), *T. aestivum* var. Prospect (PI 491568 TR04ID), and *Z. mays* ssp. mays var. B73 (PI 550473).

Brachiaria decumbens var. Basilisk (CIAT606), *M. esculenta* var. 19 (DI-2015), and *P. vulgaris* var. G19833 were obtained from the CIAT Genebank (Palmira, Valle del Cauca, Colombia).

Solanum lycopersicum var. Heinz 1706 (LA4345) was graciously provided by the C.M. Rick Tomato Genetics Resource Center (Davis, CA, United States).

Arabidopsis thaliana var. Columbia-0 and *B. distachyon* var. Bd21 were donated by the Hazen lab at the University of Massachusetts (Amherst, MA, United States).

Saccharum officinarum var. CS#725 (CC93-4112 x CC91-1987) was obtained from Cenicaña (Florida, Valle del Cauca, Colombia).

Glycine max var. Paramo 29 was purchased from Semillas del Pacifico (Cartago, Valle del Cauca, Colombia).

Coffea arabica var. Geisha was purchased from Agro Ingenio (El Chantaduro, Valle del Cauca, Colombia).

Sources of Soil

Sterile Sand

River sand was purchased in bulk from a hardware store in Palmira, Colombia, and manually sieved to a uniform consistency using a 500-μm metal sieve. Sand was then sterilized by autoclaving twice for 20 min at 121°C, and after transfer to glass jars, it was autoclaved a third time for 20 min at 121°C.

Field Soil

An agricultural mollisol was excavated from a fallow cassava field at a CIAT property near Palmira, Colombia, at GPS coordinates 3.498434, –76.354959 (**Figure 1A**). Large clods were broken into smaller fragments by crushing and then manually sieving to a uniform consistency using a 500-μm metal sieve (**Figure 1B**).

Sieved sand and soil were both submitted for physio-chemical analysis by the "Suelos y Paisajes para la Sostenibilidad" group at CIAT Headquarters (**Supplementary Table 1**: Physiochemical Soil Properties).

Experimental Setup and Plant Growth Conditions

Twenty large seeds or 0.5 g of small seeds of each accession were put in sterile 15- or 2-ml tubes and soaked for 6 h in double-distilled sterile water (**Figure 1C**). Half of these were then transferred to a sterile Petri dish containing a sterile Whatman #1 filter paper (GE HealthCare: United States) and irrigated with 3 ml of sterile water, while the other half received 3 ml of sterile water mixed with 1 g of field soil. These were incubated at 32°C in the dark for several days until germination.

From each dish, two seedlings were transplanted to corresponding jars as they germinated. Autoclaved glass jars were 13-cm tall, 7-cm wide in diameter, and filled with 100 ml of sterile sand (then autoclaved again) or with 100 ml of 1:1 soil/sterile sand, then watered once with 10 ml of sterile distilled water and sealed with a plastic lid. Jars were incubated (and never opened) in a single Panasonic MLR-352H Plant Growth Chamber set at 28°C for 12 h with 5 lm of fluorescent light and for 12 h of darkness at 22°C. Plants were grown between 2 weeks and 2 months, until they were of a significant size or until they hit the lid of the jar. Before harvesting, jar lids were removed inside a laminar flow hood, and shoots were allowed to dry off for 24 h (**Figures 1D,E**).

Harvesting Seed and Root Endospheres, Spermospheres, Phyllospheres, and Rhizospheres

To collect spermospheres and seed endospheres, 2 (maize, Phaseolus, and sunflower), 5, or 0.1 g (Arabidopsis, Brachiaria, and sugarcane) seeds of each species were placed in a 15-ml conical tube and soaked in 5 ml of double-distilled autoclaved water, in darkness for 48 h at 32°C. Tubes were then shaken

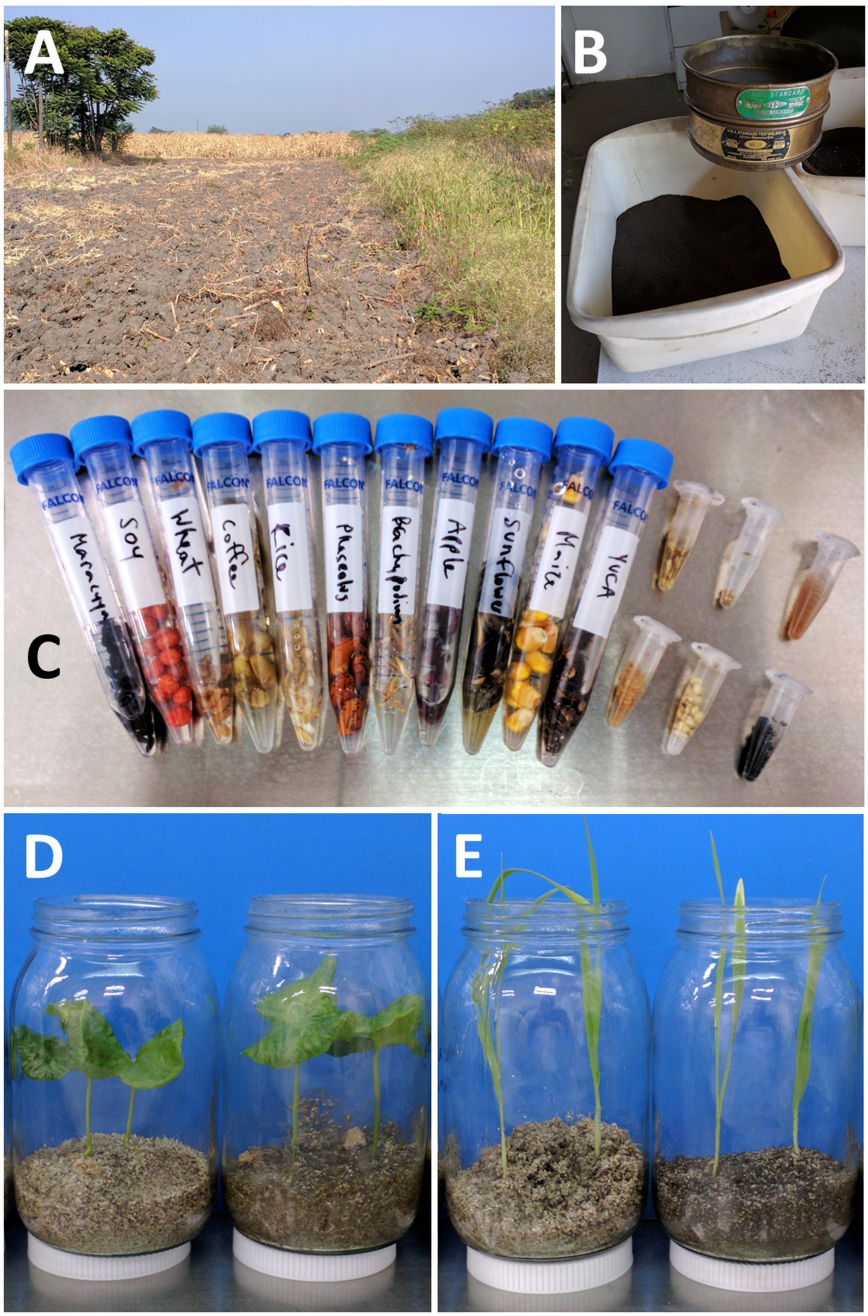

FIGURE 1 | Soil, seeds, and examples of the gnotobiotic terrariums used to grow plants in this study. **(A)** Fallow cassava field at CIAT where soil was harvested. **(B)** Cassava field soil after sieving. **(C)** Soaking in sterile water prior to extraction of seed endosphere and spermosphere DNA (yucca = cassava; maracuya and apple were later replaced with pea and barley). **(D)** Coffee at harvest after growth in sterile sand on the left and field soil mixed with sand on the right. **(E)** Barley at harvest after growth in sterile sand on the left and field soil mixed with sand on the right.

vigorously by hand and vortexed to dislodge microbes from seed surfaces, and then, supernatant liquid was decanted off into sterile conical tubes as spermosphere samples that were immediately frozen at –80°C. The remaining seeds were then surface sterilized/cleaned of DNA by soaking with agitation for 30 min in full-strength Chlorox bleach (6% Na_2HPO_4); rinsed three times in sterile, double-distilled water; and frozen at –80°C.

To collect shoot/phyllosphere material using sterile forceps and scissors, each plant was clipped just above where it emerged from the sand or soil, any remaining seed coat removed, transferred whole to a sterile 50-ml conical tube, cut into smaller pieces within the tube using sterile scissors, and then frozen at –80°C. To collect rhizosphere material, unwashed roots that had been excavated and separated with scissors from the shoot

were shaken free of any attached soil and placed into sterile 50-ml conical tubes. To these, 10 ml of sterile distilled water was added and shaken, with the resulting "muddy wash" collected in a separate 15-ml conical tube as the rhizosphere, which was immediately frozen at –80°C. The roots continued to be rinsed several more times with sterile distilled water until both the wash and root surfaces were completely clean and clear, cut into smaller pieces within the tube using sterile scissors, and then frozen at –80°C in fresh 50-ml conical tubes for later processing (Johnston-Monje et al., 2017). No surface sterilization with aggressive chemicals was attempted on either root or shoot material, just vigorous washing with distilled water.

For each of the 17 species, three repetitions/jars per substrate were sampled (pooling the two plants inside each jar) for root, shoot, and rhizosphere (306 samples). Two repetitions of seed endospheres and spermospheres from each species were also harvested (68 samples).

Sample Preparation and DNA Extraction

After thawing, rhizosphere and spermosphere washes were concentrated by centrifugation at 15,000 *g* for 15 min, generating a pellet. The supernatant was removed, and the process repeated until 3 ml of sample had been processed. The pellet was re-suspended in an additional 1 ml of spermosphere or rhizosphere wash, before proceeding with DNA extraction. In contrast, after thawing, 50-ml conical tubes containing roots, shoots, or seeds received five 6.35-mm carbon steel ball bearings and 1 ml of sterile distilled water and were then vigorously shaken by hand until the supernatant obtained the consistency of a thick soup.

Then, 400 μl of these liquid samples was transferred to a 2-ml Eppendorf tube containing five 2.3-mm zirconia/silica beads (Cat#11079125z, Biospec Products, United States) along with 500 μl of Qiagen Powerbead solution, RNAse A, Phenolics Blocker, and Solution SL (Qiagen, United States). These were shaken for 20 min in a Harbil 5G-HD 5 Gallon Shaker (Part#32940, Fluid Management, United States) and then centrifuged at 13,000 RCF for 2 min before up to 700 μl was aspirated off with a pipette and added to buffer IL. The rest of the protocol was followed as per Qiagen instructions with the DNeasy PowerPlant Pro HTP 96 Kit (Qiagen, United States).

Metagenomic Sequencing Library Preparation

In order to prepare 16S and ITS amplicons for sequencing on the Illumina MiSeq platform, a two-step PCR strategy was employed, first amplifying all 380 DNA extracts with bacterial 16S primers and fungal ITS primers (768 PCR reactions) before dual labeling them with index sequences. The initial PCR was performed with an equimolar mix of staggered universal bacterial 16S [515FB and 806RB (Lundberg et al., 2013)] or fungal ITS [ITS1F and ITS2R (Smith et al., 2020)] primers that included 19 or 20-bp 5′ tail sequences complementary to Illumina MiSeq indexing primers (**Supplementary Table 2**). Anti-chloroplast (5′-GGCTCAACCCTGGACAG-3′) and anti-mitochondria (5′-GGCAAGTGTTCTTCGGA-3′) peptide nucleic acid (PNA) blockers were added to the bacterial 16S PCR reactions to block amplification of chloroplast and mitochondria as previously described (Lundberg et al., 2013). In a total volume of 25 μl, reactions were setup with 18.3 μl of nuclease-free water, 4 μl of 5X Phusion HF buffer, 0.4 μl of 10 mM dNTPs, 0.4 μl of each forward and reverse primer at 10 mM, 0.2 μl of BSA, 0.1 μl of Phusion enzyme (NEB, United States), 0.4 μl of each PNA blocker, and 0.5 μl of template DNA (concentration unknown). Reaction conditions were 35X (denaturation at 98°C for 10 s, PNA annealing at 81°C for 10 s, primer annealing at 50°C for 10 s, and elongation at 72°C for 20 s), final elongation at 72°C for 5 min, and then a cooldown to 4°C.

Without checking for amplification success, PCR product from each of the first 768 PCR reactions was used in a second PCR whose purpose was to dual-label amplicons and add flow cell adapter sequences. These 768 different labeling reactions were conducted using 24 different forward primers (TruSeq_F), containing unique 6-bp index sequences, and 32 different reverse primers (TruSeq_R), each containing unique 6-bp index sequences (**Supplementary Table 2**). In a total volume of 25 μl, step 2 reactions were setup with 19.2 μl of nuclease-free water, 4 μl of 5X Phusion HF buffer, 0.4 μl of 10 mM dNTPs, 0.4 μl of each TSf and TSr primers at 10 mM, 0.1 μl of Phusion enzyme (NEB, United States), and 0.5 μl of unpurified PCR product from step 1 (concentration unknown). Reaction conditions were initial denaturization at 98°C for 30 s, 15X (denaturization at 98°C for 10 s and primer annealing + elongation at 72°C for 20 s), final elongation at 72°C for 5 min, and then a cooldown to 4°C.

The products of these 768 labeling reactions were checked visually for successful amplification (bacterial 16S of 428 bp and fungal ITS of 470–525 bp) on 1% agarose gels and quantity estimated using ImageJ (Schneider et al., 2012) (note: except for negative controls of water and sterile sand, which did not amplify, unsuccessful PCR reactions were repeated until there was sufficient amplicon to allow approximately equimolar amounts of all 96 labeling reactions/plate to be pooled). With this software-assisted visual estimate of amplicon quantity for each reaction within a 96-well plate, equimolar amounts of each PCR product was mixed into eight pools. Pools were concentrated with ethanol precipitation and resuspension in 10% their volume of pure water. To purify target amplicons, 200 μl of each of the eight concentrated pooled sets of PCR products were run on a 2% agarose gel, the appropriate bands excised with a scalpel, and then gel fragments extracted with an Omega Bio-Tek E.Z.N.A. gel extraction kit (Norcross, Georgia, United States). The eight purified pools were again checked visually for purity on an agarose gel, quantified using the Picogreen dsDNA quantitation assay (ThermoFisher Scientific, United States), 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, United States).

Bioinformatics

MiSeq data was demultiplexed by the commercial sequencing facility and received as one FastQ file per sample, which have been deposited at the NCBI Sequence Read Archive (SRA) under BioProject PRJNA731997. Further sequence processing was done using USEARCH 11 using the recommended settings[1].

[1] www.drive5.com

Briefly, paired-end reads were aligned and merged to form full-length sequences called "Uniques," while quality filtering was performed to remove unmatched and low-quality reads. Next, the program binned these full-length reads together at a similarity threshold of 97% and formed a reference sequence for each bin referred to as an operational taxonomic unit (OTU). Only OTUs represented by two or more raw reads were used for analysis. Bacterial 16S OTUs were assigned a taxonomic identity by USEARCH trained on the RDP training set v16 (13,000 sequences), while fungal ITS OTUs were annotated by RDP Classifier (Cole et al., 2014) trained on the RDP Warcup training set v2 (18,000 sequences). Rarefaction of OTU counts was also performed with USEARCH 11. OTU annotations, total counts, and rarefied counts were exported to Excel (Microsoft, United States) for further analysis and visualization, with statistics done by XLSTAT (Addinsoft, France) and PAST 4[2]. Based on taxonomic annotation, OTUs with lower than 15% identity to a target sequence were hand checked and excluded if they were chloroplasts, mitochondria, plant ribosomes, protists, or other non-target sequences. OTU counts were normalized by transformation into proportional abundance as recommended elsewhere (McKnight et al., 2019).

RESULTS

Plant Growth

After collecting and sieving sand and soil (**Figures 1A,B**), then soaking seeds from many different sources (**Figure 1C**), germinating seeds were planted in sealed jars with either sterile sand or field soil and left to grow for up to 2 months until they were harvested for DNA extraction. **Figure 1D** shows coffee plants growing in sterile sand and field soil, while **Figure 1E** shows barley.

Sequencing Summary

A total of 8,294,046 merged bacterial 16S sequence pairs were obtained; however, after quality control, 5,370,471 high-quality reads remained that were binned at 97% sequence identity into 1,178 OTUs. Manual inspection of OTU taxonomy revealed 102 non-target OTUs that were chloroplast or plant/fungi mitochondria, so these were discarded, resulting in 4,945,887 remaining reads. A total of 377 bacterial samples with 16S amplicons yielded read count data, ranging from 12 for maize soil shoot #2 to 80,970 for *Brachypodium* spermosphere #2. Bacterial 16S read counts averaged about 13,015 per sample, compared to the average of 3,119 non-target reads per sample, which were bioinformatically removed. To compare the diversity in different samples using Bray-Curtis dissimilarity, we rarefied the data to 3,500 reads per shoot, 3,000 reads per root, and 6,500 reads per rhizosphere (although samples with low OTU counts were included without rarefaction); OTU counts were transformed to relative proportions, then averaged across repetitions. For all other figures and tables, reads from different reps were summed together, then transformed to relative proportion.

[2]https://folk.uio.no/ohammer/past/

For fungal ITS, a total of 10,269,421 merged sequence pairs were obtained; however, after quality filtering, only 3,203,861 high-quality reads remained that were binned at 97% sequence identity into 680 OTUs. Manual inspection of 127 suspicious OTUs with low identity to fungal ITS revealed many sequences of plant, protest, or bacterial ribosome DNA, so these were excluded from analysis, leaving 2,116,837 reads. Of the 377 fungal samples with ITS amplicons, only 375 ended up returning high-quality data, with read counts ranging from just 1 for barley sand root #1 to 67,329 for coffee seed #1. Fungal ITS read counts averaged about 5,600 per sample; however, without a way to block amplification of non-target reads such as plant ITS sequences (which were bioinformatically removed), these averaged 8,058 per sample; many OTU counts for seed, spermosphere, and shoot samples came out very low. To compare the diversity in different samples using Bray-Curtis dissimilarity, we rarefied the data to 1,000 reads per shoot, 1,000 reads per root, and 2,000 reads per rhizosphere (although samples with low OTU counts were included without rarefaction); OTU counts were transformed to relative proportions, then averaged across repetition. For all other figures and tables, reads from different reps were summed together, then transformed to relative proportion.

Operational taxonomic units sequences and their taxonomy are included as **Supplementary Table 3** (bacterial 16S OTU counts/taxonomy) and **Supplementary Table 4** (fungal ITS OTU counts/taxonomy). Raw sequencing files were submitted to the NCBI SRA under BioProject PRJNA731997.

Microbes in Seeds and Spermospheres

All 68 seed and spermosphere samples yielded bacterial and fungal rDNA sequences, which were summed across biological replicates and normalized to proportional abundance (**Supplementary Data Sheet 1**). Seeds contained an average of 56 bacterial OTUs each and an average Shannon *H* index of 2.2. There was a lower fungal diversity with an average of 14.2 OTU per seeds, with an average Shannon *H* index of 1.4. Spermospheres contained an average of 133 bacterial OTUs each and had an average Shannon *H* index of 2.4. Again, fungal diversity in spermospheres was lower than that for bacteria, with an average of 22.8 OTUs per sample and an average Shannon *H* index of 1.7.

A graphical overview of phylum-level microbial taxonomy is displayed for seeds and spermospheres (**Figure 2**). Nearly all seeds were dominated by OTUs belonging to Proteobacteria and Ascomycetes, with the notable exceptions of coffee, soy, and *Brachypodium*, whose microbiomes were dominated by Firmicutes, while *Panicum*, tomato, and *Phaseolus* seed mycobiomes were made up mostly of Basidiomycetes and other unknown fungi. Bacterial diversity on seed surfaces was greater than that on interiors, but there appeared to be some influence of one to the other. The most obvious examples are bacteria in coffee, soy, and *Brachypodium* spermospheres and seeds that were both dominated by Firmicutes, while both the inside and surface of cassava, sunflower, and sugarcane seeds were nearly all Proteobacteria. The same trend did not appear to hold true for fungal populations on and inside seeds; for example, barley, sorghum, and *Arabidopsis* spermospheres contained

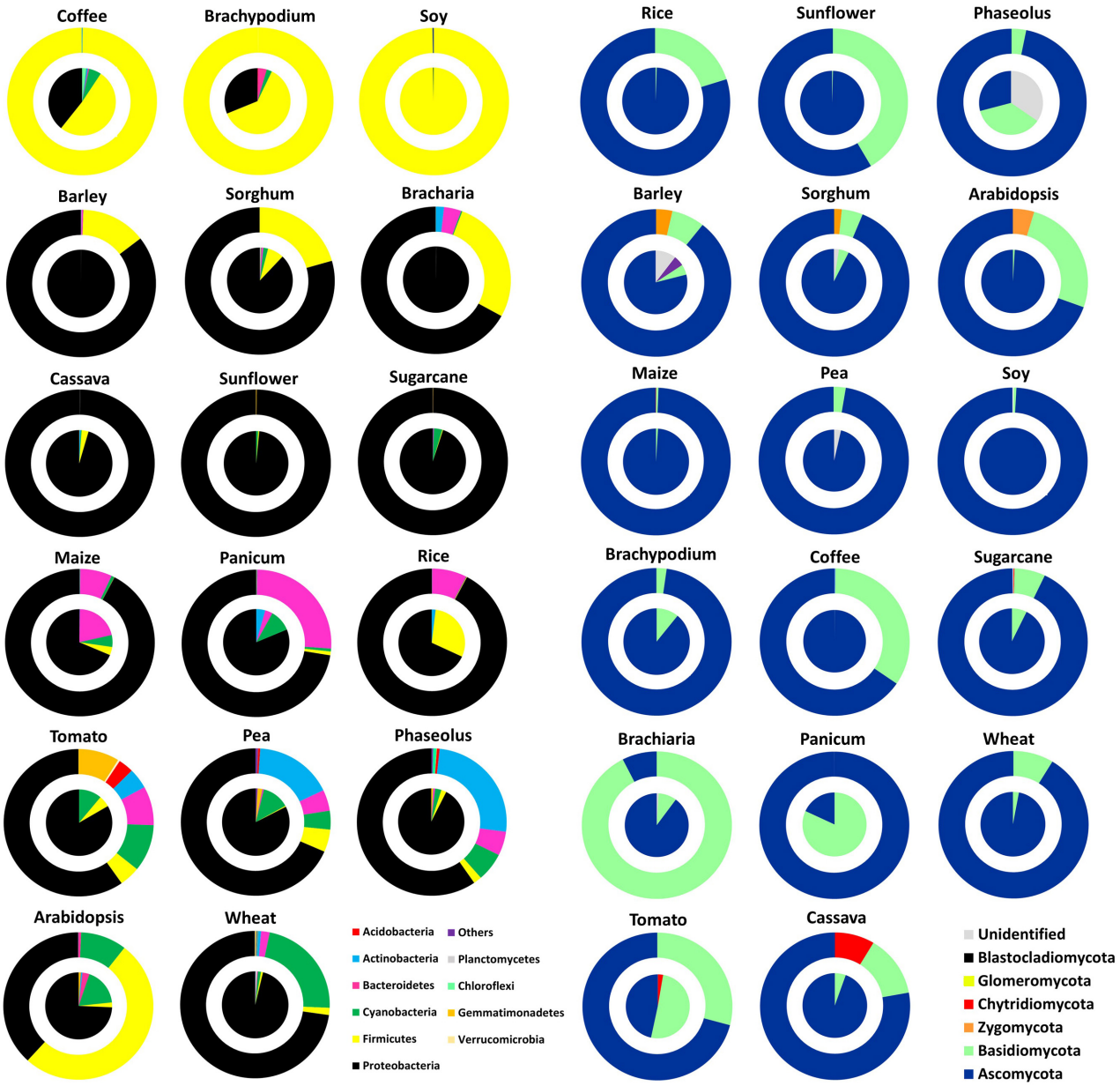

FIGURE 2 | Phylum level classification of bacterial 16S rDNA **(A)** and fungal ITS DNA **(B)** amplified from seeds (pies) and spermospheres (donuts) of the 17 different plant species used in this study. OTUs in each repetition were grouped by sample type and color classified by phylum to yield relative proportion by sample. Seeds and spermospheres were added together and sorted by agglomerative hierarchical clustering using Bray–Curtis dissimilarity of phylum distribution.

zygomycete OTUs that were not detected inside seeds; coffee spermospheres were rich in Basidiomycetes though none were detected inside seeds; Chytridiomycete sequences were found in cassava spermospheres but not inside seeds; and *Panicum* seeds were dominated by Basidiomycetes unlike spermospheres where none were observed.

In an attempt to discover common and core microbiomes of angiosperm seeds and spermospheres, **Figure 3** shows all OTUs with an occupancy in more than 60% of plant species. There were 15 and 37 bacterial OTUs common to angiosperm seeds and spermospheres, respectively (together totaling 38 OTUs), while fungi were much less common with only 4 OTUs appearing in more than 60% of samples (together totaling 5 OTUs). The only core bacteria in seeds were *Pantoea* (BactOTU1) and *Enterobacter* (BactOTU2), which also appeared in all spermospheres. *Pseudomonas* (BactOTU3) and *Bacillus* (BactOTU8) were also core to spermospheres, as was *Fusarium* (FungOTU1).

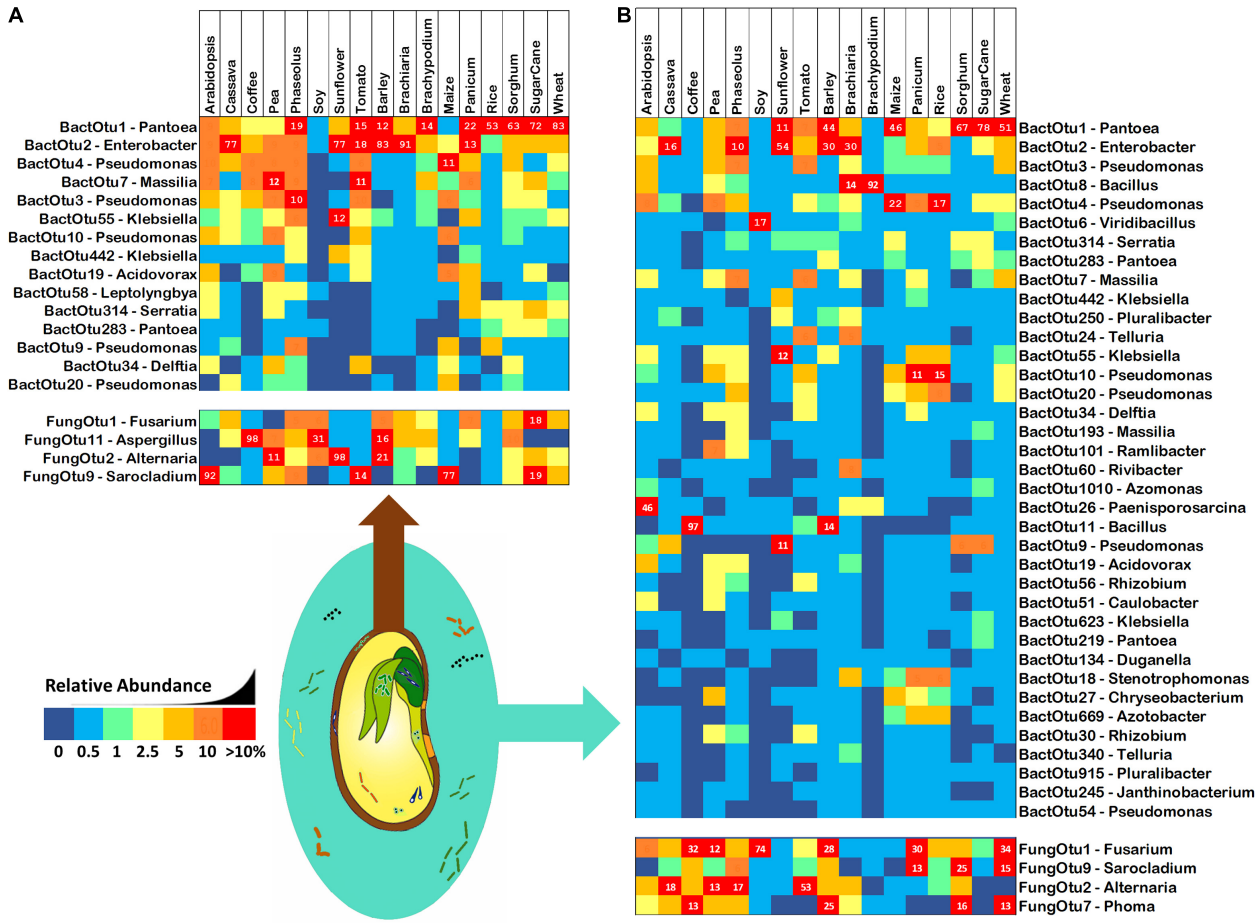

FIGURE 3 | Common bacterial 16S and fungal ITS OTUs of **(A)** seeds and **(B)** spermospheres. Common OTUs were defined as having an occupancy of more than 60% across seeds or spermospheres of the 17 different plant species. Bacterial OTUs are shown in the top blocks, fungal OTUs on the bottom blocks. Next to each OTU ID# is the predicted genus of that sequence. OTU read proportion is represented by color as shown in the legend, with red squares also showing the proportion as a number.

Vertically Transmitted Microbes in Sterile-Grown Shoots, Roots, and Rhizospheres

Seedlings growing on sterile sand inside sealed jars could only receive their microbiomes from seeds. All 306 shoot, root, and rhizosphere samples yielded sequences of bacterial and fungal rDNA. To statistically compare plants by tissue and across species and substrate, samples were rarefied (although samples with low OTU counts were included without rarefaction), normalized to proportional abundance, averaged across reps, and then ordered horizontally by Bray–Curtis dissimilarity. The 40 bacterial 16S and fungal ITS OTUs with highest occupancies are presented in descending order in roots, shoots, and rhizospheres of all 17 plant species grown on both sterile sand and field soil (**Figure 4**). As evidence of seed transmission, many of the bacterial OTUs appear in all samples regardless of substrate, while only a couple of the highest occupancy fungal OTUs appear across the board. Further evidence of seed transmission could be seen if both sterile sand and soil-grown samples of a particular species group together. For shoots, it is difficult to see whether seed or soil transmission was more important; however, bacteria populations in rice, soy, and pea samples clustered together, while fungal populations in coffee, wheat, tomato, and sugarcane were grouped into the same clade. Inside roots, seed transmission had a clear impact on endosphere microbiomes relative to soil, with 6/17 bacterial samples and 8/17 fungal root samples clustering by plant species. Except for sand and soil-grown fungal populations in sorghum rhizospheres that were put in the same clade, there was no clustering of rhizosphere samples by plant species.

To inventory the microbes in sterile sand-grown plants, OTUs were summed together across the three biological replicates and normalized (**Supplementary Data Sheet 2**). Rhizospheres contained an average of 252 bacterial OTUs each, roots contained an average of 177 bacterial OTUs each, and shoots contained an average of 255 bacterial OTUs each. There were much lower numbers of fungal OTUs observed in plants growing in sterile sand, with rhizospheres containing an

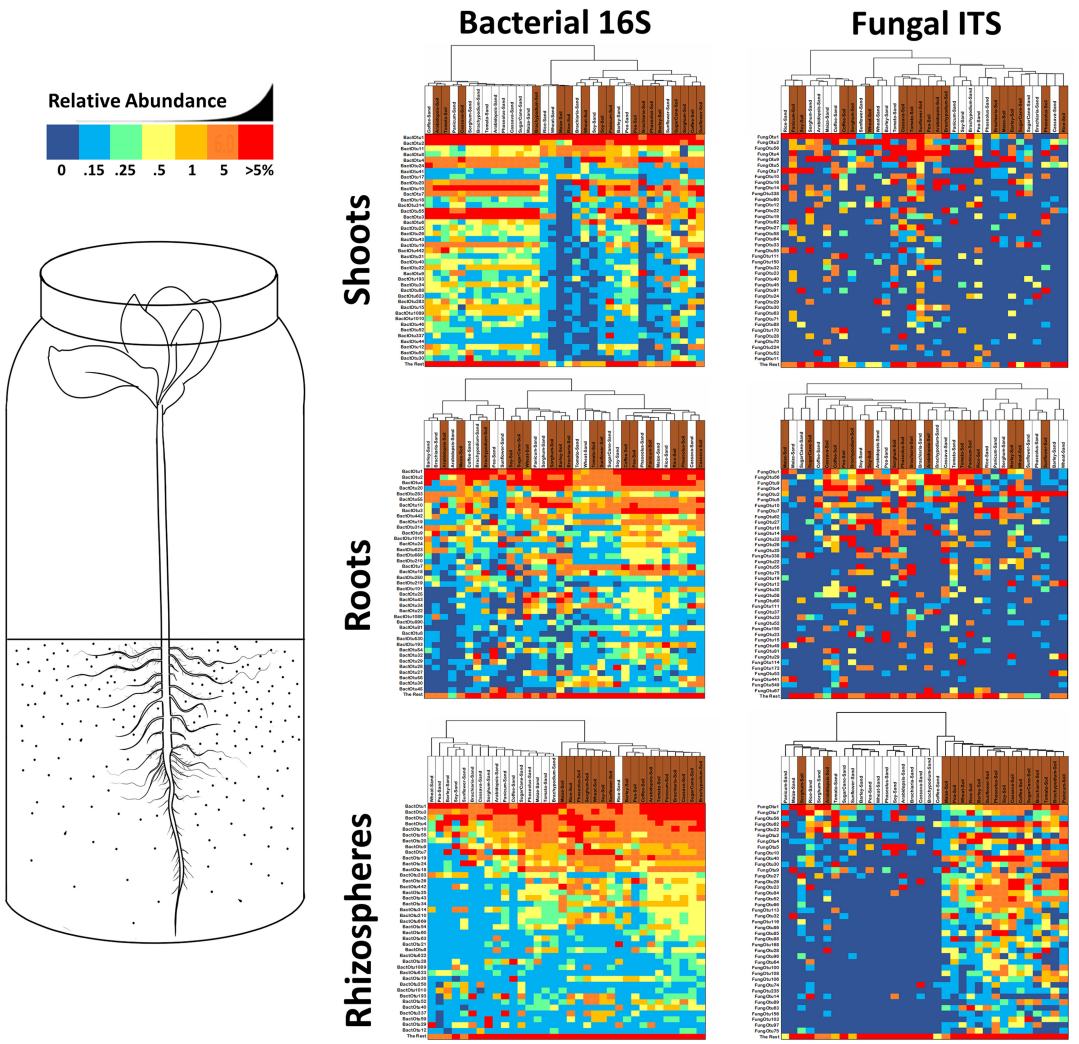

FIGURE 4 | Heatmaps of the 40 most common bacterial 16S rDNA and fungal ITS OTUs derived from Miseq analysis of rhizospheres, roots and shoots from 17 different plant species grown in sealed jars on either sterile sand or field soil. Reads were rarified for each sample (fungi—1000/roots and shoots, 2000/rhizospheres; bacteria—3000/roots, 3500/shoots, 6500/rhizospheres), averaged across repetitions, transformed into relative percentages, then clustered by Bray–Curtis dissimilarity. Samples from sand grown plants are labelled in white, samples from soil grown plants are labelled in brown. Cells are shaded by percentage value with 0% being dark blue, 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.

average of 25 fungal OTUs each, roots containing an average of 24 fungal OTUs each, and shoots containing an average of 16 fungal OTUs each. There were 41 core bacterial OTUs that occurred in all rhizospheres of sterile sand-grown plants, 20 in roots, and 56 in shoots. Of these core bacteria, there were 18 that were observed in all three sample types of all plant species, most abundant of which were the previously noted core seed OTUs *Pantoea* (BactOTU1), *Enterobacter* (BactOTU2), and *Pseudomonas* (BactOTU3 or 4) (**Table 1**). Several other OTUs of *Pseudomonas* (BactOTU9, 10, 20, and 54) appeared in all tissues of all plants, as did *Klebsiella* (BactOTU55 and 623), *Massilia* (BactOTU10), *Acidovorax* (BactOTU19), *Telluria* (BactOTU24), *Stenotrophomonas* (BactOTU18), *Rhizobium* (BactOTU30), *Methylobacterium* (BactOTU28), *Serratia* (BactOTU314), and *Pluralibacter* (BactOTU250). Unlike bacteria, there were no fungal OTUs that were observed in all plant samples grown on sterile sand, although as the most commonly observed, FungOTU1 (*Fusarium proliferatum*) was found in 16/17 of the rhizospheres, 15/17 of the roots, and 15/17 of the shoots. The next most common fungus was FungOTU2 (*Alternaria alternata*), which was observed in 16 rhizospheres, 15 roots, and 13 shoots of sand-grown plants, and then came the basidiomycete *Pseudozyma* (FungOTU56) appearing in 16 rhizospheres, 15 roots, and 14 shoots. The OTUs most commonly observed in sterile-grown plants were also usually the most abundant—BactOTU1, 2, 3, and 4 had average abundances of 22, 11, 13, and 7% in sterile sand-grown plant tissues, respectively, while

TABLE 1 | Twenty bacterial 16S and 10 fungal ITS with the highest occupancy in plants grown on sterile sand, with "common" observations shaded in green and average read percentages above 5 shaded in red.

OTU ID	Obs in rhizospheres	Obs in roots	Obs in shoots	Avg rhizosphere%	Avg roots%	Avg shoots%	Phylum	Class	Order	Family	Genus
BactOtu1	17	17	17	15.3	23.1	26.6	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea
BactOtu2	17	17	17	11.1	12.5	10.5	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
BactOtu3	17	17	17	10.1	5.5	23.8	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu4	17	17	17	6.9	8.8	6.5	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu7	17	17	17	7.1	5.4	0.6	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia
BactOtu10	17	17	17	6.3	3.1	4.4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu19	17	17	17	2.8	2.3	1.1	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax
BactOtu24	17	17	17	2.1	3.5	0.5	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Telluria
BactOtu55	17	17	17	1.5	1.6	2.7	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Klebsiella
BactOtu9	17	17	17	1.1	1.4	3.1	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu20	17	17	17	1.1	1.4	2.5	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu18	17	17	17	0.7	0.8	1.3	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
BactOtu30	17	17	17	1.4	1.3	0.1	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium
BactOtu28	17	17	17	1.0	1.4	0.3	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
BactOtu314	17	17	17	0.4	0.6	0.8	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia
BactOtu54	17	17	17	0.3	0.8	0.6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu250	17	17	17	0.3	0.3	0.2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pluralibacter
BactOtu623	17	17	17	0.2	0.5	0.2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Klebsiella
BactOtu34	17	16	17	0.1	0.2	0.3	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia
BactOtu25	16	17	17	0.1	0.2	0.4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Azotobacter
FungOtu1	16	15	15	11.5	7.9	17.4	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium
FungOtu2	16	15	13	14.0	13.3	14.9	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria
FungOtu56	16	15	14	2.3	1.5	2.6	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	Pseudozyma
FungOtu9	14	15	12	1.6	4.6	3.8	Ascomycota	Sordariomycetes	Hypocreales	Sarocladiaceae	Sarocladium
FungOtu5	11	15	9	6.2	7.6	7.9	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium
FungOtu7	12	13	10	6.1	6.3	9.9	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Phoma
FungOtu27	11	7	3	1.0	1.4	0.1	Ascomycota	Sordariomycetes	Incertae sedis	Trichosphaeriales	Nigrospora
FungOtu170	11	4	3	0.0	0.0	0.3	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Incertae sedis
FungOtu10	9	10	5	6.5	6.5	1.4	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Nectria
FungOtu14	7	8	9	2.0	1.1	4.3	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria

FungOTU1, 2, and 56 had an average abundances of 12, 14, and 2.1%, respectively.

Plants growing in sealed jars on sterile sand could only have seeds as their source of microbial inoculum; thus, it was expected that we would find evidence that 100% of all OTU diversity and read abundance came from seeds or spermospheres (**Figure 5**). By comparing seedling microbiomes to seed/spermosphere microbiomes, vertically transmitted bacteria can only explain a minority of the OTU diversity in shoots, roots, and rhizospheres, representing on average 38, 46, and 39%, respectively. The diversity of vertically transmitted bacterial OTUs in shoots, roots, and rhizospheres of *Brachypodium*, coffee, and soy plants was much lower than that in others, which might be explained by the detection of almost only Firmicutes in their seeds and spermospheres. Calculating average read abundance of vertically transmitted bacteria in shoots, roots, and rhizospheres returned 90, 84, and 81%, respectively, suggesting they predominate over other bacteria from soil or unknown provenance. Some plant samples (*Brachiaria*, barley, and rice shoots; pea rhizospheres and roots) stood out for having 98–100% of their 16S OTU reads deriving from seed-transmitted bacteria.

In plants growing on sterile sand in sealed jars, vertically transmitted fungi were expected to account for 100% of the observable OTU diversity and reads. Surprisingly, there was only evidence for seed transmission to explain about half of the diversity of fungi in shoots, roots, and rhizospheres, or on average 52, 44, and 43%, respectively. This minority of vertically transmitted fungi dominated sterile sand-grown shoot, root, and rhizosphere mycobiomes however, with an average read abundance of 72, 59, and 66%, respectively. Some unusual samples, such as *Brachiaria*

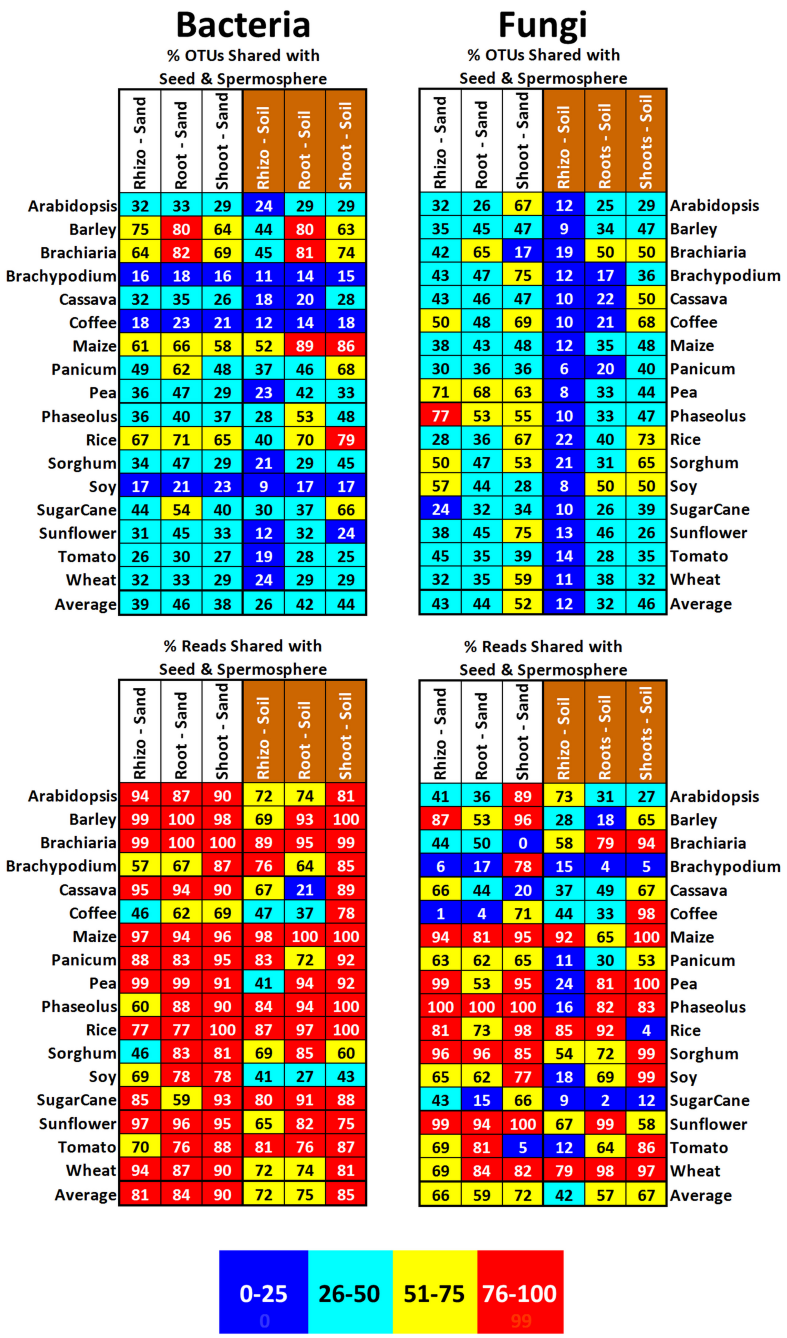

FIGURE 5 | Proportion of OTUs and reads in the shoots, roots, and rhizospheres of different plant species growing either on sterile sand (labeled in white) or field soil (labeled in brown) that were also observed in that species' corresponding seeds or spermospheres. Cells are shaded to reflect proportion, with 0–25% being blue, 26–50% being green, 51–75% being yellow, and 76–100% being red. An average across all the plant species is shown at the bottom of each column.

and tomato shoots; coffee roots; and *Brachypodium* and coffee rhizospheres, had evidence for less than 6% of their fungal reads coming from seed-transmitted fungi. These anomalies appear to have been caused by disproportionately large proportions of reads being attributed to OTUs that were not detected in the matching seed; for example, 89% of reads belonged to FungOTU45 (*Cryptococcus* sp.) in tomato shoots or 99% of FungOTU10 (*Aspergillus* sp.) in coffee rhizospheres.

Microbes in Shoots, Roots, and Rhizospheres of Soil-Grown Plants

Seedlings growing on field soil inside sealed jars could receive their microbiomes from either seeds or the substrate they

TABLE 2 | Twenty bacterial 16S and 10 fungal ITS with the highest occupancy in plants grown on soil, with common observations shaded in green and average read percentages above 5 shaded in red.

OTU ID	Obs in rhizospheres	Obs in roots	Obs in shoots	Avg rhizosphere%	Avg roots%	Avg shoots%	Phylum	Class	Order	Family	Genus
BactOtu1	17	17	17	9.3	14.1	24.3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea
BactOtu2	17	17	17	5.9	6.5	14.1	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
BactOtu3	17	17	17	13.4	2.5	11.2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu4	17	17	17	6.1	9.7	6.3	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu10	17	17	17	4.6	6.1	4.6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu55	17	17	17	2.8	2.4	5.4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Klebsiella
BactOtu7	17	17	17	3.9	1.3	1.1	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia
BactOtu9	17	17	17	1.5	2.1	1.2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu442	17	17	17	1.0	0.7	2.1	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Klebsiella
BactOtu283	17	17	17	0.2	0.3	0.4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea
BactOtu20	17	17	16	2.7	3.2	4.0	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu19	17	17	16	3.3	1.2	1.3	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax
BactOtu18	17	17	16	1.3	2.8	1.3	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
BactOtu314	17	16	17	0.3	0.5	0.7	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia
BactOtu34	17	17	16	0.7	1.6	1.5	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia
BactOtu101	17	17	16	0.5	0.4	0.9	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ramlibacter
BactOtu43	17	17	15	0.7	1.2	0.8	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
BactOtu210	17	17	15	0.5	0.7	0.4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
BactOtu30	17	16	16	0.3	0.7	0.4	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium
BactOtu28	17	15	16	0.1	0.1	0.2	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
FungOtu1	17	17	17	5.1	7.3	7.3	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium
FungOtu4	17	16	17	5.7	11.9	11.9	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium
FungOtu2	13	15	16	9.7	16.6	16.6	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria
FungOtu56	14	17	12	0.1	1.3	1.3	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	Pseudozyma
FungOtu7	16	13	12	3.8	3.4	3.4	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Phoma
FungOtu9	13	15	13	5.8	2.0	2.0	Ascomycota	Sordariomycetes	Hypocreales	Sarocladiaceae	Sarocladium
FungOtu5	14	11	13	0.1	4.4	4.4	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Penicillium
FungOtu10	15	13	10	1.2	1.6	1.6	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Nectria
FungOtu27	16	12	9	3.2	1.0	1.0	Ascomycota	Sordariomycetes	Incertae sedis	Trichosphaeriales	Nigrospora
FungOtu62	17	14	5	3.1	0.9	0.9	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Phoma

grew on. All 306 samples yielded microbial OTU sequences, which were summed across biological replicates and normalized to proportional abundance (**Supplementary Data Sheet 2**). Rhizospheres contained an average of 594 bacterial OTUs each, roots an average of 225 bacterial OTUs, and shoots contained an average of 185 bacterial OTUs. Relative to bacteria, there was a lower diversity of fungal OTUs observed in plants growing in soil, with rhizospheres containing an average of 126 fungal OTUs, roots containing an average of 37 fungal OTUs, and shoots containing an average of 24 fungal OTUs.

A total of 10 bacterial OTUs were ubiquitous among all sample types and species growing in soil, the most abundant of which were the previously noted core seed bacteria *Pantoea* (BactOTU1) and *Enterobacter* (BactOTU2) (**Table 2**). The other 8 OTUs found in all rhizospheres, roots, and shoots included *Pseudomonas* (BactOTU3, 4, 9, and 10), *Klebsiella* (BactOTU55 and 442), *Massilia* (BactOTU7), and *Pantoea* (OTU283). In total, there were 246 bacterial OTUs that occurred in all rhizospheres of soil-grown plants, 18 in roots, and 15 in shoots. Compared to bacteria, there were fewer ubiquitous fungi in soil-grown plants, with only FungOTU1 (*F. proliferatum*) occurring in all rhizospheres, roots, and shoots. *F. solani* (FungOTU4) was observed in all rhizospheres and all shoots, but only 16/17 in roots, while *A. alternata* (FungOTU2) was observed in 13/17, 15/17, 16/17 rhizospheres, roots, and shoots, respectively. FungOTU56 (*Pseudozyma* sp.) was observed in all root samples, but only 14/17 in rhizospheres and 12/17 in shoots. In total, there were 10 fungal OTUs that occurred in all rhizospheres of soil-grown plants, 2 in roots, and 2 in shoots. The OTUs most commonly observed in soil plants were again the most abundant—BactOTU1, 2, 3, and 4 had average abundances of 16, 9, 9, and 7%, while FungOTU1, 4, and 2 had average abundances

of 7, 10, and 14%. FungOTU56 was anomalous, having an average abundance of only 1%, which was even lower than that in plants grown on sterile sand.

To evaluate the impact of soil on microbiomes of these plants, OTU counts were rarefied (although samples with low OTU counts were included without rarefaction), normalized to percentages, averaged across reps, and then ordered horizontally by Bray–Curtis dissimilarity (**Figure 4**). Clustering of samples by substrate rather than plant species would indicate soil has a more important role in structuring the microbiome than vertical transmission. Bacterial and fungal populations in rhizospheres show very strong clustering by substrate, with all bacterial samples grouping together into soil and sand clades (except for sand grown rice rhizospheres), while nearly all fungal samples from soil-grown plants (except for soil grown *Arabidopsis* and sorghum) clustered together. Looking at OTU abundance across rhizospheres, it is possible to see that 30 bacterial OTUs occur in all plants on both substrates, but there is no clear trend in abundance fluctuation due to growth on soil. In contrast, fungal OTU rhizosphere abundance is dramatically increased by plant growth on soil, with the majority of fungal OTUs going from absent on sand to significantly abundant on soil. Inside roots, the influence of soil on the diversity or abundance of either bacteria or fungi was reduced in comparison to rhizosphere with only 8/17 bacterial samples and 9/17 fungal samples weakly clustering into various small clades. In shoots, 7/17 soil-grown bacterial samples grouped by substrate in one major clade, and 6/17 soil-grown fungal samples formed one major clade; however, in the rest of the samples, there was no clear soil influence on the diversity or abundance of microbes relative to seed transmission.

Despite growing on non-sterile soil, plants could be acquiring diverse and dominant microbes from their seeds (**Figure 5**). Comparing OTUs in seeds/spermospheres to those in soil-grown seedlings, vertical transmission of bacteria can only explain a minority of OTU diversity in rhizospheres, roots, and shoots, with an average 26, 42, and 44%, respectively. Again, the diversity of bacterial OTUs in *Brachypodium*, coffee, and soy seedlings was abnormally low, but this was because sequencing of their seeds/spermospheres returned mostly Firmicutes. Vertically transmitted bacteria were ecologically dominant in soil-grown rhizospheres, roots, and shoots, however, with an average read abundance of 72, 75, and 85%, respectively. Of note are some plant samples that were observed to have 97–100% of their reads deriving from seed-transmitted bacteria such as maize (all sample types), barley/*Brachiaria*/*Phaseolus*/rice shoots, and rice roots.

Seed-transmitted fungi (compared to bacteria) appear to be transmitted in similar patterns of diversity and abundance to vegetative tissues of soil-grown plants (**Figure 5**). Vertically transmitted fungi only explained a minority of the diversity of rhizosphere, root, and shoot populations, representing on average 12, 32, and 46%, respectively. This minority was quite abundant in rhizospheres, roots, and shoots, however, representing an average read abundance of 42, 57, and 67%, respectively. Some soil-grown samples had anomalous OTU read proportions; for example, *Brachypodium* and rice shoots derived less than 5% of their fungal reads from seed transmission, while 97–100% of reads in coffee/maize/pea/sorghum/soy/wheat shoots came from seed-transmitted fungi. The 98–100% of reads in sunflower and wheat root samples come from seed-transmitted fungi, while less than 4% of reads in *Brachypodium* and sugarcane roots do. Fungal diversity inside soil-grown roots and shoots (average of 31 OTUs/sample) was much smaller than that in soil-grown rhizospheres (average of 127 OTUs/sample) and often dominated by one hyperabundant fungus.

Microbes in soil-grown plants, with no evidence for provenance from seeds, presumably came instead from the substrate. To find the proportion of microbes that might be colonizing plants from the soil, we subtracted all OTUs observed in samples of sterile sand-grown plants from matching sample types of the matching plant species grown on soil (**Figure 6**). Using this method, on average, 41, 41, and 24% of bacterial OTUs in rhizospheres, roots, and shoots (respectively) could be explained as coming from soil. These soil-transmitted bacteria do not appear to heavily colonize plants, as they only represent on average 13, 13, and 2% of bacterial reads in rhizospheres, roots, and shoots. The influence of soil on fungal diversity is much stronger, with, on average, 89, 68, and 68% of fungal OTUs in rhizospheres, roots, and shoots (respectively) appearing to derive from soil. Surprisingly, these soil-transmitted fungi do not seem to be as dominant as seed-transmitted ones, as they only represent on average 56, 39, and 40% of fungal reads in rhizospheres, roots, and shoots, respectively. There was great variation of soil fungus read abundance between the plant samples; for example, over 90% of all fungal reads in all *Brachypodium* tissues seem to derive from soil, while less than 10% of any fungal reads in all maize tissues seem to derive from soil.

Common and Core Microbes, Their Abundance, and Their Provenance

To gain a global view of the microbial diversity in these plants, all samples were averaged together by type, their Shannon *H* diversity was calculated, and all core OTUs were categorized by their phylum-level taxonomy and provenance (**Figure 7**). Looking at both fungi (**Figure 7A**) and bacteria (**Figure 7C**), it was possible to see that microbial diversity of shoots went up slightly when grown on soil (fungi 3.0 to 3.1 and bacteria 2.7 to 3.0) but remained lower than that seen in either seeds or spermospheres. Microbial diversity of roots also went up modestly when grown on soil (fungi 3.4–3.5 and bacteria 3.3–4.0), increasing past that observed in seeds or spermospheres. The most dramatic effect of soil was observed in rhizospheres, where the Shannon *H* diversity index went up by a full point in fungi (3.1–4.1) and 0.9 in bacteria (3.5–4.4). Most of the changes in diversity that were observed in these samples were caused by the appearance or increase in proportion of OTUs from phyla other than Ascomycetes or Proteobacteria.

Rhizospheres had the largest number of OTUs that could be classified as common (occurring in more than 60% of samples) with 61 fungi and 541 bacteria (**Figures 7B,D**). Not surprisingly, in the rhizosphere, there was evidence that most of these common microbes were transmitted by soil (45 fungi and 263 bacteria), while only a minority had evidence of seed transmission (4 fungi

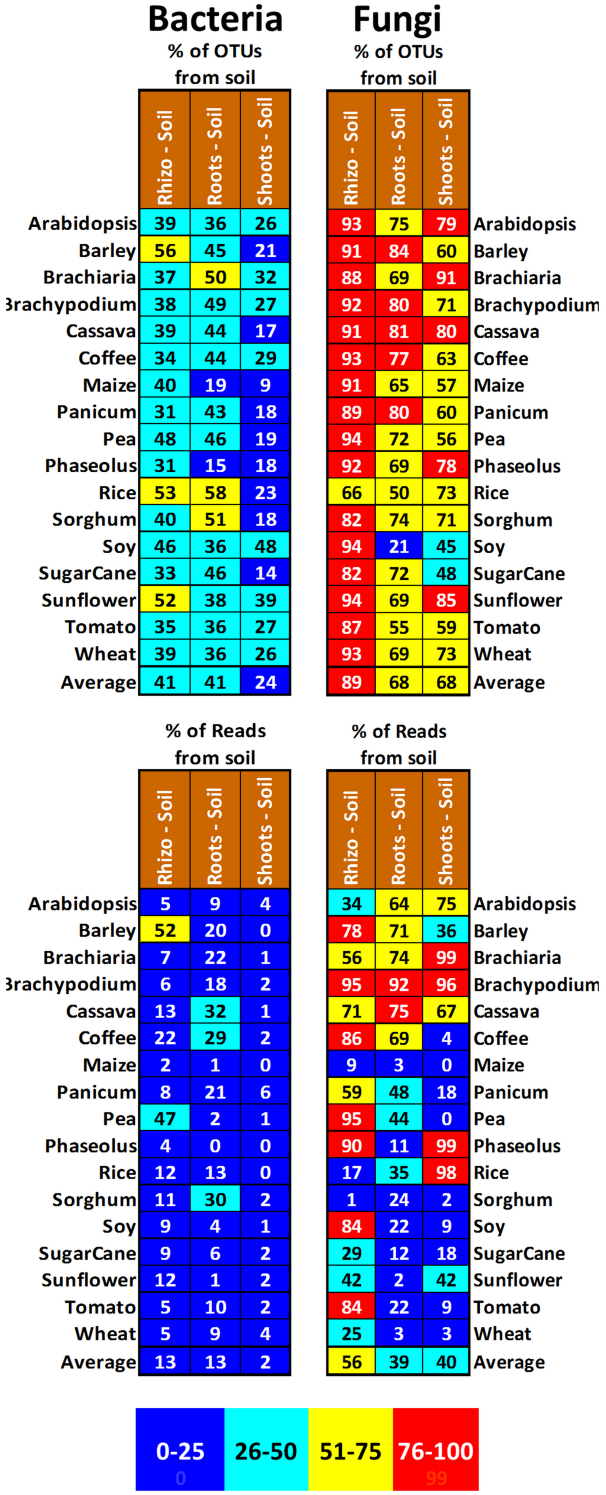

FIGURE 6 | Proportion of OTUs and reads in the shoots, roots, and rhizospheres of different plant species growing on field soil (labeled in brown) that *were not* observed in that species' corresponding shoots, roots, and rhizospheres from plants grown on sterile sand. Cells are shaded to reflect proportion, with 0–25% being blue, 26–50% being green, 51–75% being yellow, and 76–100% being red. An average across all the plant species is shown at the bottom of each column.

and 146 bacteria). Inside plant tissues, the situation was reversed, as only two out of 91 common bacterial OTUs appeared to be transmitted by soil inside roots, while 47 had evidence of seed transmission. There was a total of 10 common fungal OTUs in roots, none of which came from soil, while four had evidence of seed provenance. In shoots, no common bacterial or fungal OTU had evidence of provenance from soil, but 76 of 97 bacterial OTUs had evidence of seed provenance, while three of seven fungal OTUs appeared to come from seed. Because only seeds or soil were expected to be the source of microbes in this experiment, it was surprising that a substantial number of OTUs did not show evidence of either seed or soil provenance.

Looking at the abundance of common seed and spermosphere microbes in soil-grown plants (**Supplementary Data Sheet 2**), the 38 common seed/spermosphere bacterial OTUs together were found to represent on average 64% of rhizosphere reads (ranging from 27% in barley to 79% in maize), 67% of root reads (ranging from 14% in *Arabidopsis* to 97% in maize), and 85% of shoot reads (ranging from 40% in sorghum to 100% in maize), while the five common seed/spermosphere fungal OTUs were found to represent on average 25% of rhizosphere reads (ranging from 1% in pea to 88% in maize), 29% of root reads (ranging from 1% in Brachypodium to 95% in wheat), and 40% of shoot reads (ranging from 0.1% in Phaseolus to 99.9% in maize).

In seeds or spermospheres, only *Pantoea* (BactOTU1), *Enterobacter* (BactOTU2), *Pseudomonas* (BactOTU3), *Bacillus* (BactOTU8), and *Fusarium* (FungOTU1) appeared to be part of a core microbiome across the plant species. Because plants grown in sealed jars on sterile sand and irrigated with sterile water could only acquire their microbiomes from inside or on the surface of the seed, we also consider that any of the microbes seen in all rhizospheres, roots, or shoots were both core and seed transmitted. Despite observing few core seed/spermosphere microbes, we observed 41, 20, and 56 core (seed transmitted) bacteria found in all sand-grown rhizospheres, roots, or shoots, respectively, and zero core fungi. Pooling core OTUs from different sample types together, there were thus 63 bacteria and zero fungi that were both core and seed transmitted in sand-grown plants. Of these core seed-transmitted bacteria, there was a total of 18 OTUs that were found in all tissues of sand-grown plants, the most abundant of which were BactOTU1 (*Pantoea*), 2 (*Enterobacter*), 3/4/10 (*Pseudomonas*), 7 (*Massilia*), 19 (*Acidovorax*), 24 (*Telluria*), and 55 (*Klebsiella).* While there was no core fungus in sterile sand-grown plants, FungOTU1 (*Fusarium*) and FungOTU2 (*Alternaria*) were found in nearly all plant tissues and species. When the plants were grown on soil, there were 246, 18, and 15 core bacteria and 10, 2, and 2 core fungi found in all rhizospheres, roots, and shoots, respectively. All 63 core seed-transmitted bacteria observed in sand-grown plants continued to be observed in 100% of either rhizospheres, roots, or shoots of soil-grown plants, and of these, BactOtu1, 2, 3, 4, 7, 10, and 55 continued to be both the most abundant and ubiquitous, found in all rhizosphere, root, and shoot samples. While there had been no core fungus in sterile sand-grown plants, on soil, FungOTU1 (*Fusarium*) was now found in all sample types, while FungOTU56 (*Pseudozyma)* was core to roots and FungOTU4 (*Fusarium*) was found in all shoots.

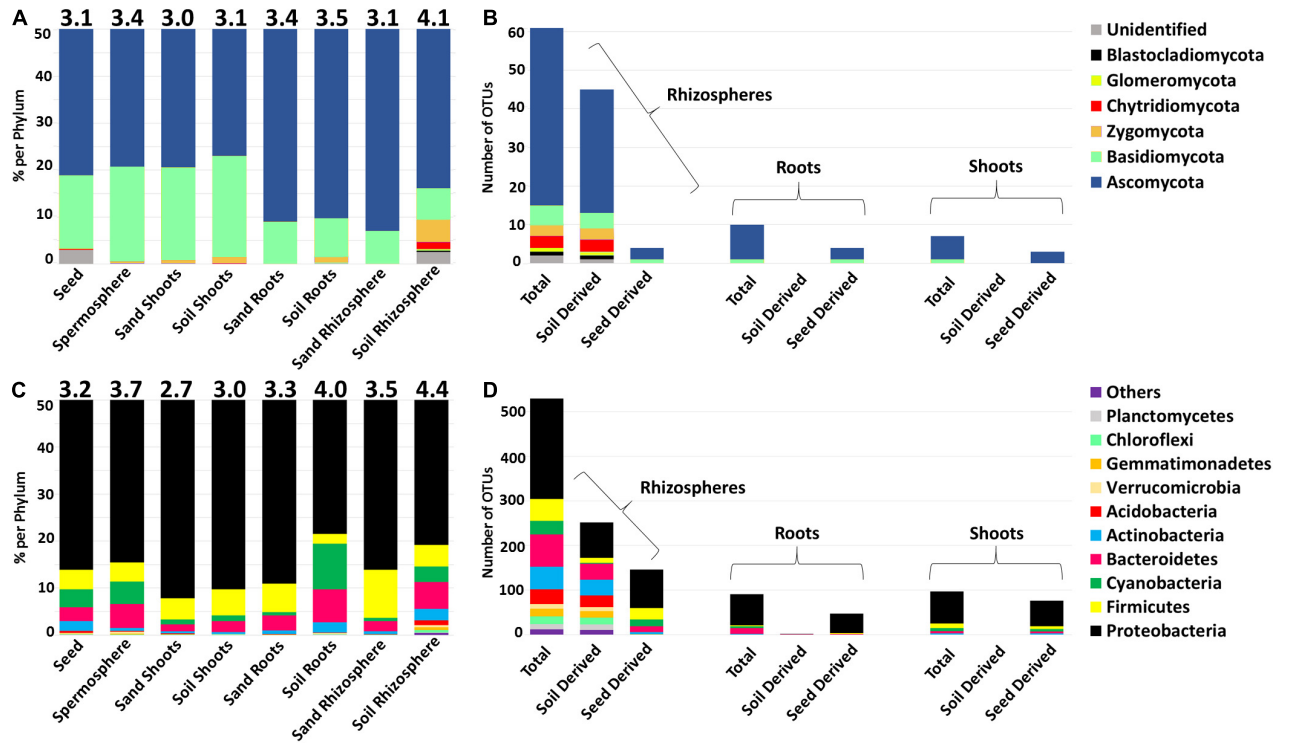

FIGURE 7 | Alpha diversity and phylum-level classification of total **(A,C)** and common **(B,D)** OTUs of fungal ITS DNA and bacterial 16S rDNA amplified from seeds, spermospheres, rhizospheres, roots, and shoots. Alpha diversity was measured using Shannon *H* index and is reported on top of each bar. The proportion of each sample type was calculated by averaging all the samples in each respective group (e.g., average of relative proportions from all seed OTUs). **(B,D)** Total common OTUs were those that were observed in more than 60% of the shoots/roots/rhizospheres of soil-grown plants, while common soil OTUs were observed in more than 60% of soil-grown samples but not in sterile sand-grown samples. Common seed OTUs were those that were observed in both >60% of soil-grown tissues and in >60% of sterile sand-grown tissues. OTUs are classified at the phylum level and shaded according to the legends at the right.

DISCUSSION

Seeds are the most direct vehicle that a parent plant might use to transmit microbes to their offspring; however, until recently, experimental science has focused on soil as the major source of the plant microbiome, rarely including sterile substrates as controls and often attempting to sterilize seeds before planting. Within this context, we wanted to characterize and compare the seed-associated microbiomes of 17 of the most important angiosperms including *Arabidopsis*, *Brachypodium*, maize, tomato, rice, and coffee. Does something resembling a core angiosperm seed microbiome exist? Equally important, we wanted to evaluate which of these microbes from natural/unsterilized seed, if any, are transferred to vegetative parts (shoot, root, and rhizosphere) of the plant. Plants were grown in hermetically sealed jars on sterile sand as a way to observe microbiome development in the absence of any other source of inoculum except seeds. Jar-grown plants were also "challenged" with farm soil to see if competition from soil microbes might displace any seed-associated microbes that may have been transmitted to vegetative parts of the plant. High-throughput sequencing was used to identify the taxonomy and relative abundance of bacteria and fungi in these samples, also allowing us to search for patterns of microbiome variation between samples and for evidence of core microbiomes shared between plants and microbial niches. Defining the core microbiomes of model crop plants has been posed as one of the world's research priorities if we hope to successfully integrate beneficial plant microbiomes into agricultural production (Busby et al., 2017).

Seed and Spermosphere Microbiomes

Considering that seed surfaces could be an important source of inoculum for vegetative surfaces, while seed endospheres may contribute to the microbiomes of vegetative endospheres, we elected to sample each separately. Every seed surface and interior contained bacterial 16S and fungal ITS sequences (**Figures 2**, **3**); however, with heavy contamination from mitochondria, chloroplast, and plant ribosome sequences and presumably low microbial titers, some seed samples generated very few bacterial or fungal reads (**Supplementary Data Sheet 1**). Others have speculated that the difficulty in detecting seed-borne microbes results because they are present in small numbers, dormant, and in viable but non-culturable states that resist DNA extraction (Rochefort et al., 2021). Bacteria that we did detect were predominantly Proteobacteria; however, coffee, *Brachypodium*, *Arabidopsis*, and soy seeds or spermospheres were dominated by Firmicutes, which might have meant

these would develop distinct Firmicutes-dominated microbiomes when grown into plants (they did not). For fungi, nearly all seeds and spermospheres were dominated by Ascomycetes, except *Panicum* seeds and *Brachiaria* spermospheres, which were richer in Basidiomycetes instead. Attempts to statistically ordinate seed and spermosphere samples by their microbiome diversity and abundance did not result in any apparent pattern, suggesting either we needed more robust data sets or that there is no phylogenetically meaningful structuring of seed microbiomes (data not shown). At the level of OTU, there was substantial commonality (found in >60% of samples) among samples, with 37 common spermosphere bacteria, compared to 15 inside seeds, and four common fungal OTUs in both seeds and spermospheres (**Figure 3**). Of these common microbes, BactOTU1 (*Pantoea*) and BactOTU2 (*Enterobacter*) were the only ones core to every surface-sterilized seed, but they also occurred in all spermosphere samples, as did BactOTU3 (*Pseudomonas*), BactOTU8 (*Bacillus*), and FungOTU1 (*Fusarium*). Other common and abundant OTUs belonged to the genus *Massilia*, *Klebsiella*, *Alternaria*, and *Sarocladium*.

Reviewed in just the last few years (Truyens et al., 2015), seed microbiomes have been found to contain a diversity of endophytic bacteria. Across 62 species of plants, reported in more than 50 different publications, 155 different bacterial genera primarily from the phyla Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes have been detected inside seed tissues, but the dominant genera reported are *Pantoea*, *Enterobacter*, *Pseudomonas*, *Acinetobacter*, and *Sphingomonas* (Hardoim, 2019). Various publications have also reported *Pantoea*, *Enterobacter*, and *Pseudomonas* as core to seeds (Johnston-Monje and Raizada, 2011; Barret et al., 2015; Leff et al., 2017; Yang et al., 2017; Rahman et al., 2018; Eyre et al., 2019; Wassermann et al., 2019b), which coincides with what we observed in this experiment. Fungi living inside seeds can be classified as clavicipitaceous endophytes, which are strictly grass seed-transmitted endosymbionts of the genera *Atkinsonella*, *Balansia*, *Balansiopsis*, *Dussiella*, *Epichloë*, *Myriogenospora*, *Parepichloë*, or as non-clavicipitaceous endophytes, which are mostly Ascomycetes and Basidiomycetes occurring in many different seed types, with the most common genera reported being *Alternaria*, *Fusarium*, *Cladosporium*, *Aspergillus*, *Rhizoctonia*, *Undifilum*, *Chaetomium*, *Colletotrichum*, *Epicoccum*, *Phialophora*, *Tricothecium*, *Cryptococcus*, and *Filobasidium* (Hardoim, 2019). *Alternaria* has been reported as core to some species of plant seeds (Leff et al., 2017; Eyre et al., 2019). Here again, the most common fungal seed endophytes we observed coincided with the non-clavicipitaceous seed endophytes most commonly reported in the literature.

Angiosperm spermospheres are dominated by many of the same microbes that we observed: Proteobacteria (*Agrobacterium*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas*), Firmicutes (*Bacillus* and *Paenibacillus*), Actinobacteria (*Microbacterium*), Ascomycetes (*Fusarium*, *Penicillium*, *Trichoderma*, *Gliocladium*, *Cylindrocarpon*, and *Cephalosporium*), Basidiomycota (*Rhizoctonia*), and Zygomycota (*Mucor*) (Nelson, 2004, 2018). It has also been shown that lettuce seeds can carry *Olpidium virulentus* (a Chytridiomycete) resting spores externally on the seed coat where they can eventually colonize the spermosphere and begin to infect the developing root (Maccarone, 2013)—we observed Chytridiomycete reads in cassava spermospheres. Similar to our results, some published core spermospheres have been described to contain *Pantoea*, *Pseudomonas*, *Massilia*, *Fusarium*, and/or *Alternaria* (Links et al., 2014; Chen et al., 2016; Klaedtke et al., 2016; Eyre et al., 2019; Chartrel et al., 2021; Moreira et al., 2021).

Bacteria and Fungi in Shoots

Microbes inside the shoot can influence movement of nutrients and sugars, while on the leaf surface, they can influence gas exchange and harvesting of light. A review of the literature shows that phyllospheres are usually reported to contain Proteobacteria, Firmicutes, Actinobacteria, and Bacteriodetes including the genera *Pseudomonas*, *Bacillus*, *Pantoea*, *Erwinia*, *Sphingomonas*, *Acinetobacter*, *Xanthomonas*, and *Gluconobacter* (Thapa and Prasanna, 2018). Core phyllosphere bacteria in a variety of plants have been observed to include *Methylobacterium*, *Pseudomonas*, *Bacillus*, *Massilia*, *Arthrobacter*, *Rhizobium*, *Pantoea*, and *Sphingomonas* (Delmotte et al., 2009; Rastogi et al., 2012; Horton et al., 2014; Ortega et al., 2016; Wallace et al., 2018; Grady et al., 2019). We observed all of these bacteria in soil-grown shoots of our experiment; however, only *Pantoea*, *Enterobacter, Pseudomonas*, *Klebsiella*, *Massilia*, and *Serratia* were part of a core shoot microbiome across plant species. In literature, the common fungal genera occurring in leaves are *Alternaria*, *Cladosporium*, *Penicillium*, *Acremonium*, *Mucor*, *Cryptococcus*, *Sporobolomyces*, *Rhodotorula*, and *Aspergillus* (Thapa and Prasanna, 2018). Core phyllosphere fungi in a variety of plants have been observed to include *Epicoccum*, *Fusarium*, *Alternaria*, *Cladosporium*, *Cryptococcus*, *Sporobolomyces*, *Udeniomyces*, *Dioszegia*, *Mycosphaerella*, *Plectosphaerella*, *Aureobasideum*, *Neoascochyta*, and *Tetracladium* (Horton et al., 2014; Sapkota et al., 2015; Bowsher et al., 2020). Seed-borne *Fusarium* has been identified as one of the dominant members of the stem endosphere mycobiome of maize (Nebert, 2018). The yeast *Pseudozyma* has been reported as the dominant fungus in and on sugarcane (Nasanit et al., 2015b) and rice leaves as well (Nasanit et al., 2015a; Laur et al., 2018; Wang et al., 2021). The only core fungi in soil-grown shoots in our experiment were both of *Fusarium* (FungOTU1 and 4). We observed *Alternaria* FungOTU2 in 16 of 17 soil-grown shoots and *Pseudozyma* in only 12.

Although the origin of phyllosphere microbes is not well established (Bulgarelli et al., 2013), it is believed that most are environmentally derived from soil, rain, dust, and contact with other organisms, with plant genotype and age playing a major role in shaping and selecting microbes (Whipps et al., 2008; Horton et al., 2014; Wagner et al., 2016). There is also evidence suggesting vertical transmission from seeds to shoots is significant: rice seeds have been shown to populate shoots with bacteria (Hardoim et al., 2012), maize seeds transmit fungi to the leaves (Nebert, 2018), and the growth of oak under axenic conditions suggests that shoots are already heavily colonized by microbes while they exist as embryos inside the seed (Abdelfattah et al., 2021). Observing

microbiomes of axenically grown plants gave us another indirect way to see which microbes might be inside seeds, since rare and previously undetectable microbes might have a chance to awaken during seedling germination, allowing them to multiply to levels that are detectable by PCR and sequencing (Shade et al., 2014). We did indeed observe in all plants that their seed can transmit both bacteria and fungi to shoots. Compared to roots and rhizospheres, shoot microbiomes in fact seem to possess the highest level of seed-derived microbes in the plant: about half of bacterial and fungal diversity in shoots seemed to come from seeds, while the majority of reads belonged to these dominant seed-derived microbes.

Ordination of shoot microbiome data did not clearly show clustering by plant species or soil, suggesting neither is a more important source of inoculum for either bacteria or fungi. Comparing soil-grown to sand-grown plants, soil was a very poor source of bacteria for shoots, although for some plants, it did serve to inoculate leaves with diverse or dominant fungi (**Figure 6**). *Brachiaria*, for example, got 91% of its fungal OTUs from soil, which represented 99% of the reads. Meanwhile, less than 1% of fungal reads in maize and pea shoots came from soil. Because shoots are physically separated from soil (as opposed to roots), perhaps plants have more of a chance to impose tight controls on the number and diversity of microbes that invade their stems and leaves. It may also be that different plants have different ecological strategies, with some practicing more stringent "biotic filtering" (the ability of a plant to restrict which endophytes may enter) than others. Possible examples of strong biotic filtering in shoots, *Bromus tectorum* or maize grown on soils containing significantly different endophytic fungal communities nevertheless develop leaf mycobiomes that are similar (Nebert, 2018; Ricks and Koide, 2019). The lack of clear clustering also leads us to speculate that our experimental setup excluded some other important variables that are important for populating shoot microbiomes, for example exposure to rain (Mechan-Llontop et al., 2021), dust-fall, or surface contact with insects, which have been observed to exert such strong effects on bacterial phyllosphere diversity that tomato leaves were practically identical to synthetic plastic surfaces nearby (Ottesen et al., 2016). The diversity of fungal endophytes in leaves of tropical forest grasses has been found to depend on dispersal limitation (Higgins et al., 2014), but our use of a filtered and homogenized soil as inoculum makes it unlikely that this was a factor in our experiment. Another important variable to consider is that hermetically sealing and growing these plants within glass jars resulted in extremely high humidity and an abnormal atmosphere, which may also have altered microbial diversity and reduced microbial abundance in phyllospheres as has been shown for laboratory- vs. field-grown lettuce (Williams and Marco, 2014).

Microbial Populations in Roots

Functioning to absorb nutrients and water while secreting biochemicals to manipulate the surrounding microbiology, roots grow into the soil where they have typically been assumed to acquire all their bacterial endophytes (Vandenkoornhuyse et al., 2015). Studies of the root microbiome of *Arabidopsis* (Lundberg et al., 2012), barley (Bulgarelli et al., 2015), rice (Edwards et al., 2015), grape (Zarraonaindia et al., 2015), and sugarcane (Yeoh et al., 2016) have shown that bacterial root endophytes are predominantly Actinobacteria, Bacteroidetes, and Proteobacteria (Bulgarelli et al., 2013). At the level of genus, these bacterial root endophytes include *Acidovorax*, *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Methylobacterium*, *Micrococcus*, *Phyllobacterium*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Stenotrophomonas*, *Streptomyces*, and *Xanthomonas* (Hallmann and Berg, 2006). We observed that the dominant/core genera of bacteria in both sterile sand- and soil-grown roots were (in descending order) *Pantoea*, *Enterobacter*, *Pseudomonas*, *Massilia*, *Acidovorax*, *Klebsiella*, and *Stenotrophomonas*. Many other bacteria such as *Rhizobium* and *Methylobacterium* were common, but did not appear in 100% of root samples. *Pantoea*, *Enterobacter*, and/or *Pseudomonas* have been identified as part of a core root microbiome in barley (Yang et al., 2017), coffee (Fulthorpe et al., 2020), tomato (Lee et al., 2019), sugarcane (Yeoh et al., 2016), *Arabidopsis* (Bulgarelli et al., 2012; Lundberg et al., 2012), and diverse seedlings (Barret et al., 2015). *Massilia* was reported as a core sugarcane root endophyte (Yeoh et al., 2016), while *Acidovorax* was identified as part of a core root microbiome across 30 species of crop plants (Fitzpatrick et al., 2018). Being legumes, pea, and soy roots were expected to be heavily colonized by rhizobia (BactOTU30), they however represented only less than 0.05% of the reads in either plant, while surprisingly, this OTU made up 21% of the reads in coffee roots growing on sterile sand.

Fungal endophytes of roots are also believed to be soil derived and thus very sensitive to the biogeography of plant growth (Bonito et al., 2014; Bokati et al., 2016; Durán et al., 2018). These communities are usually dominated by Ascomycetes (Dothideomycetes, Sordariomycetes, Leotiomycetes, Eurotiomycetes, and Pezizomycetes), Basidiomycota (Agaricales, Russulales, and Polyporales), and Zygomycota (Porras-Alfaro and Bayman, 2011). Monocots growing in grassland ecosystems have been observed to have root endospheres dominated by Dothideomycetes and specifically *Fusarium* and *Alternaria*, while in forest ecosystems, root endospheres are dominated by Leotiomycetes (Bokati et al., 2016; Jumpponen et al., 2017). The soil-dwelling Chrytidiomycete *Olpidium* has also been observed to intensively infect roots of lettuce (Maccarone, 2013), tomato (Johnston-Monje et al., 2017), melon (Stanghellini et al., 2010), and *Arabidopsis* (Durán et al., 2018). In our experiment, the dominant genera of seed-transmitted fungi in roots grown on sterile sand were *Fusarium*, *Alternaria*, *Pseudozyma*, *Sarocladium*, *Penicillium*, and *Phoma*, which also dominated soil-grown roots, although only *Fusarium* and *Pseudozyma* occurred in all samples. Both *Fusarium* and *Alternaria* have been identified as core root fungi in comparisons of poplar, oak, and pine (Bonito et al., 2014), when studying geographic influence on the *Microthlaspi* root mycoobiome (Glynou et al., 2016); in roots of mandarin orange trees (Sadeghi et al., 2019); and in root endospheres of various wild and domesticated Brassicaceae (Glynou et al., 2018). *Fusarium* has also been identified as a dominant member of the coffee root mycobiome (Fulthorpe et al., 2020), while *Alternaria* was part of a core mycobiome

among 28 different germinating seeds (Barret et al., 2015). *Pseudozyma* has been reported as the dominant fungus in and on sugarcane (Nasanit et al., 2015b) and rice leaves where it can protect the plant from pathogens by secretion of antibiotics (Nasanit et al., 2015a; Laur et al., 2018; Wang et al., 2021); however, to our knowledge, it has not been reported as a core member of plant root mycobiomes before.

Many published studies on root microbiology, having attempted to sterilize seeds and forgotten to include a sterile substrate as a negative control, nevertheless conclude that most of the root microbiome derives from soil (Vandenkoornhuyse et al., 2015). For example, a study on the recruitment of *Brassica napus* seedling microbiota, which included no sterile soil treatment and obtained very little sequencing data from seeds as opposed to soil, concluded that most of the seedling microbiome comes from soil or other unknown sources (Rochefort et al., 2021). On the contrary, our results show that seeds of all plants tested are able to transmit microbes to their roots (a core set of seed-transmitted Proteobacteria, *Fusarium*, and *Pseudozyma*), and in most cases, these microbes go on to dominate the endosphere despite being grown in microbe-rich soil. Other publications corroborate the importance of vertical transmission in establishing root microbiomes: we have shown twice before that seed-derived bacteria are the dominant members of juvenile maize root microbiomes (Johnston-Monje et al., 2014, 2016), with similar observations having been made in wheat (Walsh et al., 2021), rice (Hardoim et al., 2012), *Arabidopsis* (Truyens et al., 2016), common bean (López-López et al., 2010), barley (Yang et al., 2017; Rahman et al., 2018), sunflower (Leff et al., 2017), and diverse crops (Barret et al., 2015). It has also been noted that these seed-transmitted microbiomes may change in abundance over time, first increasing during germination (Barret et al., 2015) and later being displaced by soil-derived microbes as plants age (Yang et al., 2017). Fungi can also be transmitted by seeds to roots; for example, tomato roots grown in sterile sand contained *Fusarium*, *Alternaria*, *Penicillium*, *Phoma*, and *Cladosporium* (Johnston-Monje et al., 2017), and both sunflower seeds and young roots were dominated by Pleosporaceae (*Alternaria*), although this changed as plants aged (Leff et al., 2017).

On average, measuring by OTU diversity and abundance, both bacteria and fungi populations in roots were largely seed transmitted; however, there was a dramatic variation between some plants. For example, maize roots grown on soil had 89% of their bacterial OTUs coming from seed, while cassava had only 20%. By read abundance, fungi in sunflower roots were 99% seed transmitted, while sugarcane roots were only 2%. Plant genotype-dependent variations in root microbiomes have been often observed (Bonito et al., 2014; Bouffaud et al., 2014; Schlaeppi et al., 2014; Yeoh et al., 2017; Fitzpatrick et al., 2018; Ricks and Koide, 2019; Wang and Sugiyama, 2020) and are usually explained as variation in the plant's ability to filter or restrict entry of soil microbes, although they could also reflect variation in seed-transmitted microbial inoculum. Likewise, it has been noted that bacterial endophyte populations vary more by plant compartment than they do by the soil they are grown on (Coleman-Derr et al., 2016; Durán et al., 2018), which, rather than invoking biotic filtering, may be explained if seeds are delivering a consistent bacterial inoculum to the embryo, which then develops differently as it colonizes different organs (Abdelfattah et al., 2021). Indeed, rather than soil serving directly as a source of bacterial inoculum, there is evidence that it is variation in soil characteristics, and in particular pH, that induces the shifts in endophyte population structure, which are often observed in these studies (Hardoim et al., 2012; Barnes et al., 2016). Plant age has been shown to be an important factor in structuring the root microbiome (Wagner et al., 2016), which may also be responsible for some of the variations observed in root microbiomes that were not all sampled at the same age.

Rhizosphere Microbiomes

The first few millimeters of soil around a root is called the rhizosphere, where robust populations of up to 10^{11} microbial cells per gram live, including over 30,000 prokaryotic species, which help mineralize nutrients or protect against invasion by pathogens (Berendsen et al., 2012). Plants can influence the microbiology of the soil around them through rhizodeposition, where their roots secrete organic acids, phytosiderophores, sugars, vitamins, amino acids, nucleosides, mucilage, and even living root cap border cells (Bulgarelli et al., 2013). It has also been discovered recently that plants can directly inoculate the rhizosphere with bacteria (Johnston-Monje and Raizada, 2011), by sloughing off endophyte-filled root cap border cells (Cope-Selby et al., 2017) or by expulsing microbes out of the swollen ends of root hairs (White et al., 2018). In contrast to these recent discoveries, scientists have traditionally believed that all the rhizosphere microbiome "is recruited from the main reservoir of microorganisms present in soil" (Bakker et al., 2013), with publications on *Arabidopsis* (Lundberg et al., 2012), soy (Liu et al., 2019), rice (Edwards et al., 2015), and maize (Peiffer et al., 2013) rhizospheres reflecting this assumption. A great many publications survey the rhizosphere microbiomes of other plants, including barley (Terrazas et al., 2020), sorghum (Schlemper et al., 2017), coffee (Caldwell et al., 2015), common bean (Pérez-Jaramillo et al., 2019), sunflower (Leff et al., 2017), and pea (Turner et al., 2013). In our previous studies on bacteria in maize (Johnston-Monje et al., 2016) and fungi in tomato (Johnston-Monje et al., 2017), we have corroborated that soil adds significant microbial diversity to the rhizosphere; however, we also found that the most abundant members of the juvenile maize rhizosphere are seed-transmitted bacteria. To our knowledge, no published studies have ever directly addressed the importance of seed transmission to the rhizosphere mycobiome. In this experiment we confirmed that soil contributes to microbial diversity in the rhizosphere, and we also found that the most abundant bacteria and fungi in rhizospheres derive from seeds.

Across all plant rhizospheres grown in sterile sand, we observed 41 different core seed-transmitted bacterial OTUs, to which 205 more were added when grown in soil. Among these core seed-transmitted bacteria, 11 were the most abundant/dominant in soil-grown rhizospheres and included, in descending order: *Pantoea*, *Enterobacter*, *Pseudomonas*, *Klebsiella*, *Massilia*, *Acidovorax*, and *Stenotrophomonas*. *Pseudomonas* is a very common rhizobacteria and, along with *Massilia*, *Acidovorax*, and *Rhizobium*, is a dominant member of core rhizospheres of potato (Pfeiffer et al., 2017), tomato (Lee et al., 2019), lettuce (Schreiter et al., 2014), wheat

(Schlatter et al., 2020; Simonin et al., 2020), and maize (Walters et al., 2018). In wheat rhizospheres, it bioaccumulates over years of continuous cropping in a way that is "remarkable in view of the broad range of soil types, climates and agronomic conditions under which wheat is cultivated throughout the world" (Weller et al., 2002), building up to levels that eventually suppress the fungus *Gaeumannomyces tritici*, which causes take-all disease. It is interesting to speculate these biocontrol rhizobacteria actually derive from seeds as we observed in our experiment, rather than soil as has always been assumed. Despite being the most abundant rhizosphere bacteria in our experiment, *Pantoea* has only been reported as core in the wheat rhizosphere (Simonin et al., 2020) and was the second most abundant rhizobacteria we observed previously in juvenile maize rhizospheres (Johnston-Monje et al., 2016). *Enterobacter* has been reported as core for tomato rhizospheres (Lee et al., 2019) and as the keystone species in microbial communities on maize root surfaces (Niu et al., 2017), with the ability to travel through the endosphere, exit the roots, and colonize the surrounding soil (Johnston-Monje and Raizada, 2011).

There were no seed-transmitted fungi that colonized the rhizospheres of all 17 plant species growing on sterile sand; however, *Fusarium*, *Alternaria*, and *Pseudozyma* were present in 16/17. On soil, *Fusarium* and *Phoma* were found in all 17 rhizospheres. Of these, *Fusarium* is the only fungus regularly reported as a core rhizosphere inhabitant, being the dominant fungus on root surfaces of tomato (Lee et al., 2019), wheat (Schlatter et al., 2020; Simonin et al., 2020), maize (Cavaglieri et al., 2009), *Brachypodium* (Kawasaki et al., 2016), and sugar cane (Hamonts et al., 2018). Wheat rhizospheres have also been described to variously have *Phoma* and unidentified Chytridiomycetes as part of their core (Simonin et al., 2020), while in another study, the core wheat rhizosphere had *Alternaria* instead (Schlatter et al., 2020).

Soil significantly increased bacterial diversity in rhizospheres, however, the highest read abundance was of seed-transmitted bacteria (**Figure 5**). Only an average of 26% of bacterial OTUs came from seeds, but these were responsible for an average of 72% of the reads. These OTUs were mostly Proteobacteria of the genera *Pantoea*, *Enterobacter*, *Pseudomonas*, and *Massilia*, which we observed in seeds or spermospheres and have also been observed associated with a variety of plant seeds (Mundt and Hinkle, 1976; Adams and Kloepper, 2002; Mano et al., 2006; Ferreira et al., 2008; Kaga et al., 2009; Johnston-Monje and Raizada, 2011; Truyens et al., 2013). We have previously observed seeds transmitting dominant bacterial strains (including *Burkholderia*, *Pantoea*, and *Massilia*) into maize rhizospheres (Johnston-Monje et al., 2016). Bacterial endophytes tagged with GFP such as *Enterobacter* from maize seeds (Johnston-Monje and Raizada, 2011) and *Pantoea* from eucalyptus seeds (Ferreira et al., 2008) have also been shown to be able to colonize the endosphere, exit the root, and colonize the rhizosphere. Rice and millet seed endophytes have been later observed in rhizosphere soil (Hardoim et al., 2012; Verma and White, 2018), and seed-transmitted bacteria have been observed colonizing rhizospheres as they emerge from inside sloughed off *Miscanthus* root border cells (Cope-Selby, 2013). Seed-transmitted microbes colonizing the rhizosphere would be guaranteed first access to that habitat, perhaps creating a founder effect, blocking later colonization by less-adapted soil microbes or pathogens (Bacilio-Jiménez et al., 2001; Barka et al., 2002). Seed-transmitted rhizosphere microbes might also play an important role in plant nutrition, for example in the cardon cactus, where they help to mineralize the surrounding rock for nutrient absorption by roots (Puente et al., 2009), get intracellularly taken up by the root, and digested by the plant in a process called rhizophagy (White et al., 2018), or in grasses where dying bacteria release organic nitrogen for absorption by the plant (White et al., 2015).

Compared to bacteria, there was less seed-transmitted fungal diversity in rhizospheres, with only an average of 12%; however, these OTUs tended to become abundant, representing an average of 42% of the reads. Abundance of seed-transmitted fungal reads varied widely and unexplainably by plant, for example with sugarcane having only 9% while maize had 92%. Of these seed-transmitted rhizospheric fungi, FungOTU1 (*Fusarium*) was the most abundant, occurring in all soil-grown rhizospheres, as it did in all spermospheres. We have previously observed that tomato rhizospheres are dominated by seed-transmitted *Fusarium* (Johnston-Monje et al., 2017), but we are not aware of other examples of seed transmitted rhizospheric fungi. Seeking to protect against soil-transmitted seedling pathogens from the genera *Fusarium*, *Rhizoctonia*, *Colletotrichum*, *Cylindrocarpon*, *Pyrenophora*, and *Cochliobolus*, the plant agriculture industry commonly coats seed with fungicides but has not explicitly paid attention to the possibility of a seed-transmitted rhizosphere mycobiome (Nelson, 2018), which might make this the first publication explicitly documenting this phenomenon.

Some Caveats

Microbial detection in seeds while using PCR to amplify 16S or ITS sequences, followed by Illumina sequencing has been shown to miss as much as 50% of the sequence diversity in an environmental sample (Hong et al., 2009). Shifting patterns of microbiome diversity can be also be obscured when relying solely on the sequencing of 16S or ITS rDNA (as we have) (O'Donnell et al., 2015; Peay et al., 2016; Baltrus, 2020). For example, the frequent crop pathogen *Fusarium oxysporum* comprises a large complex of cryptic species with more than 120 different *formae speciales*, but all sharing the same ITS sequence (Michielse and Rep, 2009). Likewise, three different strains of *Pantoea ananatis* isolated from maize seeds had small but significant differences in their genomes and contrasting effects on plant growth despite sharing identical 16S rDNA (Sheibani-Tezerji et al., 2015).

Microbiomes of axenically grown plants should be 100% seed transmitted; however, this was not the case for any sample, suggesting a problem with capturing the full diversity in seeds and spermospheres. For example, soy seeds were detected to be 99% Firmicutes by abundance containing no *Pseudomonas*, however, when grown on sterile sand, they developed microbiomes similar to most other plants with all the same dominant bacteria. It may be that many seed-associated microbes are exceedingly rare and difficult to detect when they enter viable but non-culturable states such as resting spores that resist DNA extraction (Pollock et al., 2018). PCR of target amplicons may also be a limitation, as it is thought to be limited to detecting the top 99% most abundant

sequences in a population (Smalla, 2003). Primer bias also makes it impossible to amplify all the microbial sequence diversity in an environmental sample (Hong et al., 2009), or perhaps some microbial diversity was missed due to stochastic effects of seed selection for sequencing vs. germination. Ironically, sequencing microbiomes of axenically grown vegetative plants may be a better way to observe seed microbes than directly sequencing seeds, since "conditionally rare" and undetectable microbes may get a chance to awaken to more favorable conditions as seeds germinate and grow (Shade et al., 2014).

Another major caveat concerns the nature of the experimental setup, in that growing plants in hermetically sealed jars for a short period of time is not natural, though it did theoretically allow us to control all possible sources of microbial inoculum. These are juvenile plants, which may not yet have developed microbiomes corresponding to mature plants. The abundance of seed-transmitted bacteria we have observed in these young roots, shoots, and rhizospheres may be exaggerated because they have not yet had a chance to be more heavily colonized during passage through soil (Inceoğlu et al., 2011) or exposure to dust-fall or rain (Williams and Marco, 2014). Older and larger plants growing under natural conditions would also have more time to interact with other organisms such as nematodes and insects, which may vector microbes onto the plant and reduce the dominance of seed-transmitted bacteria as they are eaten and killed or displaced. Without possibility for gas exchange, air chemistry, and humidity in these jars was far from what these plants would encounter growing in a farmer's field, a fact that has been shown to alter microbial diversity and reduce microbial abundance in lettuce phyllospheres (Williams and Marco, 2014).

CONCLUSION

This experiment aimed to document the bacterial and fungal diversity in and on seeds of a panel of import plants and observe in a microbially controlled environment, how much of the seed microbiome goes on to form the plant microbiome. Seeds and spermospheres of all 17 plant species contained microbes, mostly Proteobacteria and Ascomycetes. Rhizospheres, roots, and shoots of all 17 plants grown on sterile sand also developed bacterial and fungal populations, showing that seeds are able to transmit complex microbial populations to their seedlings. All of the 63 core seed-transmitted bacteria observed in sterile sand-grown plants were also found in field soil-grown plants, and a subset of seven of these (1 *Pantoea*, 1 *Enterobacter*, 3 *Pseudomonas*, 1 *Klebsiella*, and 1 *Massilia*) were the dominant microbiome members on both types of substrate. There was no core seed-transmitted fungus; however, by tracing the fate of vertically transmitted fungi in individual plant species, it seems that some mycobiomes are also dominated by seed-transmitted fungi, especially *Fusarium* and *Alternaria*. Soil served as a minor source of bacterial diversity to plants, but a major source of diversity for fungi. The most abundant bacteria and fungi in these jar-grown seedlings came from their seeds, not the soil. Future experiments culturing these common and core microbes, cross inoculating them among plant species, and comparing their genetics and physiology may help us understand why they occur so frequently in plant seeds and how they have benefited angiosperm plant physiology over evolutionary time.

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 **Supplementary Material** (annotated sequences and OTU counts) in this publication.

AUTHOR CONTRIBUTIONS

DJ-M conceived of and designed the study, collected the all materials, performed the all wet lab experiments, conducted the all bioinformatics and statistics, and wrote the manuscript. JG aided in many aspects of the molecular biology lab work. LL-L funded the study and hosted DJ-M in his lab at CIAT. All the authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.737616/full#supplementary-material

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Vertical Transmission of Diverse Cultivation-Recalcitrant Endophytic Bacteria Elucidated Using Watermelon Seed Embryos

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Seed transmission of endophytic microorganisms is a growing research area in plant biology and microbiology. We employed cultivation versus cultivation-independent approaches on excised embryos from watermelon seeds (6–12 months in storage) and on embryo-derived *in vitro* seedlings (EIVS) to assess the vertical transmission of endophytic bacteria. Surface-disinfected watermelon seeds bore abundant residual bacteria in the testa and perisperm tissues, predominantly *Bacillus* spp. propounding the essentiality of excluding all non-embryonic tissues for vertical transmission studies. Tissue homogenates from re-disinfected seed embryos displayed no cultivable bacteria during the 1-week monitoring. Bright-field live microscopy revealed abundant bacteria in tissue homogenates and in embryo sections as intracellular motile particles. Confocal imaging on embryo sections after SYTO-9 staining and eubacterial fluorescent *in situ* hybridization (FISH) endorsed enormous bacterial colonization. Quantitative Insights Into Microbial Ecology (QIIME)-based 16S rRNA V3–V4 taxonomic profiling excluding the preponderant chloroplast and mitochondrial sequences revealed a high bacterial diversity in watermelon seed embryos mainly Firmicutes barring spore formers followed by Proteobacteria, Bacteroidetes, and Actinobacteria, and other minor phyla. Embryo-base (comprising the radicle plus plumule parts) and embryo-cotyledon parts differed in bacterial profiles with the abundance of Firmicutes in the former and Proteobacteria dominance in the latter. EIVS displayed a higher bacterial diversity over seed embryos indicating the activation from the dormant stage of more organisms in seedlings or their better amenability to DNA techniques. It also indicated embryo-to-seedling bacterial transmission, varying taxonomic abundances for seed embryos and seedlings, and differing phylogenic profiles for root, hypocotyl, and cotyledon/shoot-tip tissues. Investigations on different watermelon cultivars confirmed the embryo transmission of diverse cultivation recalcitrant endophytic bacteria. Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes formed the core phyla across different cultivars with 80–90% similarity at genus to phylum levels. Conversely, freshly harvested seeds

displayed a dominance of Proteobacteria. The findings revealed that dicot seeds such as in different watermelon cultivars come packaged with abundant and diverse vertical and seedling-transmissible cultivation recalcitrant endophytic bacteria with significant implications for plant biology.

Keywords: 16S rRNA amplicon profiling, *Citrullus lanatus* (Thumb.) Matsum. and Nakai, metagenomics, seed microbial community, plant microbiome biodiversity, cultivation recalcitrant endophytic bacteria

INTRODUCTION

Endophytic bacteria colonize plants internally without any apparent adverse effects on the host and are normally considered beneficial to the host (Hallmann et al., 1997; Afzal et al., 2019). All plants and plant parts are known to harbor bacterial endophytes with roots constituting the most widely studied region (Hardoim et al., 2015; Liu et al., 2017). Roots are also considered to be the major entry point for the organisms which traverse the cortex and reach different plant parts/organs through vascular and apoplastic channels. Other known routes of bacterial entry include the aerial plant parts such as stomata, wounds, insects, and various pollinators (Frank et al., 2017; Kandel et al., 2017). Following the realization that the majority of the soil/environmental bacteria are non-amenable to cultivation, molecular tools were applied to study the endophytic prokaryotic microbiome which revealed diverse bacterial phyla associated with the roots including several candidate phyla and some archaea (Lundberg et al., 2012; Sessitsch et al., 2012). Application of cultivation-independent molecular tools to study the endophytic bacterial diversity in the shoot tissues and in particular to the *in vitro* plant cultures which could be guarded against the external organisms revealed a huge bacterial diversity similar to the root system prevailing a cultivation recalcitrant form (Thomas and Sekhar, 2017; Thomas et al., 2017). This raised a question about the prospects of endophyte entry over and above the generally described routes.

Of late, considerable information is emerging on seed-associated microorganisms and the possibility of vertical transmission of bacterial endophytes (Nelson, 2018; Shahzad et al., 2018; Verma et al., 2019; White et al., 2019; Abdelfattah et al., 2021). Seed-associated bacteria could be of different types: (i) external on the seed surface, (ii) internal to the seed coat, or (iii) inside the embryo (Frank et al., 2017; Nelson, 2018). Seed-inhabiting bacteria possess the advantages of quick colonization at germination with some organisms possibly turning endophytic colonizers in seedlings (Cope-Selby et al., 2017; Verma et al., 2019). Studies investigating seed endophytes often used direct seeds (Barret et al., 2015), seed wash solutions (Torres-Cortés et al., 2018), mere surface-washed seeds (Adam et al., 2018), or in most instances surface-sterilized whole seeds (Khalaf and Raizada, 2016; Bergna et al., 2018; López et al., 2018). While most of the past studies adopted the cultivation-based approach (Truyens et al., 2015), some employed cultivation-independent molecular tools (Cope-Selby et al., 2017; Chen et al., 2020) with or without assessing the effectiveness of surface sterilization. In cultivation-based studies, generally Proteobacteria formed the dominant phylum with *Bacillus* spp., and other spore-forming Firmicutes also making a dominant constituent (Truyens et al., 2015; Khalaf and Raizada, 2016; Nelson, 2018).

Most studies on seed-associated bacteria did not make a discrimination between embryo inhabitants and those in other seed parts. Only the embryo-colonizing endophytes perhaps bear the advantage of vertical transmission between successive plant generations, while several bacteria could get incorporated from the seed spermosphere at germination (White et al., 2014; Shade et al., 2017; Shahzad et al., 2018). Additionally, the embryo colonizers are certain to reach different parts of new seedlings and adult plants. It is essential to distinguish the embryo-associated microbiome from other seed microbiota to elucidate the vertically transmissible microorganisms. Very few studies have factually examined the plant microbiome biodiversity confined to the embryos *per se*. A cultivation-based study on seed-endophytes in tomato employing *in vitro* grown seedlings where the seed coat could be separated post-seed germination displayed some embryo-associated bacteria that apparently arose from the activation of cultivation recalcitrant endophytic bacteria (CREB) constituting mainly Proteobacteria (Shaik and Thomas, 2019). Molecular analysis on surface-sterilized tomato seeds showed a highly diverse bacterial biome, but it was not possible to conclude whether the organisms were embryo colonizers or inhabiting other seed tissues (Thomas and Shaik, 2020). Investigations on wheat endosperm versus excised embryos revealed evident embryo colonization by a few bacterial genera (Kuźniar et al., 2020a,b). A recent internal transcribed spacer (ITS) region and 16S rRNA gene amplicon-based study of fungal and bacterial community separately in the embryo and pericarp of oak acorns showed twofold to fourfold higher microbial diversity and richness in the embryo than in the pericarp with some 20 phyla of the bacterial community in the embryos that included transient and seedling-transmitted microbiome (Abdelfattah et al., 2021).

Microscopy forms an essential tool to establish the presence of bacterial endophytes inside the tissues; this information is often lacking in the studies on endophytic microbiome. Microscopic explorations on banana and papaya shoot tissues and *in vitro* cultures indicated abundant intracellular bacteria with no obvious bacterial presence in the inter-cellular regions, which appeared contrary to the general perception about the bacterial endophytes as inter-cellular colonizers (Thomas and Sekhar, 2014; Thomas et al., 2019). The term "Cytobacts" was coined to describe such cultivation-recalcitrant cytoplasmic

Abbreviations: CREB, cultivation recalcitrant endophytic bacteria; FDW, filter-sterilized autoclaved distilled water; MS medium, Murashige and Skoog (1962) medium; NA, nutrient agar; OTU, operational taxonomic unit; SATS, spotting-and-tilt-spreading; SP-SDS, single plate-serial dilution spotting; STH, seed tissue homogenate; TSA, trypticase soy agar; VBNC, viable but non-cultivable.

colonizers which were also demonstrated in long-term actively maintained cell and callus cultures of different plant species comprising a huge taxonomic diversity as documented with fluorescent *in situ* hybridization (FISH) and 16S rRNA amplicon profiling on grape cell cultures (Thomas and Franco, 2021). The intracellular bacterial associations and the huge taxonomic diversity documented across plant species strengthened the possibility of vertical transmission of bacteria from one generation to the next.

Watermelon seeds bear the advantages of the embryo being well protected inside the hard testa and the feasibility of embryo excision excluding all external tissues. Watermelon seeds are generally dried before long-term storage bringing the moisture content down to about 7–8%. The extreme desiccation and high osmotic potential during drying and subsequent storage (ambient or low temperature) make the bacterial survival extremely difficult. Only spore-forming bacteria are generally considered to withstand such extreme conditions (Nelson, 2018). Studies addressing the seed microbiome in cucurbits (Khalaf and Raizada, 2016; Adam et al., 2018; Glassner et al., 2018) did not make the effort to separate the embryos from other seed parts. The extent of organisms that survive the desiccation and transmit to the next generation is best studied by analyzing the embryo-derived seedlings under sterile conditions. Tissue culture systems offer the feasibility of growing the embryo-derived seedlings *in vitro* protected from external organisms and also help in monitoring the distribution of true-embryo-associated bacteria in developing seedlings. This study was aimed at assessing the seed and embryo association of endophytic bacteria in stored dry seeds employing watermelon seeds and *in vitro* grown seedlings as the experimental system.

MATERIALS AND METHODS

Seed Material and Experimental Approach

Seeds of watermelon cv. "Arka Manik" (ICAR-Indian Institute of Horticultural Research, Bengaluru) were employed in detailed experiments, while other watermelon cultivars were used in extended studies. In general, "Arka" series seeds were procured from the institute seed sales counter packaged in standard polymer seed bags and stored under ambient conditions or at 16°C. After procurement, seeds were used immediately or were refrigeration stored (4°C). The seeds as per the date of packaging were of 1–6 months old, and were used within 6–12 months as mentioned under specific experiments. The seed lots were periodically checked for viability recording minimum 90% germination.

The study involved assessing the surface sterilization needs to ensure the aseptic excision of embryos, microscopic elucidation of embryo-colonization, and cultivation versus 16S rRNA metagene V3–V4 phylogenetic analysis on seed embryos and axenically grown seedlings. This was followed by V3–V4 taxonomic profiling on different parts of embryos and on seed embryos of four watermelon cultivars. All experiments were carried out under aseptic conditions with strict measures to avoid the lateral entry of microorganisms (Thomas and Sekhar, 2017). Unless mentioned differently, nutrient agar (NA) prepared in single-use γ-irradiated 90-mm plates and monitored for 4–5 days for sterility assurance after sealing in polypropylene (PP) bags was used for cultivation-based bacterial monitoring. For tissue homogenate preparation and serial dilutions, sterile 0.2-μm filtered distilled water post-autoclaving (FDW) was employed with sterility confirmation at use. The baseline to define the endophytic microbiome as CREB constituted the absence of bacterial colony growths from the tissue homogenates applied on NA or trypticase soy agar (TSA) at different serial dilutions with 36–37°C incubation for one night to trigger the growth and thereafter for 1 week under ambient conditions (at 26–30°C to reduce the chances of fast-spreading colonies) when the corresponding sample displayed high bacterial diversity/abundance as per deep sequencing.

Cultivation-Based Assessment of Seed-Associated Bacteria and Surface Sterilization Needs

An initial assessment of the extent of bacterial association with dry seeds and the surface sterilization needs was undertaken through different disinfection steps and monitoring the seed wash solutions pre- and post-chemical treatments using 6-month refrigeration-stored watermelon "Arka Manik" seeds. Precisely, 100 seeds were vortexed for 20 min in 10 ml FDW (0.01% Tween-20) in a 50-ml Falcon tube, and the colony-forming unit (CFU) released was assessed through single plate-serial dilution spotting (SP-SDS) (Thomas et al., 2015) of wash solutions on NA employing four replications. Seeds were further rinsed five times in FDW (100 μl per seed) with the wash solution monitoring through 10 μl sample spotting on NA. After this step, 25 seeds each were taken through three treatments: (T1): six washings using FDW with the first step in 0.01% Tween-20, then 1 min treatment with 90% ethanol; (T2): T1 followed by chemical disinfection for 5 min employing NaOCl (4% available chlorine; Fischer Scientific); or (T3): T1 succeeded by 0.1% $HgCl_2$ (0.1% Tween-20) treatment, with wash solution monitoring by spotting as above. After six rinses in FDW, seeds were aseptically dried on tissue paper and imprinted on NA. The dried seeds were decoated aseptically using a sterile nail cutter, and the seed coat tissues were assessed for any cultivable bacteria after an extended manual homogenization (15–20 min) in a mortar (50 mg ml^{-1} FDW) followed by SP-SDS. The excised seed embryos (25 each) with the adhering perisperm membrane were monitored for external bacteria after 20 min gentle vortexing in FDW (0.01% Tween-20) followed by six rinses (2.5 ml each time). Finally, the seed embryos were homogenized in a mortar (100 mg ml^{-1} FDW), and the bacterial CFU was assessed through spotting-and-tilt-spreading (SATS) (Thomas et al., 2012) on NA, which allowed 100 μl samples, and through SP-SDS on TSA, a cost-saving reliable method. The plates were monitored for 1 week at 36–37°C for one night followed by 30°C, as above. As per the outcome, the disinfection method employing NaOCl (4% chlorine) was tested again with 1- and 12-month refrigeration-stored seed lots. Surface-sterilized embryos were cultured on

sugar-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) gelled with 0.1% Phytagel (Sigma Chemical Company, St. Louis, MO, United States) to assess the effect of the chemical treatments on seed germination. Based on the results, excision of seed embryos followed by their surface sterilization with perisperm exclusion as per T2 above was adopted to study the embryo-associated microbiome.

Microscopic Observations on Seed Wash Solutions, Tissue Homogenates, and Tissue Sections

Seed soak/wash solutions from surface-sterilized watermelon seeds, seed testa homogenate, perisperm membrane, and the embryo sections were examined under bright field/phase contrast using a Leica DM2000 microscope, and still images/movie were captured under high magnification (×1,000) as described elsewhere (Thomas and Sekhar, 2014). Further, thin sections from dry seed embryos were prepared with a cryostat microtome (Thermo Scientific Microtome FSE) or with a razor blade after 1–2 h soaking in FDW and were examined in a Leica LB-02 epi-fluorescence microscope after staining with SYTO-9 and propidium iodide employing the Live/Dead bacterial staining kit (Molecular Probes) as per Thomas and Sekhar (2014). Razor-thin sections prepared from FDW-soaked embryos were further examined under confocal scanning laser microscope (Nikon Confocal A1, 90i) after staining with SYTO-9 with the imaging done under 488 nm green channel.

Fluorescent *in situ* hybridization was undertaken on fixed tissue of watermelon seeds (4% formaldehyde solution) as per Rossmann et al. (2012), adopting the basic protocol of Pernthaler et al. (2001) using 5′ Cy3 labeled Eub338 probe and Eub338ns control probe. The imaging was done under 543.5 nm channel using the NIS element 3.2.3 program (Nikon). Eub338 FISH images were captured under the same settings where no signal was observed with the Eub338ns probe.

Cultivation Versus 16S rRNA Amplicon Profiling of Seed Embryo/Seedling-Associated Bacteria

Embryos excised from dry seeds were disinfected with NaOCl (4% chlorine) as per the optimized procedure with CFU monitoring of all wash solutions. The embryos were cleared off the perisperm tissue, treated with filter-sterilized 2% $Na_2S_2O_3$ (10 min) to remove chloramines, washed twice in FDW, and imprinted on NA to ensure proper surface sterilization. After aseptic weighing, 50 embryos were homogenized in a mortar (1 ml FDW/embryo; approximately 40–50 mg per embryo), and the decimal dilutions (10^0 to 10^5) of the homogenate were applied on NA as per SATS and on TSA through SP-SDS. The plates were incubated at 37°C (NA) or 30°C (TSA) to provide wider growing conditions.

Watermelon Seed Embryos Versus *in vitro* Seedlings

This study involved DNA extracted from excised and surface-sterilized seed embryo (MG-37) and 2-week-old seed embryo-derived seedlings (MG-41) of watermelon cv. Arka Manik. The *in vitro* seedlings were confirmed to be free from cultivable bacteria through their indexing on NA and TSA (Thomas and Sekhar, 2017). DNA was extracted from the milky seed embryo homogenate or the *in vitro* grown index-negative seedlings (after testing for any cultivable bacteria) employing PowerFood (PF) microbial DNA isolation kit (MOBIO Laboratories, Inc., Carlsbad, CA, United States). After preliminary quantity and quality assessments, the DNA was submitted to M/s Xcelris Labs Ltd., Ahmedabad[1] for 16S rRNA gene taxonomic profiling. 16S rRNA gene amplicon libraries were prepared by M/s Xcelris targeting the V3–V4 hypervariable region as per the standard Illumina 16S Metagenomic Sequencing Library preparation protocol. Library preparation, PCR amplification, amplicon purification, paired-end sequencing on Illumina MiSeq platform (2 × 300 bp), quality filtrations, chimera screening, stitching, and the operational taxonomic units (OTU) picking were undertaken as described by Thomas and Sekhar (2017) with bioinformatics support from the service provider. For taxonomic assignment, QIIME bioinformatics analysis tool was employed based on sequence similarity within the reads in Greengenes database through *de novo* approach excluding singletons (<2 reads). Two rounds of QIIME analyses were undertaken to avoid the majority of reads corresponding to chloroplast and mitochondrial 16S rRNA with the second round analysis (QIIME analysis II) excluding the plant sequences and unassigned reads from the sequence files as described in detail elsewhere (Thomas and Sekhar, 2017).

Whole Embryos Versus Embryo-Base and Shoot Tip + Cotyledon Parts of Embryos

In this study, 20 re-disinfected whole seed embryos were employed in comparison with another 20 embryos where the embryo-base (comprising the radicle and plumule which is to develop as the new seedling) and cotyledon parts were segregated and used for DNA isolation. The DNA samples after the quality and quantity assessments were taken through 16S rRNA gene taxonomic profiling as above.

16S V3–V4 Taxonomic Profiling of Watermelon Seedling Root, Shoot, and Hypocotyl Parts

The root, hypocotyl, and the remaining shoot tissues comprising the shoot tip and the cotyledons from 2-week-old *in vitro* grown seedlings post-surface sterilization and after assessing the effectiveness of surface sterilization were employed here. The tissue homogenates after serial dilutions were plated separately on NA to assess the cultivable bacterial population. DNA was extracted from the three tissue homogenates separately using the PF kit, and the samples were submitted to M/s Xcelris Labs (after the quality and quantity assessments) for the 16S V3–V4 taxonomic profiling.

16S V3–V4 Taxonomic Profiling on Seed Embryos of Four Different Watermelon Cultivars

Seeds of four watermelon cultivars, "Arka Manik," "Arka Muthu" (ICAR-IIHR), "Madhubala" F1 Hybrid, and "SS455"

[1] www.xcelrislabs.com

(Nunhems, Bangalore) stored at 4°C were employed here. The former two had small black seeds, while the latter two showed large bold seeds. Seed embryos were gathered after seed decoating and surface sterilization and were cleared off the perisperm membrane. The embryo homogenates were tested for cultivable bacteria on NA and TSA through SP-SDS. The embryo homogenate suspension was stored at 4°C for 1–2 h to allow the large particles to settle down. The supernatant was subjected to three rounds of spinning, and the DNA was extracted from the pellet employing the PF kit. The 16S rRNA V3–V4 taxonomic profiling was performed as above.

Experiment Setup and Statistical Procedures

For surface sterilization trials, 100 seeds of different lots were employed. For cultivation versus cultivation-independent assessment of embryo microbiome, the homogenate derived from about 50 excised seed embryos (homogenized in 1 ml FDW per seed embryo) was used. Cultivation-based studies were targeted at getting an estimate of the cultivable bacteria which employed four replications for different serial dilutions. Deep-sequencing studies were undertaken on DNA samples pooled from different seed lots with a single replication per sample. The deep-sequencing data generated in this study have been deposited with the National Center for Biotechnology Information/Sequence Read Archive (NCBI/SRA).

Accession Numbers

The metagenome data generated have been deposited with NCBI/SRA under the project title "Cucurbit Seed, Embryo and Seedling Microbiome" with the bioproject ID: PRJNA564696, BioSample accession nos. SAMN12726399 to SAMN12726402 (MG37-MG40); SAMN12726475 to SAMN12726478 (MG41-44) and SAMN12726555 to SAMN12726558 (MG45-48) as indicated in the respective tables.

RESULTS

Seed Bacterial Load and Surface Sterilization Needs

Preliminary observations on watermelon "Arka Manik" seeds surface-sterilized with NaOCl showed residual bacteria in the seed coat as per the monitoring of wash solutions. Surface sterilization involving ethanol soaking and NaOCl treatment also did not eliminate all the external bacteria. Monitoring the seed external and internal bacterial load after the three treatments, namely, repeated FDW washing followed by 1 min ethanol soaking (T1), T1 followed by 5 min NaOCl treatment (T2), and T1 succeeded by 5 min 0.1% $HgCl_2$ (T3), indicated a large amount of seed external bacteria (1.0×10^4 CFU/seed), a part of which (4.4×10^3 CFU/seed) was released with mere water-rinsing which comprised largely spore-forming *Bacillus* spp. (**Table 1** and **Supplementary Figure 1**).

The next monitoring step involved drying the above surface-disinfected seeds in a vertical airflow cabinet, removing the hard seed coat aseptically with the help of a nail cutter, and then assessing the testa-homogenate and the embryo parts for any surviving bacteria. The seed testa from surface-sterilized seeds showed substantial bacterial CFU (445–557 per seed), dominantly *Bacillus* spp. The embryos excised from surface-disinfected seeds (with the intact perisperm) bore externally 10^3 to 10^4 CFU per embryo as per the six recurrent vortexing washes. The tissue homogenate from perisperm-bearing washed embryos again showed 10^3 to 10^4 CFU g^{-1} tissues. Thus, the "supposedly surface-sterilized seeds" harbored a high share of cultivable bacteria inside the seed coat and on the perisperm-bearing embryos. The bacterial population that emerged from the seed testa, embryo washes, or the embryo homogenate varied with the disinfectant employed: slower-growing non-sporulating Gram-positive colony types dominated the NaOCl (4% chlorine) treated set, the $HgCl_2$ (0.1%) treated set showed a high population of fast growing *Bacillus* spp., while the mere ethanol-treated seeds showed an intermediate population; the reason for this differential outcome was not understood.

Embryo Disinfection and Cultivation-Based Assessment of Embryo-Associated Bacteria

The above observations proved that it was essential to go for seed decoating and detailed surface sterilization of excised embryos to ensure the removal of all bacteria external to it. Accordingly, three FDW rinses of excised embryos followed by 90% ethanol (1 min) and 10 min NaOCl (4% chlorine) treatment and six FDW rinses was arrived at as the standard disinfection procedure. This treatment also facilitated the removal of embryo-adhering perisperm (**Figure 1**). With this procedure, no bacteria were detected in the final wash solutions, in embryo imprints, or in the PCR employing bacterial 16S rRNA universal primers on the two last wash solutions. The homogenate from disinfected embryos did not display any bacterial CFU on NA or TSA during the 1 week of observation except for the grainy raised appearance at the direct-homogenate applied spots re-streaking of which to fresh medium did not elicit any colony growth for another week. Surface-sterilized embryos showed > 90% germination on MS medium indicating that they were viable and healthy. A few bacterial colonies emerged upon the extended incubation of nutrient plates for 2–4 weeks which were not pursued since the emphasis in this study was mainly elucidating the gross embryo association by endophytic bacteria.

Microscopic Observations on Seed Wash Solutions and Tissue Homogenates

The original seed-vortexed FDW (100 µl per non-disinfected seed) showed a few mobile bacterial cells under live bright field microscopy (×1,000) upon loading 10 µl samples under a 22 × 22 mm cover-glass (**Supplementary Movie 1**). On the other hand, the seed testa homogenate (50 mg ml^{-1}) and the perisperm tissue from surface-sterilized seeds displayed much more/abundant motile bacterial cells (**Supplementary Movies 2**, **3**). Razor-thin tissue sections prepared from re-surface-sterilized embryos (post 1–2 h FDW soak) also exhibited

TABLE 1 | Bacterial CFU detected with 6-month refrigeration-stored seeds of watermelon "Arka Manik" adopting different surface disinfection methods.

Step	Particulars			CFU per seed
A	Direct seed washing: 100 seeds from a 6-month-old seed lot were vortexed for 20 min in 10 ml FDW (0.01% Tween-20) and the solution monitored for bacterial CFU			4,400
B	Seed washes 2–6: Seeds were further rinsed five times in 10 ml FDW (100 μl per seed)			
	Rinse 1			517
	Rinse 2			296
	Rinse 3			282
	Rinse 4			197
	Rinse 5			93
	Total CFU removed/seed (A + B)			5,685
C	Surface-disinfection treatment followed by the monitoring of six wash solutions	T1: Mere 90% ethanol soaking for 60 s	T2: Ethanol (90%) treatment (60 s) followed by 5 min NaOCl treatment (4% available chlorine)	T3: Ethanol (90%) followed by 5 min 0.1% $HgCl_2$ (0.1% Tween-20)
	CFU per seed after seed coat separation			
D	Seed testa homogenization (average 50 mg per seed; in 1 ml FDW) and homogenate CFU monitoring	498 ± 54.07	555 ± 61.7	447 ± 58.5
			Average = 500	
E	Vortexing the perisperm-bearing seed embryos excised from surface disinfected seeds (2 min at top speed) and CFU monitoring	527 ± 78.5	600 ± 40.8	975 ± 120.2
			Average = 700	
F	Monitoring the six sequential washes of excised embryos (with perisperm)	In effect 10^3 to 2×10^3 CFU was left behind with the seed coat or periderm membrane per seed irrespective of the surface sterilization procedure adopted (average 1,500)		
G	Preparation of embryo homogenate from surface-disinfected seeds and CFU monitoring (after step C)	10^4 CFU g^{-1} embryo tissue	10^3 CFU g^{-1} embryo tissue	10^4 CFU g^{-1} embryo tissue
	Population pattern in "G"	Intermediate to T2 and T3	Mix of sporulating and non-sporulating Gram-positive bacteria	Spore-forming *Bacillus* spp.

CFU, colony-forming unit; FDW, filter-sterilized autoclaved distilled water.

FIGURE 1 | Excision of watermelon seed embryos after seed decoating and surface sterilization, the removal of perisperm tissues, and monitoring the seed embryo homogenate for cultivable bacteria. **(A)** Whole dry seeds, **(B)** removed seed coat, **(C)** seed embryo with perisperm, **(D)** perisperm tissue removed during sodium hypochlorite-mediated surface sterilization, and **(E)** surface-sterilized embryo with the perisperm tissue removed.

copious motile cocci inside and around the disturbed tissues (**Supplementary Movie 4**). Tissue homogenate from surface-sterilized embryos (100 mg ml^{-1}) appeared as a thick milky suspension with no obvious particle motility but upon dilution (1:10 or 1:100) displayed profuse motile bacteria along with passively moving plastids, mitochondria, and possibly starch grains and other cellular inclusions (**Supplementary Movie 5**). The embryo homogenate under phase contrast showed phase-bright plastids (≥5 μm) and mitochondria (2–3 μm) along with abundant fine rods and cocci (**Supplementary Figure 2**), which apparently corresponded to diverse bacteria as elucidated subsequently through 16S rRNA amplicon profiling.

Microscopy and Fluorescent *in situ* Hybridization on Tissue Sections

Confocal imaging with SYTO-9 staining on perisperm tissue displayed abundant green fluorescing bacteria (**Figure 2A**). Ultra-thin cryo-sections of seed embryos showed vague SYTO-9 signal (data not shown), while direct embryo sections prepared with a razor blade (after 1–2 h FDW soaking of disinfected embryos) displayed plentiful green-fluorescing bacteria along the cell periphery and in the cytoplasm (**Figure 2B**). Confocal movie microscopy on SYTO-9 stained seed embryo sections indicated abundant bacteria in the intracellular matrix across different vertical planes (**Supplementary Movie 6**) in line with bright-field microscopy. FISH employing Eub338 probe displayed abundant bacteria in the intracellular matrix (**Figure 2C**) with no signal detected in the Eub338ns control (**Figure 2D**).

Cultivation Versus 16S Taxonomic Profiling on Watermelon Seeds and Seedlings

Watermelon Seed Embryos Versus *in vitro* Seedlings

This study involved seed embryos and 2-week old seed embryo derived *in vitro* seedlings of watermelon cv. Arka Manik, the latter that were confirmed to be free from cultivable bacteria through their indexing on NA and TSA almost 10 days after culturing the surface-disinfected seed embryos on MS medium. DNA extracted from the milky seed embryo homogenate (sample MG-37) and the index-negative seedlings (MG-41) yielded good amplicon libraries with the 16S rRNA V3–V4 primers (**Table 2**). After filtering out the reads corresponding to plant and no blast hit sequences as per the QIIME analysis I, QIIME round II analysis on stitched quality reads showed diverse bacteria associated with both seed embryos and *in vitro* grown seedlings. Firmicutes formed the dominant phylum in both samples with similar OTU shares (78.2 and 74.1%, respectively). Seed embryos showed Proteobacteria, Bacteroidetes, and Actinobacteria as the next major phyla, while seedlings displayed an increase in Proteobacterial OTU share, a reduction in Bacteroidetes and Actinobacteria, and the emergence of six additional phyla that were not observed in seed embryos albeit in small shares (**Figure 3A**). Class level distribution showed 11 constituents in seed embryos and an additional 12 constituents in seedlings again in minor shares (data not shown). Clostridia formed the major class in both seed embryo and seedling samples (72.1 and 68.5%, respectively), with more β-Proteobacteria emerging in seedlings compared with the higher share of γ-Proteobacteria observed with seed embryos. At the family level, the seed embryos showed 26 constituents with Ruminococcaceae as the main constituent followed by Lachnospiraceae, Alcaligenaceae, Enterobacteriaceae, Clostridiaceae, and others, while seedlings displayed notably more diversity (62 constituents) with the dominance of Ruminococcaceae followed by others (**Figure 3B**). The two samples showed 23 common families with three additional families in seed embryos and 39 extra families in seedlings. At the genus level, undefined Ruminococcacea appeared as the dominant constituent in both samples followed by undefined Enterobacteriaceae, undefined Clostridiaceae, *Faecalibacterium*, and others in seed embryos. Seedlings displayed *Achromobacter*, undefined Clostridiales, *Dorea*, [*Eubacterium*], *Coprococcus*, etc., as the major constituents. Thus, the seedlings displayed

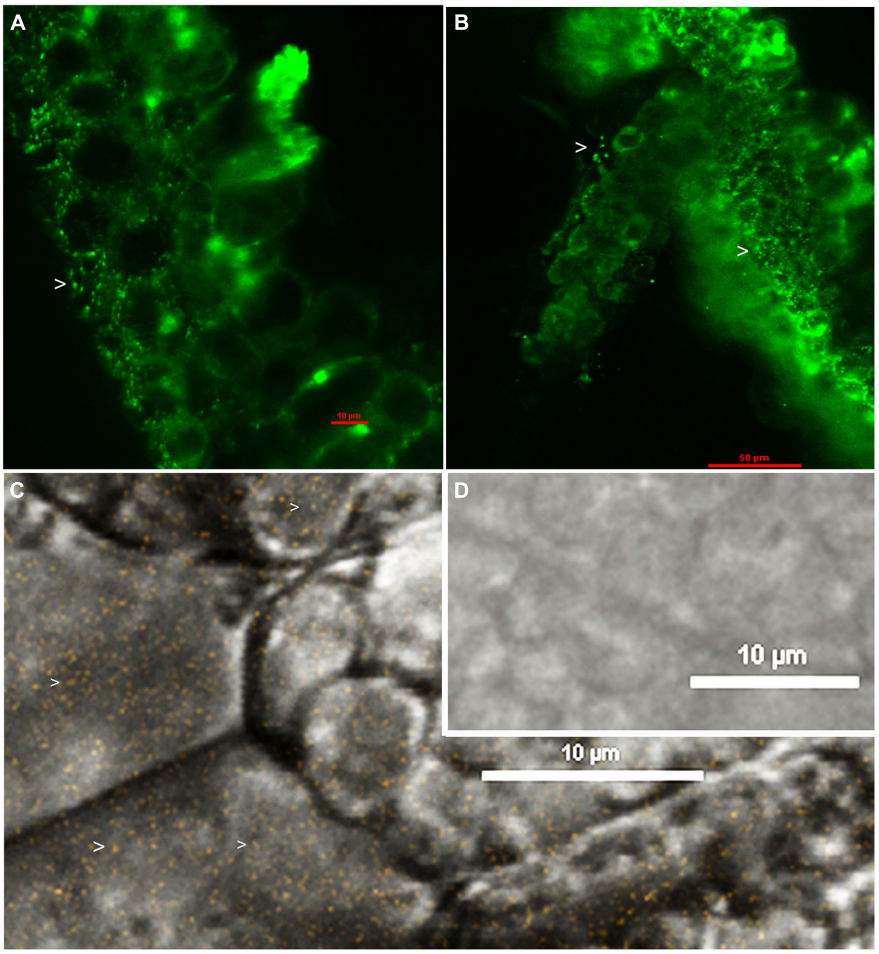


FIGURE 2 | Confocal imaging after SYTO-9 staining, or FISH with Eub338 probe on seed tissue sections of watermelon "Arka Manik": Perisperm tissue stained with SYTO-9 under 488 nm green channel **(A)**, seed embryo stained with SYTO-9 under 488 nm green channel **(B)**, FISH on seed embryo tissues of watermelon with Cy3 labeled Eub338 displaying abundant fluorescing bacteria under 543.5 nm channel **(C)**, and FISH negative control with Eub338ns **(D)**. Arrow heads show bacterial cells.

a considerably higher diversity compared with seed embryos with 33 common genera, 15 additional genera in seed embryos, and 50 more in seedlings (**Supplementary Dataset 1**). The results altogether indicated the prevalence of diverse bacteria as embryo-colonizing endophytes and their transmission to new seedlings with taxonomic realignments with respect to the deciphered population. It was significant to note that spore-forming Firmicutes (*Bacillus* and *Paenibacillus* spp.) were not documented in the seed embryos while they formed very minor share in seedlings.

Whole Seed Embryos, Embryo Base, and Cotyledons

In this study, 20 re-disinfected whole seed embryos were employed (MG-38) in comparison with another 20 embryos where the embryo base (radicle-plumule part; MG-39) and cotyledon part (MG-40) were segregated before DNA isolation. After filtering out the reads corresponding to plant and no blast hit sequences as per the QIIME analysis I (**Supplementary Table 1**), QIIME analysis II on stitched quality reads showed 0.97 million reads for the three samples with 1,825 to 2,444 OTUs. Overall, the embryo base which is to give rise to the root and shoot systems showed more diversity than the cotyledon part, while the whole embryos stood in between (**Figure 4**). The whole embryos showed an abundance of Firmicutes (88.3%) followed by Proteobacteria as documented earlier for the seed embryo. The embryo base also showed dominant Firmicutes (66.9%) followed by Proteobacteria, Actinobacteria, and Bacteroidetes. The cotyledon part, on the other hand, displayed majorly Proteobacteria (87.1%), while the rest formed mainly Firmicutes (12.1%). The same trend continued at class level with Clostridia constituting the major share for both whole embryo and embryo base which included mainly Ruminococcaceae, and Lachnospiraceae families. The cotyledon part on the other hand largely showed β-Proteobacteria contributed almost exclusively by Alcaligenaceae.

A large share of OTUs under Clostridia and Proteobacteria remained unassigned to any specific genera. The prominent defined genera in whole embryos and the embryo base

TABLE 2 | Data statistics for seed embryos (MG37) and 2-week-old in vitro seedlings (MG41) of watermelon "Arka Manik" samples as per QIIME analysis.

Sample name	Seed embryo (MG-37)	*In vitro* seedling (MG-41)
DNA concentration (μg/μl)	33.8	23.0
QIIME analysis I		
Number of reads	854,516	722,438
Total data (Mb)	419	341
Mean sequence length	245.18	236.63
Guanine and cytosine (GC)	54%	54%
Stitch reads	774,782	588,705
Mean sequence length of stitch read	457.15	454.9
Number of reads after quality check (QC)	774,501	532,944
Number of OTUs	3,414	6,079
Shannon alpha diversity	4.27	2.77
Number of observed species	4,017	7,095
Taxonomy at phylum level		% OTUs
Cyanobacteria/chloroplast[a]	37.56/37.56	1.30/1.30
Proteobacteria/mitochondria[b]	20.59/15.7	1.16/1.09
Firmicutes	36.56	0.27
Actinobacteria	2.28	–
Bacteroidetes	2.87	0.01
No blast hit	0.14	97.25
QIIME analysis II after removing reads assigned to chloroplast, mitochondria, and unassigned		
Number of reads removed	441,975	191,434
Total data (Mb)	213	77
Number of stitched reads	362,241	57,701
Number of reads after QC	361,962	1,940
OTUs	3,087	214
Alpha diversity: Shannon index	5.67	5.09
Observed species	3,465	211

OTU, operational taxonomic unit; QIIME, Quantitative Insights Into Microbial Ecology.
[a] *Class level.*
[b] *Mitochondria at family level.*

under Firmicutes included *Faecalibacterium*, *Blautia*, *Coprococcus*, *Soehngenia*, *Dialister*, *Ruminococcus*, *Clostridium*, *Lachnobacterium*, *Roseburia*, and *Anaerostipes*. Other major defined genera included *Achromobacter*, *Kerstersia*, and *Sutterella* under Proteobacteria, *Bacteroides* under Bacteroidetes, and Bifidobacterium under Actinobacteria. The results overall indicated the prevalence of a rich bacterial diversity in watermelon seed embryos with differential population in embryo-base and seed-cotyledon parts and high phylogenic diversity in the radicle-plumule part which is to develop to the new seedlings.

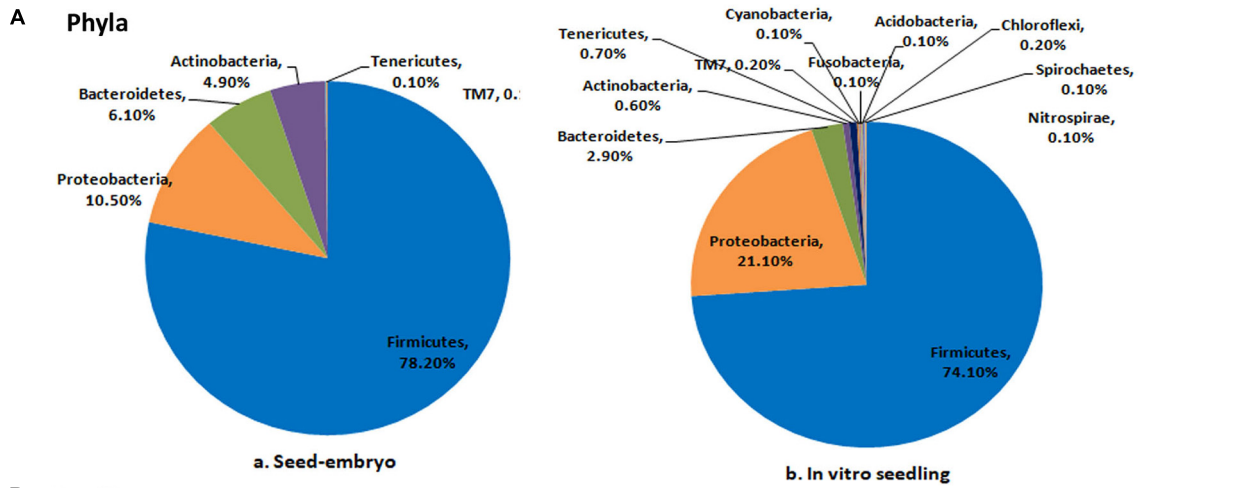
Root, Hypocotyl, and Cotyledon Parts of *in vitro* Grown Seedlings

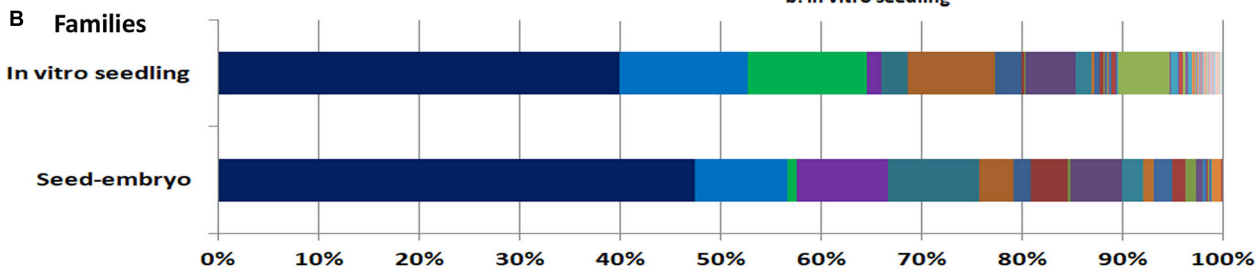
To assess the transmission of bacterial endophytes to the next generation cycle, seedlings derived from the surface-disinfected seed embryos were employed. *In vitro* seedlings growing on Phytagel gelled MS medium after 10 days of embryo culturing were indexed/tested on NA, TSA, and agar-gelled MS medium, and the root, hypocotyl, and cotyledon tissues from individual seedlings were stored singly in 2-ml tubes at −20°C to ensure that only seedlings without any cultivable bacterial association were selected to avoid the over-representation of such bacterial OTUs. Tissues from such 20 seedlings were pooled to three composite samples for the said three parts. QIIME analysis II filtering out the plant sequences showed distinct taxonomic profiles for the three seedling regions (**Figure 5**). Root tissues displayed the maximum taxonomic diversity (eight phyla) followed by cotyledon (five phyla) and hypocotyl (four phyla). The distribution of major phyla in the root and cotyledon parts appeared similar (Firmicutes 52.0% and 58.8%; Proteobacteria 41.6% and 40.0%). Conversely, hypocotyl showed dominantly Proteobacteria (85.8%) followed by Firmicutes (11.2%). Root tissues also showed a notable share of Tenericutes. At the class level, root tissues showed Clostridia and Erysipelotrichi under Firmicutes, while cotyledon showed a high share of Clostridia. While γ-Proteobacteria formed a major class for the hypocotyls and roots, the cotyledon part showed more of β-Proteobacteria.

At the genus level, both roots and cotyledon parts displayed high diversity (41 and 43 genera, respectively), compared with the hypocotyls (20 genera). Nine genera appeared common to the three samples that constituted 80.1, 64.4, and 88.3% OTUs, respectively, for root, cotyledon, and hypocotyl parts. These included *Halomonas* (γ-Proteobacteria), *Achromobacter* (β-Proteobacteria), [*Eubacterium*], *Coprococcus*, [*Ruminococcus*], *Streptococcus*, *Tissierella_Soehngenia*, undefined Ruminococcaceae, and undefined Clostridia, all belonging to Firmicutes. While the shoot region displayed a high share of *Halomonas* and undefined Ruminococcaceae, the cotyledon part showed a high share of *Achromobacter*. The hypocotyl tissue exhibited high abundance of *Halomonas* and *Achromobacter*. *In vitro* grown seedlings displayed altogether 70 constituents at genus level across the three segments despite the fact that they were protected from all external organisms. This indicated that diverse bacteria are carried by the embryo which in turn transmit to the root and shoot parts of seedlings with differential distribution in various seedling parts. Significantly, *Bacillus* spp. and the related genera of spore formers did not form notable constituents in any part of the seedlings.

16S rRNA Taxonomic V3–V4 Profiling on Different Watermelon Cultivars

Embryo homogenates from the four watermelon cultivars ("Arka Manik," "Arka Muthu," "Madhubala," and "SS-455") did not show any bacterial colony growth on NA/TSA during the 1-week period of observation. This study employed a fresh lot of seeds of "Arka Manik," while the others were from refrigeration-stored seeds of >6 months. Employing the upper part of the milky embryo homogenates after 1 h standing at 4°C, the DNA yields appeared better than in the previous instance (74.0–110 ng μl^{-1}). Illumina sequencing yielded 0.66–0.91 million reads per sample with 0.59–0.84 million stitched reads (**Supplementary Table 2**). QIIME analysis II excluding the plant sequences gave rise to 3,677–26,157 stitched high quality bacteria-corresponding reads with the OTUs in the range of 395 ("Arka Muthu") and 1,574 ("SS-455"). Firmicutes formed the main phylum in



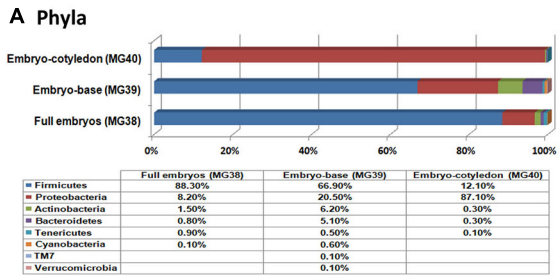


	Seed-embryo	In vitro seedling
Firmicutes; f_Ruminococcaceae	47.20%	40.60%
Firmicutes; f_Lachnospiraceae	9.20%	13.10%
Proteobacteria; f_Alcaligenaceae	0.90%	12.00%
Proteobacteria; f_Enterobacteriaceae	9.10%	1.40%
Firmicutes; f_Clostridiaceae	9.00%	2.80%
Firmicutes; f_undefined	3.40%	8.80%
Firmicutes; f_Veillonellaceae	1.70%	2.60%
Actinobacteria; f_Coriobacteriaceae	3.70%	0.30%
Firmicutes; f_[Tissierellaceae]	0.20%	0.10%
Firmicutes; f_Erysipelotrichaceae	5.20%	5.10%
Bacteroidetes; f_Bacteroidaceae	2.00%	1.70%
Actinobacteria; f_Bifidobacteriaceae	1.10%	0.20%
Bacteroidetes ;f_Prevotellaceae	1.80%	0.60%
Firmicutes; f_Peptostreptococcaceae	1.30%	0.30%
Bacteroidetes; f_Porphyromonadaceae	1.10%	0.20%
Firmicutes; f_Lactobacillaceae	0.60%	0.10%
Firmicutes; f_Christensenellaceae	0.10%	0.20%
Tenericutes; f_undefined	0.10%	0.10%
Firmicutes; f_Streptococcaceae	0.20%	0.30%
Bacteroidetes; f_S24-7	0.10%	0.30%
Bacteroidetes; f_[Paraprevotellaceae]	0.20%	0.10%
Firmicutes; f_[Mogibacteriaceae]	0.10%	0.10%
Proteobacteria; f_Oxalobacteraceae	0.10%	0.10%
Bacteroidetes; f_Rikenellaceae	0.90%	
Bacteroidetes; f_[Odoribacteraceae]	0.10%	
TM7; f_undefined	0.10%	
Proteobacteria; f_Halomonadaceae		5.30%
Proteobacteria; f_Bradyrhizobiaceae		0.10%
Proteobacteria; f_Sphingomonadaceae		0.60%
Fusobacteria;f_Fusobacteriaceae		0.10%
Proteobacteria; f_undefined		0.10%
Proteobacteria; f_Bacteriovoracaceae		0.40%
Proteobacteria; f_Desulfobacteraceae		0.20%
Proteobacteria; f_undefined		0.30%
Tenericutes; f_undefined		0.40%

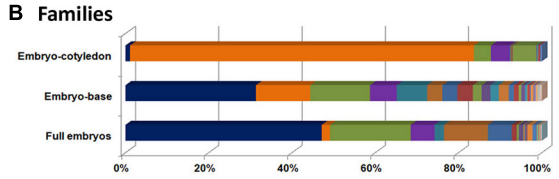
	Seed-embryo	In vitro seedling
Acidobacteria; f_undefined		0.10%
Actinobacteria; f_C111		0.10%
Actinobacteria; f_Microbacteriaceae		0.10%
Bacteroidetes; f_SB-1		0.10%
Bacteroidetes; f_Flavobacteriaceae		0.10%
Chloroflexi; f_Anaerolinaceae		0.10%
Chloroflexi; f_undefined		0.10%
Chloroflexi; f_undefined		0.10%
Cyanobacteria; f_undefined		0.10%
Cyanobacteria; f_Synechococcaceae		0.10%
Firmicutes; f_Bacillaceae		0.10%
Firmicutes; f_Paenibacillaceae		0.10%
Firmicutes; f_Thermoactinomycetaceae		0.10%
Firmicutes; f_Peptococcaceae		0.10%
Nitrospirae; f_[Thermodesulfovibrionaceae]		0.10%
Proteobacteria; f_Rhodobacteraceae		0.10%
Proteobacteria; f_undefined Alphaproteobacteria		0.10%
Proteobacteria; f_undefined Alphaproteobacteria		0.10%
Proteobacteria; f_undefined Betaproteobacteria		0.10%
Proteobacteria; f_undefined Betaproteobacteria		0.10%
Proteobacteria; f_Neisseriaceae		0.10%
Proteobacteria; f_Desulfobulbaceae		0.10%
Proteobacteria; f_Desulfovibrionaceae		0.10%
Proteobacteria; f_undefined Deltaproteobacteria		0.10%
Proteobacteria; f_[Marinicellaceae]		0.10%
Spirochaetes; f_undefined		0.10%
TM7; f_F16		0.20%
Tenericutes; f_Anaeroplasmataceae		0.10%
Tenericutes; f_Mycoplasmataceae		0.10%
Tenericutes; f_undefined		0.10%

FIGURE 3 | Distribution of phylogenetic groups as per 16S rRNA metagene V3–V4 region taxonomic profiling of DNA derived from the seed embryo versus *in vitro* derived seedlings of watermelon cv. Arka Manik at phylum **(A)** and family **(B)** levels.

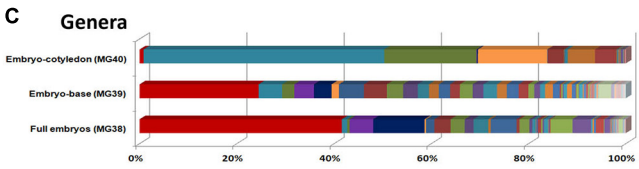
three cultivars similar to the observations documented earlier with "Arka Manik." Conversely, cv. Arka Manik in this trial showed Proteobacteria as the dominant phylum which possibly arose from the use of a fresh seed lot as documented with other fresh seed samples also (unpublished data). The four phyla including Firmicutes, Proteobacteria, Actinobacteria, and



	Full embryos (MG38)	Embryo-base (MG39)	Embryo-cotyledon (MG40)
Firmicutes	88.30%	66.90%	12.10%
Proteobacteria	8.20%	20.50%	87.10%
Actinobacteria	1.50%	6.20%	0.30%
Bacteroidetes	0.80%	5.10%	0.30%
Tenericutes	0.90%	0.50%	0.10%
Cyanobacteria	0.10%	0.60%	
TM7		0.10%	
Verrucomicrobia		0.10%	



	Full embryos	Embryo-base	Embryo-cotyledon
Firmicutes; f_Ruminococcaceae	47.00%	31.20%	1.10%
Proteobacteria; f_Alcaligenaceae	2.00%	13.00%	82.50%
Firmicutes; f_Lachnospiraceae	19.30%	14.30%	4.10%
Proteobacteria; f_Enterobacteriaceae	5.70%	6.40%	4.50%
Firmicutes; f_Clostridiaceae	2.30%	7.30%	0.20%
Firmicutes; f_UC Clostridiales	10.50%	3.60%	0.20%
Firmicutes; f_Veillonellaceae	5.70%	3.60%	0.10%
Actinobacteria; f_Coriobacteriaceae	1.20%	3.70%	0.20%
Firmicutes; f_[Tissierellaceae]	0.50%	2.10%	5.70%
Firmicutes; f_Erysipelotrichaceae	1.00%	2.10%	0.10%
Bacteroidetes; f_Bacteroidaceae	0.10%	2.00%	0.20%
Actinobacteria; f_Bifidobacteriaceae	0.30%	2.40%	0.10%
Bacteroidetes; f_Prevotellaceae	0.30%	1.20%	0.10%
Firmicutes; f_Peptostreptococcaceae	0.30%	1.20%	0.40%
Bacteroidetes; f_Porphyromonadaceae		0.60%	
Bacteroidetes; f_Rikenellaceae		0.90%	0.10%
Firmicutes; f_Lactobacillaceae	0.10%	0.50%	
Firmicutes; f_Christensenellaceae	1.20%	0.10%	
Tenericutes; f_UC Mollicutes	0.90%	0.00%	0.10%
Firmicutes; f_Streptococcaceae	0.10%	0.50%	
Bacteroidetes; f_S24-7	0.10%		
Bacteroidetes; f_[Odoribacteraceae]		0.20%	
Bacteroidetes; f_[Paraprevotellaceae]	0.20%	0.10%	
Cyanobacteria;f_UC 4C0d-2	0.10%	0.50%	
Firmicutes; f_Enterococcaceae		0.10%	0.20%
Firmicutes; f_[Mogibacteriaceae]	0.20%	0.10%	
Proteobacteria; f_Succinivibrionaceae	0.20%		
Proteobacteria; f_Pasteurellaceae	0.10%	0.40%	0.00%
TM7; f_UC TM7-3		0.10%	
Tenericutes; f_Anaeroplasmataceae		0.40%	
Actinobacteria; f_Micrococcaceae		0.10%	
Bacteroidetes; f_UC Bacteroidales	0.10%		
Cyanobacteria; f_Pseudanabaenaceae		0.20%	
Proteobacteria; f_Rickettsiaceae		0.10%	
Proteobacteria; f_Oxalobacteraceae			
Proteobacteria; f_Desulfovibrionaceae	0.10%	0.10%	
Proteobacteria; f_Shewanellaceae		0.10%	
Proteobacteria; f_Halomonadaceae		0.10%	
Proteobacteria; f_Pseudomonadaceae		0.10%	
Proteobacteria; f_Xanthomonadaceae		0.10%	
Spirochaetes; f_Spirochaetaceae	0.10%		
Verrucomicrobia; f_Verrucomicrobiaceae		0.10%	

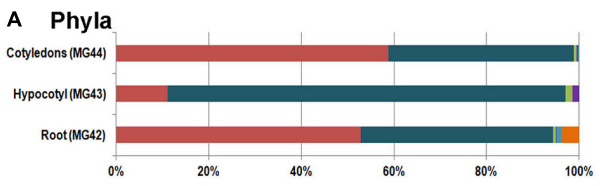


	Full embryos (MG38)	Embryo-base (MG39)	Embryo-cotyledon (MG40)
01. UC Ruminococcaceae (Firmicutes)	41.30%	24.30%	0.80%
02. UC Alcaligenaceae-UC (Betaproteobacteria)	1.10%	4.80%	49.30%
03. Achromobacter (Betaproteobacteria)	0.50%	2.50%	18.90%
04. UC Enterobacteriaceae (Gammaproteobacteria)	4.80%	4.00%	0.10%
05. UC Clostridiales (Clostridia)	10.50%	3.60%	0.20%
06. Kerstersia (Betaproteobacteria)	0.30%	1.50%	14.20%
07. UC Clostridiaceae (Clostridia)	1.70%	5.10%	0.10%
08. UC Lachnospiraceae (Clostridia)	3.30%	4.70%	3.30%
09. Faecalibacterium (Firmicutes)	2.90%	3.30%	0.10%
10. Blautia (Clostridia)	1.80%	3.00%	0.10%
11. Coprococcus (Clostridia)	3.00%	2.30%	0.60%
12. Tissierella_Soehngenia (Clostridia)	0.50%	2.00%	5.60%
13. Dialister (Clostridia)	5.30%	2.30%	0.00%
14. Proteus (Gammaproteobacteria)	0.60%	2.00%	4.40%
15. Ruminococcus (Clostridia)	2.00%	2.60%	0.10%
16. Clostridium (Clostridia)	0.60%	2.20%	0.10%
17. UC Coriobacteriaceae (Actinobacteria)	0.70%	2.80%	0.20%
18. Bacteroides (Bacteroidetes)	0.10%	2.00%	0.20%
19. Bifidobacterium (Actinobacteria)	0.30%	2.40%	0.10%
20. Roseburia (Clostridia)	0.70%	2.00%	0.10%
21. Prevotella (Bacteroidia)	0.30%	1.20%	0.10%
22. UC Peptostreptococcaceae (Clostridia)	0.30%	1.20%	0.40%
23. Oscillospira (Clostridia)	0.80%	1.00%	0.10%
24. Rhodospirillum (Betaproteobacteria)	0.10%	1.60%	0.10%
25. Lachnospira (Clostridia)	0.20%	1.40%	0.10%
26. Dorea (Clostridia)	0.20%	0.30%	0.10%
27. Anaerostipes (Clostridia)	4.50%	0.30%	
28. Lachnobacterium (Clostridia)	3.80%	0.20%	
29. Bulleidia (Erysipelotrichi)	0.30%	0.70%	
30. Catenibacterium (Erysipelotrichi)	0.30%	1.00%	
31. Collinsella (Coriobacteriia)	0.40%	0.80%	
32. Butyrivibrio (Clostridia)	1.60%	0.10%	
33. Lactobacillus (Bacilli)	0.10%	0.50%	
34. UC Christensenellaceae (Clostridia)	1.20%	0.10%	
35. UC Veillonellaceae (Clostridia)	0.00%	0.70%	
36. Mitsuokella (Clostridia)	0.10%	0.20%	
37. Streptococcus (Bacilli)	0.10%	0.50%	
38. Megasphaera (Clostridia)	0.10%	0.40%	
39. [Eubacterium] (Erysipelotrichi)	0.10%	0.30%	
40. Slackia (Coriobacteriia)	0.10%	0.10%	
41. UC Bacteroidales (Bacteroidia)	0.10%	0.00%	
42. Butyricimonas (Bacteroidia)	0.00%	0.20%	
43. [Prevotella] (Bacteroidia)	0.10%	0.10%	
44. UC Cyanobacteria (4C0d-2: Cyanobacteria)	0.10%	0.50%	
45. [Ruminococcus] (Clostridia)	0.10%	0.10%	
46. UC [Mogibacteriaceae] (Clostridia)	0.20%	0.10%	
47. Klebsiella (Gammaproteobacteria)	0.20%	0.30%	
48. Haemophilus (Gammaproteobacteria)	0.10%	0.30%	
49. UC Erysipelotrichaceae (Erysipelotrichi)	0.10%	0.10%	
50. Succinivibrio (Gammaproteobacteria)	0.20%		0.20%
51. UC Mollicutes (Mollicutes)	0.90%		
52. UC Bacteroidales (Bacteroidia)	0.10%		
53. Veillonella (Clostridia)	0.20%		
54. p-75-a5 (Erysipelotrichi)	0.10%		
55. Desulfovibrio (Deltaproteobacteria)	0.10%		
56. Treponema (Spirochaetes)	0.10%		
57. Sutterella (Betaproteobacteria)		2.60%	
58. Parabacteroides (Bacteroidia)		0.60%	
59. UC-TM7 (TM7-3)		0.10%	
60. UC-Anaeroplasmataceae (Mollicutes)		0.40%	
61. Rothia (Actinobacteria)		0.10%	
62. UC Rikenellaceae (Bacteroidia)		0.80%	
63. Enterococcus (Bacilli)		0.10%	
64. Alistipes (Bacteroidia)		0.10%	
65. Pseudanabaena (Synechococcophycidea)		0.20%	
66. Bilophila (Deltaproteobacteria)		0.10%	
67. Citrobacter (Gammaproteobacteria)		0.10%	
68. Halomonas (Gammaproteobacteria)		0.10%	
69. UC Pasteurellaceae (Gammaproteobacteria)		0.10%	
70. Pseudomonas (Gammaproteobacteria)		0.10%	
71. Akkermansia (Verrucomicrobiae)		0.10%	

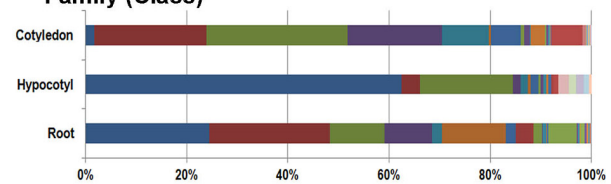
FIGURE 4 | Distribution of phylogenetic groups as per 16S rRNA metagene V3–V4 region taxonomic profiling of DNA derived from the whole excised seed embryos of watermelon "Arka Manik," seed embryo base and seed embryo cotyledons at phylum **(A)**, class **(B)**, and family **(C)** levels.

Bacteroidetes constituted a core microbiome of 96.9–99.6% OTUs in the four cultivars (**Figure 6A**). Minor shares of 17 other phyla were seen in one or more cultivars. At the class level, the four cultivars showed OTU distribution under 40 constituents (**Supplementary Dataset 2**) with 10 common classes constituting ≥ 95% OTUs (**Figure 6B**).

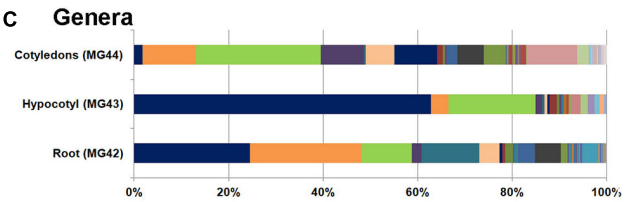
The OTUs were distributed under 23, 58, 26, and 72 families, respectively, for the above four cultivars with 16 core families constituting 98.1% ("Arka Manik"), 83.9% ("Arka Muthu"), 95.1% ("Madhubala"), and 61.9% ("SS-455") OTUs in them (**Figure 6C**). Alcaligenaceae, Clostridiaceae, Ruminococcaceae, and Lachnospiraceae were seen across the four cultivars as major families, while Xanthomonadaceae, Sphingomonadaceae, and a few other families accounted for the difference in "SS-455." Genus level distribution indicated a huge bacterial diversity spanning across 159 units with 19 core genera accounting for 75.4, 68, 69.3, and 57.7% OTUs, respectively, in the four cultivars (**Figure 6D**). The core genera included unclassified Alcaligenaceae, *Rhodospirillum* (β-Proteobacteria), *Proteus* (γ-Proteobacteria), and several unclassified groups. The maximum amount of diversity was observed under class Clostridia with 31 genera and 14 core genera. Only negligible amounts of spore-forming genera (0.1 *Bacillus* spp. in "Arka Manik," and 0.5% in "Arka Muthu," 0.2% *Paenibacillus* in "Arka Muthu," and 0.1% *Lactobacillus* in "SS-445") were documented in the embryo tissues.



	Root (MG42)	Hypocotyl (MG43)	Cotyledons (MG44)
Firmicutes	52.80%	11.20%	58.80%
Proteobacteria	41.60%	85.80%	40.00%
Actinobacteria	0.60%	1.50%	0.50%
Fusobacteria	0.10%	1.50%	0.30%
Bacteroidetes	1.20%		0.30%
Tenericutes	3.50%		
Acidobacteria	0.10%		
Gemmatimonadetes	0.10%		



	Root	Hypocotyl	Cotyledon
Halomonadaceae (Gammaproteobacteria)	24.60%	62.40%	1.80%
Ruminococcaceae (Clostridia)	23.80%	3.60%	22.00%
Alcaligenaceae (Betaproteobacteria)	10.90%	18.30%	27.80%
Lachnospiraceae (Clostridia)	9.40%	1.50%	18.70%
UD Clostridiales (Clostridia)	2.00%	1.50%	9.10%
Erysipelotrichaceae (Erysipelotrichi)	12.60%	0.50%	0.50%
Clostridiaceae (Clostridia)	2.00%	1.50%	5.90%
UD RF39 (Mollicutes)	3.50%	0.00%	0.00%
Streptococcaceae (Bacilli)	1.70%	0.50%	0.60%
[Tissierellaceae] (Clostridia)	0.10%	0.50%	1.20%
Peptostreptococcaceae (Clostridia)	0.70%	0.50%	0.10%
Nocardioidaceae (Actinobacteria)	0.20%	0.50%	2.80%
Veillonellaceae (Clostridia)	0.20%	0.50%	
Comamonadaceae (Betaproteobacteria)	0.00%	0.50%	
Enterobacteriaceae (Gammaproteobacteria)	5.60%		0.30%
Prevotellaceae (Bacteroidia)	0.40%		0.40%
Christensenellaceae (Clostridia)	0.20%		0.30%
Oxalobacteraceae (Betaproteobacteria)	0.10%		0.10%
Pasteurellaceae (Gammaproteobacteria)	0.10%		0.10%
Corynebacteriaceae (Actinobacteria)		1.00%	6.20%
Bacteroidaceae (Bacteroidia)	0.80%		
Coriobacteriaceae (Coriobacteriia)	0.40%		
Fusobacteriaceae (Fusobacteriia)	0.10%		
UD Alphaproteobacteria (Alphaproteobacteria)	0.20%		
UD Acidobacteria (iii1-8)	0.10%		
[Odoribacteraceae] (Bacteroidia)	0.10%		
Paenibacillaceae (Bacilli)	0.10%		
Turicibacteraceae (Bacilli)	0.10%		
UD Gemmatimonadetes (Gemmatimonadete)	0.10%		
Brucellaceae (Alphaproteobacteria)	0.10%		
Methylobacteriaceae (Alphaproteobacteria)	0.10%		
Sphingomonadaceae (Alphaproteobacteria)			0.70%
Xanthomonadaceae (Gammaproteobacteria)			0.20%
Bifidobacteriaceae (Actinobacteria)			0.10%
Porphyromonadaceae (Bacteroidia)			0.10%
S24-7 (Bacteroidia)			0.10%
Bacteriovoracaceae (Deltaproteobacteria)			0.00%
Enterococcaceae (Bacilli)			0.10%
Lactobacillaceae (Bacilli)			0.10%
Campylobacteraceae (Epsilonproteobacteria)			0.10%
Alteromonadaceae (Gammaproteobacteria)			0.10%
Idiomarinaceae (Gammaproteobacteria)			0.10%
Alcanivoracaceae (Gammaproteobacteria)			0.10%
Shewanellaceae (Gammaproteobacteria)		2.00%	
Leptotrichiaceae (Fusobacteriia)		1.50%	
Bradyrhizobiaceae (Alphaproteobacteria)		1.50%	
Moraxellaceae (Gammaproteobacteria)		1.00%	
[Mogibacteriaceae] (Clostridia)		0.50%	

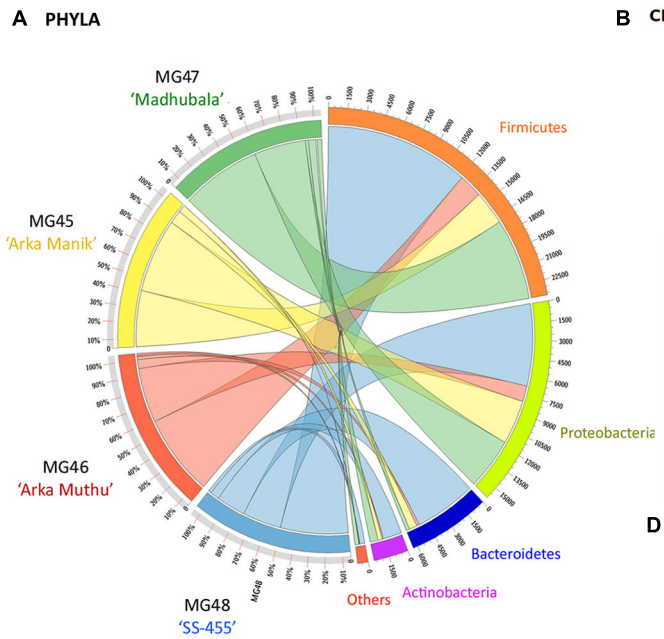


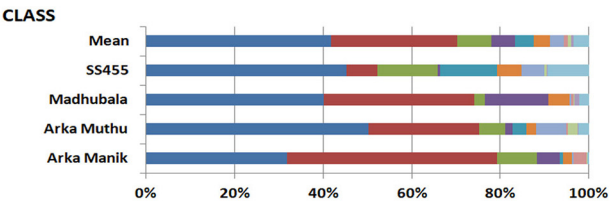
	Root (MG-42)	Hypocotyl (MG-43)	Cotyledons (MG-44)
Halomonas (Proteobacteria)	24.60%	62.40%	1.80%
UD Ruminococcaceae (Firmicutes)	23.70%	3.60%	11.20%
Achromobacter (Proteobacteria)	10.70%	18.30%	26.10%
UD Clostridiales (Firmicutes)	2.00%	1.50%	9.10%
[Eubacterium] (Firmicutes)	12.30%	0.50%	0.40%
Coprococcus (Firmicutes)	4.40%	0.50%	6.00%
[Ruminococcus] (Firmicutes)	0.60%	0.50%	8.90%
UD Clostridiaceae (Firmicutes)	0.50%	1.50%	1.20%
Streptococcus (Firmicutes)	1.70%	0.50%	0.50%
Tissierella_Soehngenia (Firmicutes)	0.10%	0.50%	0.40%
UD Peptostreptococcaceae (Firmicutes)	0.70%	0.50%	
Dialister (Firmicutes)	0.20%	0.50%	
Dorea (Firmicutes)	3.70%		2.20%
UD Enterobacteriaceae (Proteobacteria)	5.50%		5.50%
Clostridium (Firmicutes)	1.40%		4.60%
UD Lachnospiraceae (Firmicutes)	0.20%		0.20%
Bacteroides (Bacteroidetes)	0.80%		0.10%
UD Coriobacteriaceae (Actinobacteria)	0.30%		0.30%
Anaerostipes (Firmicutes)	0.60%		0.10%
UD Alcaligenaceae (Proteobacteria)	0.10%		0.80%
Kerstersia (Proteobacteria)	0.20%		0.50%
Proteus (Proteobacteria)	0.10%		0.50%
UD Christensenellaceae (Firmicutes)	0.20%		0.10%
Oscillospira (Firmicutes)	0.10%		0.20%
Fusobacterium (Fusobacteria)	0.10%		0.30%
Blautia (Firmicutes)		0.50%	1.20%
UD [Mogibacteriaceae] (Firmicutes)		0.50%	0.10%
Prevotella (Bacteroidetes)	0.40%		
UD RF39 (Tenericutes)	3.50%		
Propionicimonas (Actinobacteria)	0.20%		
Bulleidia (Firmicutes)	0.20%		
UD Alphaproteobacteria (Proteobacteria)	0.20%		
UD iii1-8 (Acidobacteria)	0.10%		
Collinsella (Actinobacteria)	0.10%		
Butyricimonas (Bacteroidetes)	0.10%		
Paenibacillus (Firmicutes)	0.10%		
Turicibacter (Firmicutes)	0.10%		
Christensenella (Firmicutes)	0.10%		
UD Gemmatimonadetes (Gemmatimonadetes)	0.10%		
Pseudochrobactrum (Proteobacteria)	0.10%		
Methylobacterium (Proteobacteria)	0.10%		
Oxalobacter (Proteobacteria)	0.10%		
Pasteurellaceae (Proteobacteria)	0.10%		
Shewanella (Proteobacteria)		2.00%	
UD Leptotrichiaceae (Fusobacteria)		1.50%	
Balneimonas (Proteobacteria)		1.50%	
Corynebacterium (Actinobacteria)		1.00%	
Acinetobacter (Proteobacteria)		1.00%	
UD Nocardioidaceae (Actinobacteria)		0.50%	
Ruminococcus (Firmicutes)			10.60%
Pseudomonas (Proteobacteria)			2.50%
Bifidobacterium (Actinobacteria)			0.10%
Anaerovorax (Firmicute)			0.30%
UD [Tissierellaceae] (Firmicutes)			0.20%
Sedimentibacter (Firmicutes)			0.50%
Rhodospirillum (Proteobacteria)			0.50%
UD Pseudomonadaceae (Proteobacteria)			0.30%
Wohlfahrtiimonas (Proteobacteria)			0.60%
Scardovia (Actinobacteria)			0.10%
UD Porphyromonadaceae (Bacteroidetes)			0.10%
Vagococcus (Firmicutes)			0.10%
Lactobacillus (Firmicutes)			0.10%
Lactococcus (Firmicutes)			0.10%
Janthinobacterium (Proteobacteria)			0.10%
Arcobacter (Proteobacteria)			0.10%
Candidatus Endobugula (Proteobacteria)			0.10%
Pseudidiomarina (Proteobacteria)			0.10%
Klebsiella (Proteobacteria)			0.10%
Alcanivorax (Proteobacteria)			0.10%
UD Xanthomonadaceae (Proteobacteria)			0.10%

FIGURE 5 | Distribution of phylogenetic groups as per 16S rRNA metagene V3–V4 region taxonomic profiling of DNA derived from the root, hypocotyls, and cotyledon tissues of 2-week old *in vitro* grown seedlings of watermelon "Arka Manik" at phylum **(A)**, family **(B)**, and genus **(C)** levels.

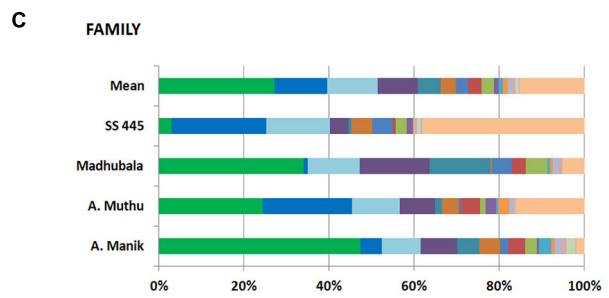
DISCUSSION

Seeds in dicotyledonous plants grow to maturity well protected inside fruit tissues. Under natural conditions, the fruit degenerates releasing the mature seeds to the environment. Under organized cultivation, the seeds are processed and utilized soon after, or maintained in storage. Thus, the seeds could bear the organisms inherited from the mother plant, if any, besides those acquired from environment (Nelson, 2018; Rodríguez et al., 2018). As the seed germinates, microorganisms from three sources get to associate with it: (i) external microbiota from the seed spermosphere, (ii), seed internal microbiome from the testa and perisperm, and (iii), the true embryo colonizers (Nelson, 2018). While the former two categories of microorganisms could converge as endophytes in new seedlings, the microbiome transmitted from the parent through the ovum plus that contributed by pollen (Maniranjan et al., 2017) which reaches the embryo alone could qualify as vertically transmitted organisms that are certain to be passed on to the seedlings (Abdelfattah et al., 2021). The present investigations reveal a vast diversity of bacterial phyla and genera prevailing inside watermelon seed embryos with clear vertical transmission prospects and assured colonization of new seedling (Abdelfattah et al., 2021). The quantum of such deep-seated cultivation-recalcitrant microbes

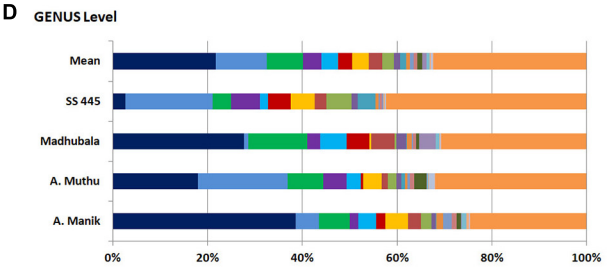




	Arka Manik	Arka Muthu	Madhubala	SS455	Mean
1. Clostridia	31.90%	50.30%	40.10%	45.30%	41.90%
2. Betaproteobacteria	47.40%	25.00%	34.10%	6.90%	28.35%
3. Bacteroidia	8.90%	5.80%	2.40%	13.60%	7.68%
4. Erysipelotrichi	5.30%	1.70%	14.20%	0.60%	5.45%
5. Gammaproteobacteria	0.60%	3.10%	0.10%	12.80%	4.15%
6. Actinobacteria	2.00%	2.10%	4.80%	5.60%	3.63%
7. Alphaproteobacteria	0.40%	6.80%	0.30%	5.10%	3.15%
8. Coriobacteriia	2.90%	0.50%	0.60%	0.10%	1.03%
9. Bacilli	0.20%	2.10%	0.20%	0.50%	0.75%
10. Mollicutes	0.10%	0.30%	1.00%	0.20%	0.40%
11. Others	0.30%	2.30%	2.20%	9.30%	3.53%



	A. Manik	A. Muthu	Madhubala	SS 445	Mean
1. Alcaligenaceae (Burkholderiales)	47.30%	24.40%	34.10%	3.00%	27.20%
2. Clostridiaceae (Clostridiales)	5.10%	21.00%	1.00%	22.30%	12.35%
3. Lachnospiraceae (Clostridiales)	9.20%	11.30%	12.10%	14.90%	11.88%
4. Ruminococcaceae (Clostridiales)	8.50%	8.20%	16.50%	4.40%	9.40%
5. Erysipelotrichaceae(Erysipelotrichales)	5.30%	1.70%	14.20%	0.60%	5.45%
6. Bacteroidaceae (Bacteroidales)	4.80%	3.90%	0.40%	5.00%	3.53%
7. Bifidobacteriaceae (Bifidobacteriales)	1.90%	0.50%	4.70%	4.90%	3.00%
8. [Tissierellaceae] Clostridiales	3.90%	4.50%	3.20%	0.60%	3.05%
9. f_(Clostridiales)	2.80%	1.30%	5.00%	2.50%	2.90%
10. Enterobacteriaceae (Enterobacteriales)	0.50%	2.40%	0.10%	1.50%	1.13%
11. Coriobacteriaceae (Coriobacteriales)	2.90%	0.50%	0.60%	0.10%	1.03%
12. Peptostreptococcaceae (Clostridiales)	0.90%	2.60%	0.60%	0.10%	1.05%
13. Veillonellaceae (Clostridiales)	1.40%	0.90%	1.40%	0.10%	0.95%
14. S24-7 (Bacteroidales)	1.40%	0.50%	1.00%	0.70%	0.90%
15. Porphyromonadaceae (Bacteroidales)	2.10%	0.10%	0.10%	0.80%	0.78%
16. [Mogibacteriaceae] (Clostridiales)	0.10%	0.10%	0.10%	0.40%	0.18%
17. Others	1.90%	16.10%	4.90%	38.10%	15.25%



	A. Manik	A. Muthu	Madhubala	SS 445	Mean
01. c_Betaproteobacteria; f_Alcaligenaceae; g_	38.60%	17.90%	27.70%	2.60%	21.70%
02. c_Clostridia; f_Clostridiaceae; g_	4.90%	19.00%	0.90%	18.50%	10.83%
03. c_Clostridia; f_Ruminococcaceae; g_	6.50%	7.50%	12.50%	3.90%	7.60%
04. c_Clostridia; f_Lachnospiraceae; g_	1.80%	4.90%	2.70%	6.10%	3.88%
05. c_Clostridia; g_Roseburia	3.80%	3.00%	5.60%	1.60%	3.50%
06. c_Actinobacteria; g_Bifidobacterium	1.90%	0.50%	4.70%	4.90%	3.00%
07. c_Bacteroidia; g_Bacteroides	4.80%	3.90%	0.40%	5.00%	3.53%
08. c_Clostridia ;f_;g_	2.80%	1.30%	5.00%	2.50%	2.90%
09. c_Clostridia; g_Blautia	2.10%	1.80%	0.40%	5.30%	2.40%
10. c_Clostridia; g_Coprococcus	0.90%	1.10%	2.10%	1.30%	1.35%
11. c_Clostridia; g_Clostridium	0.20%	0.80%	0.10%	3.70%	1.20%
12. c_Bacteroidia; f_S24-7; g__	1.40%	0.50%	1.00%	0.70%	0.90%
13. c_Clostridia; g_Oscillospira	1.80%	0.50%	0.30%	0.30%	0.73%
14. c_Betaproteobacteria; g_Rhodospirillum	1.10%	0.90%	0.60%	0.30%	0.73%
14. c_Clostridia; f_Peptostreptococcaceae; g_	0.90%	2.60%	0.60%	0.10%	1.05%
15. c_Clostridia ;g__Ruminococcus	0.10%	0.20%	3.50%	0.20%	1.00%
16. c_Clostridia; g__Megasphaera	1.10%	0.20%	0.80%	0.10%	0.55%
17. c_Clostridia; g_Dorea	0.50%	0.10%	0.20%	0.30%	0.28%
18. c_Gammaproteobacteria; g_Proteus	0.10%	1.20%	0.10%	0.20%	0.40%
19. c_Clostridia; f_[Mogibacteriaceae];g_	0.10%	0.10%	0.10%	0.10%	0.10%
20. Others	24.60%	32%	30.70%	42.30%	32.40%

FIGURE 6 | Distribution of phylogenetic groups as per 16S rRNA metagene V3–V4 region taxonomic profiling of DNA derived from the surface-sterilized seed embryos of four watermelon cultivars at phylum **(A)**, class **(B)**, family **(C)**, and genus **(D)** levels.

elucidated in this study is much higher than the generally documented diversity (Hameed et al., 2015; Robinson et al., 2016; Adam et al., 2018) in line with the recent report on oak seed embryos which also demonstrate the differential transmittance of microbiome to the roots and phyllosphere of new seedlings (Abdelfattah et al., 2021). These intimately plant-associated microbes will hold considerable significance in deciding the colonization competence, survival, and functioning of the native rhizospheric microbiome as well as any laterally applied inocula.

The major limitation while studying the vertical transmission of endophytic bacteria is the intricacy in dissecting out the embryos distinctly and growing the seedlings away from

environmental microorganisms. Several past studies have addressed the seed microbiome and seed endophytes most of which employed mere surface-sterilized seeds with no discrimination between the embryo and the external tissues (Khalaf and Raizada, 2016; Bergna et al., 2018). Most plant species that have been the subjects of seed microbiome studies showed the firm attachment of embryo to the seed coat such as in *Arabidopsis*, legumes, maize, cotton, brassicas, tobacco, etc. Microscopic observations in this study have shown a substantial bacterial load in watermelon seed testa and perisperm tissues even after extensive surface sterilization steps. Monitoring the wash solutions and seed imprints in this study ensured effective surface sterilization. Past cultivation-based studies on embryos excised from surface-sterilized seeds often showed a significant amount of spore-forming bacteria (Truyens et al., 2015; Nelson, 2018). The re-disinfected embryos in this study displayed no readily cultivable bacteria on two common bacteriological media for a reasonable period of 1 week which indicated that the organisms survived as CREBs.

Considering the literature available on seed microbiome, very few studies have considered the seed embryo tissues distinct from other seed parts. Analyzing the seed endosphere of four rice cultivars after surface sterilization of de-hulled seeds (without discriminating embryo and endosperm tissues) through cultivation versus cultivation-independent PCR-denaturing gradient gel electrophoresis (DGGE), Hameed et al. (2015) observed a total of 20 distinct PCR-DGGE bands most of which corresponded to γ-Proteobacteria (50%) followed by *Bacilli* (25%) and β-Proteobacteria (10%). In wheat, where the embryo and endosperm were separated out, Robinson et al. (2016) found the bacterial association only with the endosperm and not in the embryo. Cultivation-independent analysis of bacterial biome distinctly in the endosperm and germ tissues of wheat seeds showed several beneficial bacterial genera in the endosperm tissues and a lesser diversity in the germ tissues (Kuźniar et al., 2020a). Further exploration on seeds of different wheat cultivars demonstrated the embryo transmission of about 20 genera with 10 genera shared with the 35 genera documented for the endosperm (Kuźniar et al., 2020b) and the differential distribution of various genera in different organs (root, coleoptile, and leaf) of field plants (Kuźniar et al., 2020a,b). The easy decoating of watermelon seeds unlike wheat seeds which needed extended water-soaking for the embryo dissection (with possible modifications in microbial profiles), excluding the maternal perisperm membrane, allowed a clear conclusion on vertical transmission of diverse endophytic bacteria. The study employing oak acorn excised embryos and seedlings raised under special conditions showed a high microbial diversity and spatial partitioning of fungal and bacterial communities within both seed and seedling indicating vertical inheritance, niche differentiation, and divergent transmission routes for the establishment of root and phyllosphere communities (Abdelfattah et al., 2021). The observations also amend the present understanding depicting soil/environment as the main source of endophytic microorganisms.

This study was directed at bringing out the maximum amount of taxonomic diversity including the minor OTUs to understand the true seed transmission of endophytes rather than the common practice of elucidating the functional aspects of major associates. Firmicutes formed the dominant phylum inside the dry seed embryos followed by Proteobacteria, Actinobacteria, and Bacteroidetes along with minor shares of a number of other phyla including several candidate phyla that lack the cultured relatives. On the other hand, embryos from seeds which were not in long-term storage showed a predominance of Proteobacteria. This appeared the case for freshly harvested seeds which did not go through the desiccation (Thomas, unpublished data). This indicated that during seed drying, the share of Proteobacteria goes down leading to a larger share of Firmicutes. Class Clostridia formed the major constituent under Firmicutes which was understandable considering the anaerobic conditions prevailing inside dry seeds. *In vitro* grown seedlings showed a different phylogenetic profile from seed embryos indicating a taxonomic realignment with seed germination and seedling growth and a variable distribution within different seedling parts. The variable taxonomic profiles for the embryos of different watermelon cultivars and for different seedling parts suggested the prevalence of a much higher diversity of bacterial biome which is perhaps dynamic and varying as per the growth phase or the prevailing conditions such as fresh or older seeds, dry or soaked seeds, etc.

Spore-forming bacterial genera are commonly documented as seed-associated organisms and often considered as seed endophytes in cucurbits (Khalaf and Raizada, 2016) and other crops (Truyens et al., 2015; Nelson, 2018; Kuźniar et al., 2020b). Considering the low moisture content and the high osmotic potential on and inside dry seeds, endospore formation is considered as an important feature for seed colonizers (Truyens et al., 2015; Cope-Selby et al., 2017; Shahzad et al., 2018). Cultivation-based studies on seed testa and perisperm tissues of watermelon in this investigation indicated a huge share of *Bacillus*/other spore formers (often documented with the surface-sterilized seeds) which clearly emanated from seed external tissues. Deep-sequencing studies on seed embryos showed only very negligible share of spore-forming genera (≤1%) across different watermelon cultivars similar to the 16S rRNA V4 profiling study on surface-washed seeds of different pumpkin genotypes where *Bacillus* sp. constituted a very minor (∼1%) share (Adam et al., 2018). Molecular studies on tomato seeds also showed *Bacillus* spp. as a minor constituent, while *Bacillus* and *Paenibacillus* spp. formed major cultivable bacteria (Bergna et al., 2018; Thomas and Shaik, 2020). Deep-sequencing-based studies on surface-sterilized tomato seeds further showed a huge bacterial taxonomic diversity with very identical OTU profiles for two cultivars, while spore-forming Firmicutes formed only a very low share (Thomas and Shaik, 2020). Thus, the observations with the excised cucurbit embryos suggested that spore-forming genera do not form major vertically transmissible organisms.

It was also worth noting the survival of diverse genera of Gram-negative Proteobacteria and Bacteroidetes and non-spore-forming Actinobacteria inside dry embryos under high desiccation. Several bacteria are known to enter viable but non-cultivable (VBNC) state with them turning cultivable upon the return of suitable conditions (Barer et al., 1993; Ramamurthy et al., 2014). The term "cultivation recalcitrant

endophytic bacteria" (Thomas and Shaik, 2020) best describes such endophytic bacteria, some of which could be brought to cultivation with specialized media or in the presence of host tissue constituents (Thomas, 2011; Thomas and Franco, 2021). It is common to observe the activation of normally uncultivable bacteria to cultivation during micropropagation or other tissue culture applications as documented with banana (Thomas et al., 2008), watermelon (Thomas, 2011), etc. Recent observations with tomato seeds cultured *in vitro* where the seed coat was removed post-germination showed the gradual activation of different bacteria that constituted mainly Gram-negative Proteobacteria and Bacteroidetes and some Gram-positive non-spore-forming Actinobacteria (Shaik and Thomas, 2019). Cultivation-independent 16S V3–V4 taxonomic profiling on surface-sterilized seeds of tomato had shown a huge diversity of CREB which included mainly Proteobacteria followed by Firmicutes, Actinobacteria, and Bacteroidetes. *Bacillus* and other spore formers appeared as predominant seed external associates with a very minor share of OTUs recorded in molecular analysis (Thomas and Shaik, 2020).

How the organisms reach the inside of the seeds is a vital aspect. Endophytes are known to gain entry inside plants mainly through roots and through natural openings and wounds from phyllosphere and other aerial plant parts from where they colonize the vascular stream and reach various plant organs (Compant et al., 2011; Hardoim et al., 2015). Bacterial endophytes are considered to be transmitted inside seeds from vegetative parts through various routes such as vascular connections traversing the micropyle, colonizing the shoot meristems that transforms to floral parts, through horizontal movement inside the fruits, or through pollen (Truyens et al., 2015; Berg and Raaijmakers, 2018; Nelson, 2018). Obligate and strict vertical transfer of bacteria is considered unlikely in plants (Frank et al., 2017). In melon, some amount of bacteria is considered to enter the fruit from vegetative parts and from there to seeds in the early stages of seed development wherein the thin envelope enclosing the embryo is considered to act as a barrier for bacteria in the later stages of seed maturation (Khalaf and Raizada, 2016).

Transmission of endophytic bacteria through pollen has been established in different plant species (Maniranjan et al., 2017), which allows their direct passage to the embryo. In our assessment, vertical transmission through gametes or seeds essentially needs the organisms to be able to colonize the intracellular niche of tissues that contribute to pollen or ovum development. Endophytic bacteria are considered primarily colonizers in the intercellular region (Hardoim et al., 2015; Alibrandi et al., 2018). Microscopic observations on banana and papaya have indicated abundant cytoplasmic colonization by endophytic bacteria with their terming as "Cytobacts" (Thomas and Sekhar, 2014; Thomas et al., 2019) in which case the intracellular bacteria could move to the gametophytes through mitosis and meiosis. Intracellular bacteria have also been documented in the meristem of pine (Pirttilä et al., 2000) that could move to floral tissues and reproductive units (Frank et al., 2017) and also in axenically grown pineapple and orchids (Esposito-Polesi et al., 2017). More recent studies implying cell cultures of grapevine and other plant species have shown abundant and diverse Cytobacts across plant species with their origin ascribable to the field source tissues (Thomas and Franco, 2021) with clear indication of vertical transmission across generations (Thomas et al., unpublished data). It is also possible that bacteria from the stigma get incorporated to the embryo at fertilization (Mitter et al., 2017).

The seed embryo microbiomes are likely participating in various plant processes including growth promotion, host defense, and metabolic pathways. It is understood that functional elucidation of seed endophytes is not practically easy considering that the associated organisms are diverse and uncultivable, their dynamic and variable nature, inability to focus on single organisms at the exclusion of others, and variable population structure depending on the organ and the developmental stage of seedlings (Shaik and Thomas, 2019). The functional elucidations would warrant concerted efforts by different research groups. The recent report that the individual seeds of bean and radish were associated with a dominant bacterial taxon which in turn was highly variable between plants and within seeds of the same plant is worth noting (Chesneau et al., 2021). The embryo-associated bacteria have the advantage of being able to establish and spread to different parts of seedlings at germination before the externally associated microorganisms make their way inside seedlings. Some endophytic bacteria can also get out of the plant and colonize the rhizosphere (Johnston-Monje and Raizada, 2011), which also applies to embryo-derived endophytes (Abdelfattah et al., 2021). The interactive effects between the internal versus external organisms at seed germination, with seedling growth and the selective acquisition of organisms from the spermosphere or rhizosphere, are worthy of in-depth investigations.

It is now certain that the embryos are coming packaged with a series of endophytic bacteria which certainly have the edge over external organisms (Khalaf and Raizada, 2016). Seed-associated microbes can improve seed germination, promote seedling health, enhance plant growth, and mitigate stress (Shahzad et al., 2018). As the concept of holobiome highlights the inseparable significance of the microbiome in developmental and other physiological behavior of the individual (Kim and Lee, 2020), which has been well proven by the studies on the gut microbiome, the phytobiome could be a panacea for solving the emerging problems in crop production. Keeping in mind that plant microbiome is proposed as a platform for realizing the next green revolution (Rodriguez and Durán, 2020), deciphering the native/seed transmitted endophytes could be of greater significance. Thus, this study would be pivotal in widening our understanding of the structure and transmission of plant microbiome and gathering insights for their roles in plant growth and health promotion.

In summary, the deep sequencing and microscopy-based investigations on watermelon seed embryos revealed abundant and enormously diverse bacteria colonizing the seed embryo tissues and transmitted to the seedlings, and in all probability vertically to the next cycle. The extent of bacterial diversity documented within the seed embryos clearly excluding the seed coat parts was unprecedented unlike as documented in the earlier published reports. The results here indicate that the seeds/embryos come packaged with their microbiome which spread to different parts of the developing seedling/plant unlike

the earlier understanding that the plants mostly acquired the desirable endophytic microorganisms from the soil/rhizosphere. This embryo colonization by bacteria might be facilitated by the intracellularly associated diverse "Cytobacts." It calls for more in-depth investigations to understand the entry routes of endophytes inside seed embryos and how the organisms get activated/multiply and distribute themselves to the root/shoot tissues of the new plant besides their functional roles. The well-protected seed embryos inside the seed coat in watermelon and other cucurbits with their prominent embryos and the feasibility of removing the testa form ideal candidates to study vertically transmitted bacterial endophytes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

PT: conceiving the idea, conduct of the experiments, data analysis and interpretation, and manuscript preparation. PS: undertaking confocal microscopy and FISH (fluorescent *in situ* hybridization). Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.635810/full#supplementary-material

Supplementary Figure 1 | Assessing seed external bacterial load and the efficacy of three surface sterilization treatments (ethanol treatment; ethanol + NaOCl, ethanol + $HgCl_2$) through bacterial monitoring on nutrient agar (NA) after different treatment steps with *Bacillus/other* spore-forming bacteria detected after different steps. **(A)** SP-SDS of seed first wash solution at the rate of 100 μl per seed, with the lowest dilution on the top left side and highest dilutions on the right lower side **(B)** monitoring of six washes through spotting 10 μl solutions, **(C)**, bacterial monitoring after ethanol step and five washes, **(D)** SP-SDS of seed testa homogenate, **(E)** SP-SDS of excised embryos dispersed in FDW, **(F)** monitoring the six sequential washes of excised embryos with perisperm, and **(G)** SP-SDS of embryo homogenate.

Supplementary Figure 2 | Seed embryo homogenate from surface-sterilized seed embryos of watermelon under phase contrast (100× objective) displaying starch grain aggregates (s), large phase bright plastids of > 5 μm size (p) and mitochondria of 2–3 μm size (m) along with fine bacterial cells of ≤ 1 μm (b) in the background.

Supplementary Table 1 | Data Statistics for watermelon Seed Embryos-Bulk (MG38), Embryo base (MG39) and Embryo cotyledons (MG40) from 40 seeds each as per QIIME analysis I and II.

Supplementary Table 2 | DNA yields and 16S rRNA V3-V4 region based Illumina sequencing and QIIME- bioinformatics analysis on four watermelon cultivars.

Supplementary Dataset 1 | OTU abundance at genera level in watermelon seed embryo (MG37) and *in vitro* derived seedlings (MG41).

Supplementary Dataset 2 | OTU abundance at class level in different cultivars of watermelon.

Supplementary Movie 1 | Bright field microscopy (1000×) on the original seed-vortexed FDW (100 μl per non-disinfected seed) showing few mobile bacterial cells (10 μl sample loaded under a 22 × 22 mm cover-glass).

Supplementary Movie 2 | Bright field microscopy (1000×) on the seed-testa homogenate (50 mg ml^{-1}) from surface sterilized seeds displaying abundant motile bacterial cells (10 μl sample loaded under a 22 × 22 mm cover-glass).

Supplementary Movie 3 | Bright field microscopy (1000×) on perisperm tissue from surface sterilized seeds (one per 50 μl) displaying abundant motile bacterial cells (10 μl sample loaded under a 22 × 22 mm cover-glass).

Supplementary Movie 4 | Razor thin tissue sections prepared from re-surface sterilized embryos (post 1–2 h FDW-soak) exhibiting copious motile bacterial cocci inside and around the disturbed tissues.

Supplementary Movie 5 | Milky tissue homogenate from surface sterilized embryos of 'Arka Manik' watermelon (10 mg ml^{-1}) displaying profuse motile bacteria along with passively moving plastids and mitochondria under bright field microscopy (1000×).

Supplementary Movie 6 | Confocal Movie-microscopy on SYTO-9 stained seed-embryo sections showing abundant bacteria in the intracellular matrix across different vertical planes.

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The remaining author declares 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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Genetic Diversity of the Symbiotic Fungus *Epichloë festucae* in Naturally Occurring Host Grass Populations

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Epichloë festucae is a common symbiont of the perennial and widely distributed cool season grass, *Festuca rubra*. The symbiosis is highly integrated involving systemic growth of the fungus throughout above-ground host parts and vertical transmission from plant to its offspring *via* host seeds. However, the nature of symbiosis is labile ranging from antagonistic to mutualistic depending on prevailing selection pressures. Both the loss of fungus in the maternal host lineage and horizontal transmission through sexual spores within the host population may partly explain the detected variation in symbiosis in wild grass populations. Epichloë species are commonly considered as pathogens when they produce sexual spores and partly castrate their host plant. This is the pathogenic end of the continuum from antagonistic to mutualistic interactions. Here we examined the population genetic structure of *E. festucae* to reveal the gene flow, importance of reproduction modes, and alkaloid potential of the symbiotic fungus in Europe. *Epichl*oë-species are highly dependent on the host in survival and reproduction whilst benefits to the host are largely linked to defensive mutualism attributable to fungal-origin bioactive alkaloids that negatively affect vertebrate and/or invertebrate herbivores. We detected decreased genetic diversity in previously glaciated areas compared to non-glaciated regions during the last glacial maximum period and found three major genetic clusters in *E. festucae* populations: southern, northeastern and northwestern Europe. Sexual reproduction may have a higher role than expected in Spanish *E. festucae* populations due to the predominance of unique genotypes and presence of both mating types in the region. In contrast, asexual reproduction *via* host seeds predominates in the Faroe Island and Finland in northern Europe due to the presence of biased mating-type ratios and large dominant genotypes in the *E. festucae* populations within the region. A substantially larger variation of alkaloid genotypes was observed in the fungal populations than expected, although the variability of the alkaloid genotypes within populations is considerably lower in northern than Spanish populations in southern Europe. *E. festucae* populations consist of different combinations of alkaloid classes from the gene clusters of ergot alkaloid and indole-terpenes, and from pyrrolopyrazine

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alkaloid gene. We suggest that the postglacial distribution history of the host grass, prevailing reproduction strategies of *E. festucae*, and local selection pressures likely explain a large part of the genetic variation observed in fungal populations among geographic regions. The identified alkaloid genotypes can be used by turfgrass breeders to improve resistance against herbivores in red fescue varieties and to develop new sustainable cultivars in Europe.

Keywords: alkaloid production, *Epichloë festucae*, *Festuca rubra*, ergot alkaloid, indole-diterpene, pyrrolpyrazine, reproductive modes, genetic population structure

INTRODUCTION

Microbes are ubiquitous and involved in all biogeochemical processes supporting the evolutionary trajectories from the origin of life. Earliest free-living microbes enabled terrestrial life on Earth by producing atmospheric oxygen (Wellman and Strother, 2015), and since then reciprocal adaptations and counter adaptations between microbes and higher organisms have driven adaptive radiation of species (Janzen, 1980; Thompson, 1994, 2005; Saikkonen et al., 2020). As adaptive capacity of microbes is unparalleled, microbes still sustain and organize biodiversity globally. For example, plant symbiotic bacteria and fungi are vital for host plant fitness during the primary succession as well as in hostile and extreme environments (Zilber-Rosenberg and Rosenberg, 2008; Nissinen et al., 2012; Kumar et al., 2017). Many of these symbiotic interactions are mutually beneficial and characterized by evolutionary outcomes such as species-specificity and vertical transmission of the microbial partner from host plant to its offspring, which will in turn select for benign association. However, vertically transmitted microbes could have a greater chance of destabilizing or constraining the symbiosis because it is often associated with the loss of contagious spread and the independent phase of the life cycle. Loss of contagious spread by sexual spores results in genetic host specificity, decreased sexual reproduction and recombination potential, and increased genetic uniformity of the microbe (Frank, 1994, 1996a,b; Nowak et al., 1994; Doebeli and Knowlton, 1998; Herre et al., 1999; Saikkonen et al., 1999, 2004). Thus, interactions between hereditary microbes and plants are context dependent and ranging from antagonistic to mutualistic (Saikkonen et al., 1998, 2010a).

Here we examine the genetic structure of fungal symbiont, *Epichloë festucae* Leuchtmann, Schardl, and Siegel (Clavicipitaceae, Hypocreales, Ascomycota), commonly associated with the widely distributed cool-season perennial grass, *Festuca rubra* L. (Poaceae, subfamily Pooideae) (Dirihan et al., 2016; von Cräutlein et al., 2019). Transmission and reproductive modes of *E. festucae* provide unique opportunities to study how life history traits of the fungal partner may translate into adaptable genetics determining the ecology of symbiotum. A single filamentous *E. festucae* genotype typically forms systemic and asymptomatic association throughout the above ground parts of the host plant, including the developing seeds (Clay and Schardl, 2002; Tadych et al., 2014). In addition to asexual distribution *via* vertical transmission to the seeds, *E. festucae* can occasionally spread horizontally by sexual spores within grass populations (Clay and Schardl, 2002). A similar mixed strategy involving both sexual and asexual reproduction is characteristic of numerous haploid fungal symbiotic microorganisms (Milgroom, 1996, 1997). As *E. festucae* is a heterothallic obligate out-crosser with two different mating types, fertilization requires dispersal of spermatia (male gametes) to an unfertilized fruiting body, a stroma, of opposite mating type vectored by phylogenetically distinct clade of anthomyiid flies (*Botanophila* ssp.) (Leuchtmann and Michelsen, 2016). This allows sexual crossing and perithecial development on the stroma surface (Bultman and White, 1988; Bultman and Leuchtmann, 2003). The stroma envelops an inflorescence and prevents seed development of the enclosed florets, causing a syndrome known as a choke disease (White et al., 1991; Schardl, 2010; Tadych et al., 2014). A mature stroma bears numerous perithecia with elongated asci, which produce filiform wind-dispersed haploid ascospores that mediate transmission to new hosts by infecting the host ovule (White, 1988). The choking stromata in *F. rubra* have been observed only in few flowering stems in Spain (Zabalgogeazcoa et al., 1999), and a stroma is usually formed in few tillers of an individual host resulting in simultaneous asexual and sexual reproduction efforts of the fungus in a grass population (Schardl, 2001). Thus, *E. festucae* has only limited recombination potential and opportunities of contagious spread linking its fitness tightly to the fitness of the host grass and aligning the coevolution of the interaction toward mutually beneficial cooperation (Clay, 1998; Saikkonen et al., 1998, 2004, 2016). However, empirical evidence has revealed that the interaction between *Epichloë*-species and their host grass, similarly to all biological interactions, are context dependent and labile but can be mutualistic in some environments (Saikkonen et al., 1998; Cheplick and Faeth, 2009; Decunta et al., 2021).

Epichloë-species are highly dependent on the host grasses whilst the benefits from the interaction remains conditional to the host. In most cases the specific Epichloë species or isolate is either directly or indirectly linked to defensive mutualism attributable to alkaloids (Saikkonen et al., 2006, 2010a; Clay, 2009). Ecological consequences, however, may vary depending on the alkaloid profile of symbiota determined by the genotype of the fungus and prevailing environmental conditions (Morse et al., 2007; Schardl et al., 2012, 2013a; Saikkonen et al., 2013, 2016). The alkaloids providing defense against herbivores fall into four classes - ergot alkaloids, indole-diterpenes, lolines and pyrrolopyrazines as peramine - that differ in deterrence and toxicity to invertebrate

and vertebrate herbivores (Schardl et al., 2012, 2013b,c; Berry et al., 2019). Peramine can deter insects, lolines are insecticidal whereas ergot alkaloids and indole-diterpenes are well known for their toxicity to vertebrate grazers (Clay et al., 1985; Tanaka et al., 2005; Schardl et al., 2006, 2007, 2013b,c; Crawford et al., 2010; Berry et al., 2015; Saikkonen et al., 2016).

In concordance with the reputed context-dependency between hereditary microbes and their host plants, our earlier studies have revealed that the natural populations of *F. rubra* are formed by structured mosaics of *Epichloë*-free and *Epichloë*-symbiotic grass individuals (Zabalgogeazcoa et al., 1999, 2006; Saikkonen et al., 2000; Arroyo Garcia et al., 2002; Wäli et al., 2007; Dirihan et al., 2016; Leinonen et al., 2019; von Cräutlein et al., 2019). In addition to postglacial colonization history of the species and their local coadaptation to prevailing selection forces, highly integrated morphological and life history traits seem to determine the geographic variation in the frequencies of *Epichloë*-symbiotic grasses. Numerous empirical studies, reviews and meta-analyses have demonstrated that herbivory best explains high *Epichloë* frequencies in grass populations. We have been sampling and monitoring *F. rubra* populations in relation to occurrences of *E. festucae* in Spain, Switzerland, Greenland, Faroe Islands, Iceland, Norway and Finland from south to north during the last 20 years (Dirihan et al., 2016). Herbivory appears to be important force promoting the symbiosis in our study populations in Faroe Islands, Northern Finland and Spain as the highest *Epichloë* frequencies are subjected to heavy grazing pressure by sheep, reindeer and cattle, respectively. Thus, considerably lower numbers of *E. festucae* infections has been found in all the other studied regions without the presence of intense herbivory. Yet an unanswered question is whether the distribution of *E. festucae* is primarily determined by herbivory selection operating on the symbiotum rather than the distribution history of the host grass and founder effect, i.e., coincidental distribution history of *E. festucae*-symbiontic host grasses.

In this study, we examine genetic population structure and importance of reproduction modes, and predict alkaloid production potential of *E. festucae* across Europe using nuclear microsatellite (SSR) markers as well as mating type and alkaloid gene markers. Nuclear microsatellite markers enable us to make inferences about population structure, gene flow and genetic drift based on the patterns of genetic diversity within and among populations and regions. Moreover, SSR and mating type gene markers provide estimates of recombination potential and reproduction modes (asexual vs. sexual) based on the genotype frequencies, the structure of multilocus genotypes and mating type ratios (Milgroom, 1995). Alkaloid gene markers provide insights into ecological importance of alkaloid production in the studied populations. Accordingly, we predict that geographic variation and population differentiation detected should be structured and resemble each other if *E. festucae* is primarily spread *via* host grass seeds and herbivory defines recent and present phenotypic selection on the symbiotum. Furthermore, we hypothesize that genetic diversity should decrease toward the edges of *E. festucae* range in Europe due to potential genetic drift and strong selection. We also expect to detect the highest genetic diversity near areas that remained ice-free and in glacial refugias during the last glacial maximum period, as detected in the host grass *F. rubra* populations (van Zijll de Jong et al., 2008; von Cräutlein et al., 2019).

MATERIALS AND METHODS

Plant Material, Fungal Isolation, and DNA Extraction

The plants used in this study were originally collected as a part of research examining the occurrence and ecological importance of *Epichloë festucae* in wild populations of *Festuca rubra* L. s.l. across Europe in 2011 (Dirihan et al., 2016; Leinonen et al., 2019; von Cräutlein et al., 2019; Saikkonen et al., 2020; Vázquez de Aldana et al., 2020). The initial plants were split and a copy of each individual was maintained in pots with a mixture of peat and sand in the greenhouses at the Ruisalo Botanical Garden of Turku University. Splitting grass plants is a common way of generating identical genetic copies of both the host and the endophyte as the endophyte systemically infects the above ground plant tissue. The initial plants were tested for endophyte infection with methods described in Dirihan et al. (2016). In this study, we focused on three European regions of the host distribution extreme range with the highest occurrence of *E. festucae* infections (Dirihan et al., 2016). We examined a total of 240 individual *Epichloë*-infected plants originating from 15 natural populations from six islands in the Faroe Islands ($n = 71$), from six populations located in two different habitat types, meadows ($n = 73$) and riverbanks ($n = 30$), in Finland, and from three populations located in two different habitat types, Mediterranean oak forest ($n = 22$) and semiarid oak grassland ($n = 45$), in Spain ($n = 66$). More detailed information on the plants, occurrence of associated *Epichloë*-fungus and collection sites, including population geographic locations, coordinates, altitudes, habitat features and estimate of grazing intensity, can be found in **Table 1**, Dirihan et al. (2016); von Cräutlein et al. (2019). The frequencies of *Epichloë*-individuals in the studied populations ranged from 5% to 81% and the number of individual isolates varied accordingly (**Table 1**; Dirihan et al., 2016).

Epichloë festucae was isolated from the plants in 2013 and 2014. Three leaves from each tiller were selected from pots and tillers were surface sterilized. A leaf was cut in five segments and inoculated on autoclaved Petri dishes containing 5% potato dextrose agar (PDA). Plates were stored at room temperature until mycelium emerged, after which a small sample of mycelium were transferred to a new PDA plate on a piece of sterilized cellophane. Total genomic DNA was extracted from pure cultures of mycelium growth using the E.Z.N.A Plant DNA Kit (Omega Bio-Tek, Norcross, GA, United States) according to the procedures described in von Cräutlein et al. (2014). The same DNA samples were used for the analyses based on the SSR mating type and alkaloid gene markers.

Genetic Population Structure and Reproduction Modes

Genetic structure and the amount of clonality in the 240 *E. festucae* isolates were investigated using 14 polymorphic SSR markers, which were developed based on the searches for

TABLE 1 | Collection sites, habitat features and occurrences of *Epichloë festucae* in host *Festuca rubra* populations based on Dirihan et al. (2016), von Cräutlein et al. (2019).

Population code	Geographic site	Population site	Geographic coordinates	Altitude (m a.s.l.)	Habitat	Grazing pressure	Endophyte infection%
FAS1	The Faroe Islands	Mykines	N 62°5′51″ W 7°40′56″	125	Meadow	High (sheep)	68
FAS2	The Faroe Islands	Vidoy	N 62°22′3″ W 6°32′32″	148	Meadow	High (sheep)	44
FAS3	The Faroe Islands	Sandoy	N 61°50′11″ W 6°51′21″	69	Meadow	High (sheep)	21
FAS4	The Faroe Islands	Nolsoy	N 62°1′15″ W 6°41′8″	55	Meadow	High (sheep)	5
FAS5	The Faroe Islands	Vagar	N 62°6′59″ W 7°26′43″	246	Meadow	High (sheep)	25
FAS6	The Faroe Islands	Eysturoy	N 62°17′24″ W 7°2′10″	316	Meadow	High (sheep)	54
MS1K	Finland	Kevo 1	N 69°38′6″ E 27°5′1″	91	Meadow	High (reindeer)	56
MS2K	Finland	Kevo 2	N 69°43′56″ E 27°12′0″	85	Meadow	High (reindeer)	75
KS3	Finland	Kevo 3	N 69°45′32″ E 26°59′19″	107	Meadow	High (reindeer)	50
RBS1	Finland	Kevo 4	N 69°54′36″ E 27°1′48″	73	Riverbank	High (reindeer)	45
RBS2	Finland	Kevo 5	N 69°56′41″ E 26°43′22″	85	Riverbank	High (reindeer)	20
RBS3	Finland	Kevo 6	N 69°56′11″ E 26°27′45″	106	Riverbank	High (reindeer)	23
SPGD	Spain	Garganta de los Infiernos	N 40°12′1″ W 5°45′11″	768	Mediterranean oak forest	Medium (cattle, goat, sheep)	81
SPLV	Spain	Salamanca 1	N 40°56′20″ W 6°7′7″	863	Semiarid oak grassland, dehesas	High (cattle)	67
SPPOR	Spain	Salamanca 2	N 40°58′24″ W 5°57′34″	812	Semiarid oak grassland, dehesas	High (cattle)	59

≥10 mono- and dinucleotide repeats, and for ≥8 tri-, tetra-, penta-, and hexanucleotide repeats in the unplaced genomic scaffold sequences of *E. festucae* (for section "Materials and Methods" see von Cräutlein et al., 2014). The forward primers of each SSR primer pair were end-labeled with two different phosphoramidite fluorescent dyes, either HEX or 6-FAM. The samples were analyzed by multiplexing markers (2-4 primer pairs/reaction) with different labels and expected fragment sizes. Allele sizes ranged from 92 to 340 bp depending on the primer pairs (**Supplementary Table 1**). The details of PCR amplifications are described in von Cräutlein et al. (2014). Each genotyping plate included negative and positive controls and samples from several populations. The PCR products were run on an ABI 3130xl DNA Sequencer using GeneScan 500 ROX Size standard (Applied Biosystems) at the Institute of Biotechnology, University of Helsinki, Finland. Peak Scanner version 1 software were used (Applied Biosystems) to assign the allelic sizes of the amplified fragments. The detailed information on SSR markers, including e.g., names and locations of markers in the *E. festucae* chromosomes, are described in **Supplementary Table 1** and whole SSR data set with host plant IDs in **Supplementary Table 2**. As *E. festucae* is haploid, the samples were expected, and did produce one allele per locus. However, in the rare exception where multiple alleles were observed in at least one SSR locus of Faroe Islands (five isolates), Finland (six isolates), and Spain (six isolates), the samples were not included in the study.

Mating Type and Alkaloid Gene Variation

The genetic loci involved in alkaloid biosynthetic pathways essential for the production of ergot alkaloids (*EAS*), indole-diterpenes (*IDT*) and lolines (*LOL*) are complex gene clusters in *Epichloë* taxa, whereas the pyrrolopyrazine alkaloids (*PPZ,* previously referred as *PER*) production is dependent on the alleles of the *perA* gene (Tanaka et al., 2005; Schardl et al., 2012, 2013b; Berry et al., 2015, 2019). The genes encoding different alkaloid classes have recently been identified (Schardl et al., 2013b) allowing us to predict alkaloid production based on presence or absence of a key alkaloid genes within the pathway (Takach et al., 2012; Schardl et al., 2013b,c; Charlton et al., 2014; Takach and Young, 2014; Berry et al., 2015; Shymanovich et al., 2015; Vikuk et al., 2019). These genes are upregulated in planta (Young et al., 2006, 2015; Chujo and Scott, 2014) and the gene clusters are devoid of known pathway specific regulatory genes, unlike other fungal secondary metabolite clusters that often contain a gene that encodes a regulatory

TABLE 2 | Genotype and genetic diversity and indices of association by populations, by regions (in bold) and by genetic groups (Bayesian Analysis of Population Structure, $K = 4$) based on all the isolates of nuclear microsatellite data set ($n = 240$) in *Epichloë festucae*.

		Genotype diversity				Genetic diversity				Index of association		
	No. of isolates	No. of MLG	No. of eMLG	No. of unique MLG	No. of common MLG	*P* (%)	Ne	Np	uh	I_A	r_d	shared *p*-Value
SSR												
by populations												
FAS1	18	7	5	4	3	42.9	1.118	0.07	0.086	nt	nt	nt
FAS2	10	7	7	4	4	28.6	1.269	0	0.154	nt	nt	nt
FAS3	10	4	4	1	3	92.9	1.668	0.07	0.427	nt	nt	nt
FAS4	5	2	2	0	2	7.1	1.034	0	0.029	nt	nt	nt
FAS5	9	5	5	1	4	21.4	1.117	0	0.081	nt	nt	nt
FAS6	19	8	5.4	5	3	71.4	1.231	0.29	0.152	nt	nt	nt
Faroe Islands	**71**	**23**	**22**	**15**	**8**	**100**	**1.34 (1.69)**	**0.57**	**0.197 (0.346)**	**4.65**	**0.364**	**0.001**
MS1K	21	4	3	2	2	14.3	1.080	0.07	0.045	nt	nt	nt
MS2K	25	7	3.9	5	2	35.7	1.068	0.14	0.051	nt	nt	nt
KS3	27	8	4.2	4	4	85.7	1.168	0.14	0.128	nt	nt	nt
RBS1	14	5	4.1	1	4	85.7	1.200	0	0.168	nt	nt	nt
RBS2	10	4	4	3	1	21.4	1.065	0.14	0.054	nt	nt	nt
RBS3	6	1	1	0	1	0	1.000	0	0	nt	nt	nt
Finland	**103**	**19**	**13.4**	**15**	**4**	**92.9**	**1.11 (1.47)**	**0.50**	**0.085 (0.298)**	**4.08**	**0.364**	**0.001**
SPGD	22	18	9.1	15	3	100	2.372	1.36	0.473	4.544	0.359	0.001
SPLV	23	23	10	23	0	100	2.963	1.36	0.632	0.967	0.075	0.001
SPPOR	21	20	9.8	19	1	100	2.831	1.00	0.566	1.273	0.100	0.001
Spain	**66**	**61**	**61**	**57**	**4**	**100**	**3.75 (3.85)**	**6.43**	**0.682 (0.690)**	**1.05**	**0.083**	**0.001**
TOTAL	240	103	-	86	17	100	3.33	10.71	0.676	3.43	0.270	0.001
by genetic groups												
Far	69	22	7.9	14	8	71.4	1.22 (1.40)	0.43	0.124 (0.206)	0.329	0.043	0.002
Fin	104	19	4.7	14	5	92.9	1.09 (1.30)	0.79	0.068 (0.204)	0.624	0.065	0.001
Sp1	14	10	10	7	3	35.7	1.41 (1.49)	0.57	0.133 (0.154)	nt	nt	nt
Sp2	53	52	13.9	51	1	100	3.99 (3.98)	5.64	0.688 (0.692)	1.033	0.082	0.001

In brackets are shown the genetic diversity indices (Ne, uh) calculated in clone corrected data set for Faroe Islands (MLG n = 23), Finland (MLG n = 19) and Spain (MLG n = 61) and for genetic groups (BAPS, K = 4). MLG, multilocus genotypes; eMLG, expected MLG based on rarefaction; P%, percentage of polymorphic loci; Ne, average effective numbers of alleles; Np, average numbers of unique alleles; uh, unbiased genetic diversity; I_A and rd: Indices of association: clone corrected data; nt, not tested because of too low number of MLGs for the analysis.Occur only in one region.

function in the form of pathway specific transcription factors (e.g., aflR required for aflatoxin production; Woloshuk et al., 1994).

The presence of selected key genes from the loci for alkaloid production and mating type idiomorphs (genes *mtAC* and *mtBA*) were examined in a total of 198 *E. festucae* isolates originating from six populations in the Faroe Islands ($n = 60$), six populations in Finland ($n = 91$) and three populations in Spain ($n = 46$) (**Table 1**).

A multiplex PCR method was used to determine the mating type (*A* or *B*) and key genes present at each alkaloid loci (Charlton et al., 2014). The primers including two additional primers for *IDT* genes (*idtK* and *idtF*) used for mating type and alkaloid gene profiling, expected product sizes and six different multiplex sets are described in Supplementary Material in Charlton et al. (2014). The PCR amplification methods are described in Charlton et al. (2014). PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide by UV transillumination. The combination of two samples, MS2K-35 and SPGD-31 was positive for all examined alkaloid genes and used as a positive control on each PCR plate. The presence of a nonsense mutation and inframe stop codon in the first exon of *idtF* gene were determined in isolates that contained the *IDT* genes required for terpendole C or lolitrem B productions. The primers *idtF-M-F* (5′-GGGCCATCCTATCTTACAC-3′) and *idtF-M-R* (5′-ACGAAGCCTTGAATCCAC-3′) were designed based on the *idtF* gene sequences with and without the mutation (GenBank accession numbers: EU530694 and MF464362). The PCR product of each alkaloid gene locus was sequenced using the following methods: the PCR products were separated on a 1.2% agarose gel, extracted from the gel and purified with E.Z.N.A. Gel Extraction Kit (Omega, Bio-Tek). The purified PCR products were submitted to Macrogen Inc., for Sanger sequencing with

both upstream and downstream primers. The obtained sequences were visualized and manually corrected using Chromas version 2.6.5 (2018). The sequence similarity searches were performed in GenBank using BLAST sequence analysis tool (NCBI). All sequences showed the closest match (100% similarity) with *E. festucae* and the alkaloid gene region in question. The sequences were submitted to the European Nucleotide Archive. Accession numbers are available in **Supplementary Table 3** for the positive controls of the alkaloid genes and for the presence or absence of deletion in the *idtF* gene in the set of samples.

The presence or absence of a set of key alkaloid genes within the alkaloid class pathway detected here are described in Charlton et al. (2014). Ergovaline was expected to be produced, if five examined *EAS* genes (*dmaW, easC, easA, cloA*, and *lpsB*) produced PCR bands of the expected size, and chanoclavine was expected if only *dmaW* and *easC* were present. Peramine, a pyrrolpyrazine-1-one, was assumed to be produced, if all three markers including *perA5′*, *perAT2* and *perAR*, produced PCR bands, and if the reductase domain (*perA*R) was absent, then pyrrolpyrazine-1, 4-dione (PPZ-1 diones) were expected (Berry et al., 2019). Ergovaline and peramine chemotypes of some of the *F. rubra* – *E. festucae* symbionts in the present study (n = 27) determined by Vázquez de Aldana et al. (2020) were compared with the alkaloid gene profiles obtained in this study to confirm prediction of the alkaloid production. The first stable indole-diterpene intermediate, paspaline, is predicted if *idtG* and *idtQ* are both present. Isolates that can produce early pathway terpendoles, such as terpendoles E and I, also contain a functional *idtF*, and isolates that can produce late pathway terpendoles, such as terpendole C, contain *idtF* and *idtK* in addition to *idtG* and *idtQ*. Lolitrem B (LTB), the end product of IDT biosynthesis in *E. festucae,* was predicted to be produced, if in addition to the genes mentioned above also *idtJ* produced a PCR band. The prerequisite of the production of terpendole C and lolitrem B was also that the sequence of *idtF*-gene was functional, without the deletion in the first exon of the gene that causes an inframe stop codon (Young et al., 2009; Shi et al., 2017; Yi et al., 2018). In addition, presence of *idtP* gene was checked from our unpublished data in 109 of the *F. rubra* – *E. festucae* combinations in the present study. The host samples used in alkaloid gene, *idtF* mutation and *idtP*-gene detections are provided in **Supplementary Table 4**. Lolines were predicted to be produced, if examined *LOL* genes (*lolC, lolA, lolO*, and *lolP*) produced PCR bands.

The number of multilocus alkaloid gene genotypes (aMLG) based on the presence (1) or absence (0) of each key alkaloid gene was determined using haploid binary data and multilocus options within populations, across populations within regions and across the whole data set. For the alkaloid gene data set, pairwise PHi_{PT} values were used to estimate population pairwise differentiation levels within regions, two populations, FAS4 and RBS3, were excluded from the analysis, because of low numbers (<5) of isolates per population using the GenAlex version 6.5. (Peakall and Smouse, 2006, 2012).

Statistical Analysis of Nuclear Microsatellite Markers

The number of multilocus SSR genotypes (nMLG), the number of expected genotypes based on rarefaction (eMLG) and random association among loci (indices of associations: I_A and r_d; clone corrected data, 999 permutations) were computed for each population, across Finnish, Faroese and Spanish populations within regions and across the whole data set. Tests of random association among loci were not performed for the Faroese and Finnish populations due to lack of statistical power because of a low sample size after clone correction (Fincham and Day, 1963). Minimum spanning networks (MSN) using Bruvo's distance were computed across all populations (Bruvo et al., 2004). The analyses were computed with R 3.5.1 (R Core Team, 2016) package *poppr* (v2.8.5.; Kamvar et al., 2014).

To study the genetic relationships of isolates, a Bayesian Analysis of Population Structure (BAPS) software, version 6.0, was used by applying a non-spatial mixture clustering analysis at individual level (sampling unit) with linked loci option (Corander and Tang, 2007; Corander et al., 2008) using multilocus SSR data set including all the isolates in order to represent the distribution of allele frequencies in this randomized sample set (n = 240). The partition of optimal K numbers, which refer to number of groups into which the SSR data can be clustered, was conducted by performing 150 iterations of K from 2 to 30, which resulted in the number of genetically diverged clusters in optimal partition to be 15 [log(marginal likelihood) value = −1901,6]. Fixed K model was used, because BAPS identified several small clusters (nine clusters with an average of 2.7 individuals). The number of clusters for the fixed K model was determined based on the uppermost hierarchical levels of genetic structure shown in a UPGMA tree based on the Kullback-Leibler divergence matrix. The individual level mixture clustering analysis with linked option was conducted using the fixed K mode with 150 iterations of K = 4.

Genetic diversity indices of the SSR were calculated with two data sets by using all isolates (n = 240) and using all unique MLGs within regions (n = 103) with haploid data option and are based on the numbers of allele frequencies at each locus. The percentage of polymorphic loci (P%), the average effective numbers of alleles (Ne), the average numbers of unique alleles (Np) and unbiased genetic diversity (uh) estimates were calculated for each locus (n = 14), over the entire sample set (n = 240), over the MLGs (n = 103), for each region (n = 3), for each population (n = 15) and for each genetic group obtained by BAPS analysis (K = 4). A principal coordinate analysis (PCoA) was used to plot the major patterns in SSR data sets based on the whole data set (n = 240) and separately for each region (Faroe, Finland, and Spain) without clone corrections in relation to the genetic similarities of the isolates using pairwise individual-by-individual haploid genetic distance matrixes. Tests for significance were run with 999 random permutations. Analyses were conducted using GenAlex v. 6.5.

A hierarchical analysis of molecular variance (AMOVA; Weir and Cockerham, 1984; Excoffier et al., 1992; Weir, 1996) was used to estimate the degree of differentiation among regions

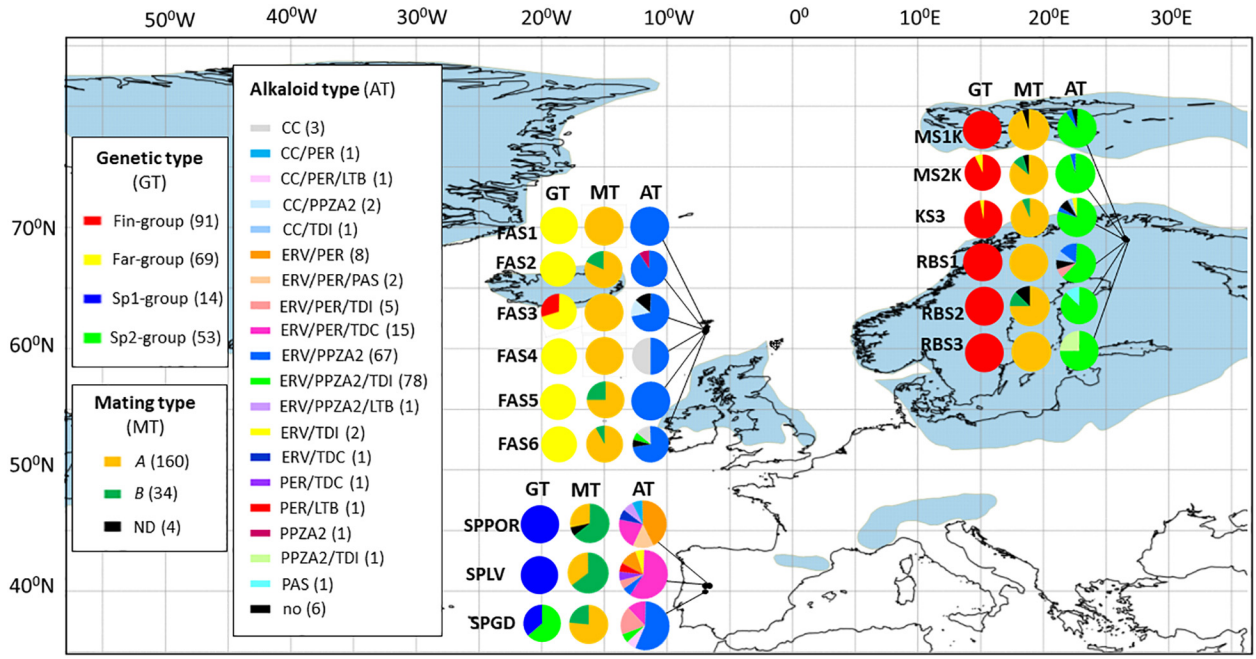

FIGURE 1 | The frequencies of genetic (GT), mating (MT), and alkaloid types (AT) of *Epichloë festucae* isolates are visualized in the fifteen populations located in the Faroe Islands, Finland, and Spain. The genetic types are presented according to the Bayesian partition of the genetic groups (*K* = 4: *Fin-*, *Far-*, *Sp1-*, *or Sp2-group*), mating types (*A* or *B*) according to the presence of mating type genes (*mtAC*, *mtBA*, ND, not determined) and alkaloid types according to the predicted alkaloid production based on the alkaloid markers (in brackets are indicated the numbers of isolates within the alkaloid type). No = no alkaloid production predicted. Detailed information about alkaloid types are shown in **Table 5**. Blue regions indicate permanent ice cover during the last glacial period (25,000–15,000 years ago; Ray and Adams, 2001).

and populations, and pairwise Fst values were used to estimate population differentiation levels among populations, regions, habitats within region and genetic clusters (K = 4) in SSR data set (n = 240, all isolates included). Moreover, the degree of differentiation among regions were also estimated with the data set with unique MLGs (n = 103). The analysis was conducted using Arlequin software, version 3.5 (Excoffier and Lischer, 2010). The significance of the fixation indices was run with 999 non-parametric permutations.

RESULTS

Population Structure

The Bayesian mixture analysis was conducted using fixed K mode of K = 4 [log (marginal likelihood) value = −2194.4]. At K = 4, the isolates are mainly distributed according to the geographical regions (**Figure 1**). *Fin*-group (n = 104) represents mainly Finnish isolates (n = 101) and three isolates from the Faroe Islands (FAS3). *Far*-group (n = 69) contains mainly Faroese isolates (n = 67) and two isolates from Finnish populations (KS3, RBS1). The Spanish isolates were partitioned in one large and one smaller genetic group. *Sp1*-group includes fourteen isolates from SPGD population. *Sp2*-group (n = 53) represents all isolates from SPLV (n = 23) and SPPOR (n = 21) but also eight isolates from SPGD and one isolate from the Faroe Islands (FAS6).

Consistent with the results of Bayesian mixture analysis, a clear geographic pattern of three groups was detected among all the Finnish, Faroese and Spanish isolates (n = 240) according to the principal coordinate analyses based on pairwise individual-by-individual haploid genetic distance matrixes, in which the first and second axes explained 44.5% and 18.6% of the variation, respectively (**Figure 2A**). In Faroe Islands, three individuals differed from the main group, which was highly mixed with individuals from different populations, the first and second axes explained 35.4% and 16.7.% of the variation, respectively (**Figure 2B**). In Finland, two individuals differed from the main group, which was also mixed with individuals from different populations, the first and second axes explained 36.2% and 27.7% of the variation, respectively (**Figure 2C**). In Spain, individuals were distributed in one small and one large group and individuals of the larger groups were moderately mixed, the first and second axes explained 19.3% and 13.2% of the variation, respectively (**Figure 2D**).

Genetic Diversity and Population Differentiation

Relatively low levels of genetic variation indices and numbers of private alleles were detected in most of the Finnish and Faroese populations, although the levels of genetic variation differed widely among the populations (**Table 2**). In contrast, relatively high levels of genetic variation indices and high numbers of

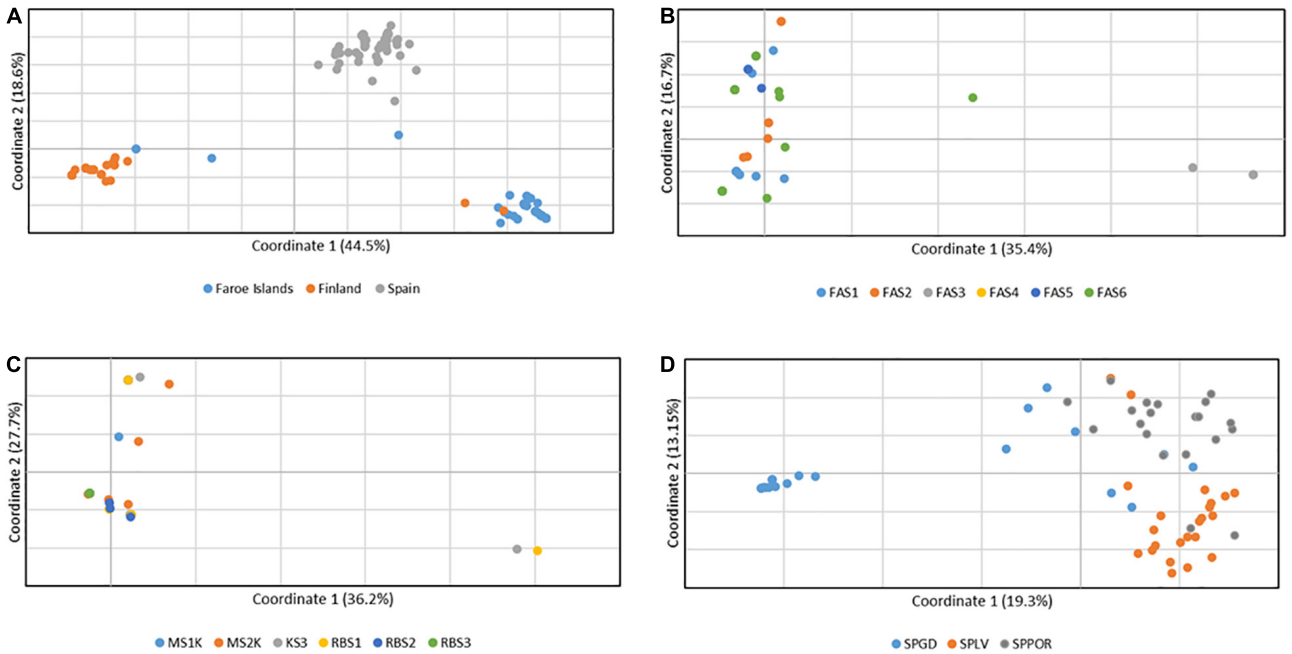

FIGURE 2 | The results of principal coordinate analysis (PCoA) based on pairwise individual-by-individual haploid genetic distance matrixes of the nuclear microsatellite data **(A)** among all the Finnish, Faroese and Spanish *Epichloë festucae* isolates (n = 240), **(B)** among Faroese isolates (n = 71), **(C)** among Finnish isolates (n = 103), and **(D)** among Spanish isolates (n = 66). A clear geographic pattern of three groups corresponds to the genetic groups identified by Bayesian mixture analysis (*Far, Fin*, and *Sp*). The Spanish genetic groups, *Sp1* and *Sp2*, identified by Bayesian mixture analysis, are separated in the PcoA analysis among Spanish isolates.

private alleles were found in all Spanish populations (**Table 2**). Accordingly to the regions, genetic diversity estimates differed between the geographic locations of genetic groups (BAPS clusters) being highest in Spanish *Sp2*-group and clearly lower in *Far*- and *Fin*-groups (**Table 2**).

Overall using all the isolates (n = 240), the AMOVA analysis estimated that 66.6% of the genetic variation occurs among regions, 36.1% within populations and only 7.3% among populations within regions indicating high differentiation among the regions and low differentiation among the populations (**Table 3**). Based on the data set of unique MLGs (n = 103) for the regions, 44% of the genetic variation occurs among populations and 56% within populations (**Table 3**).

In the Faroe Islands (using all the Faroese isolates), most of the variation (73.1%) was detected within populations and moderate levels of genetic differentiation (26.9%) among the six populations (**Table 3**). The population pairwise Fst values varied from 0.004 to 0.447 being on average 0.25 ± 0.14 and significant differentiation levels were found among 67% of the population pairs ($p < 0.05$; n = 10; **Supplementary Table 5A**).

In Finland (using all the Finnish isolates), majority of the variation (95.5%) was within populations and only low level (4.5%) genetic differentiation was found among the six populations (**Table 3**). No significant differentiation was detected between the populations located in meadow (n = 3) and riverbank (n = 3) habitats (Fst = 0.067, p = 0.175, see **Table 1**). The population pairwise Fst values varied from 0.011 to 0.202 being on average 0.065 ± 0.060 and 20% of population pairs showed significant differentiation between populations ($p < 0.05$; n = 3; **Supplementary Table 5A**).

In Spain (using all the Spanish isolates), most of the variation (73.6%) was detected within populations and moderate level genetic differentiation (26.4%) observed among the three populations (**Table 3**). A significant differentiation was detected between the populations located in Mediterranean forest (SPGD) and dehesa grassland (SPLV and SPPOR) habitats (Fst = 0.295, $p < 0.001$, see **Table 1**). The population pairwise Fst values are shown in **Supplementary Table 5A**.

Among the genetic groups (K = 4, including all the isolates, n = 240), AMOVA analysis revealed that 75% of the genetic variation is distributed among the genetic groups and 25% within clusters indicating very high genetic differentiation among the groups (**Table 3**). Using the data set of unique MLGs for the region (n = 103), 53.3% of the genetic variation occurs among populations and 46.7% within populations (**Table 3**). The pairwise Fst values of genetic groups are shown in the data sets with all the isolates and with MLGs in **Supplementary Table 5B**.

Reproduction Modes

The clonal structure of the isolates according to populations within regions is visualized in **Figure 3**. In total, 103 fungal multilocus nuclear genotypes (nMLG) were observed in the SSR data set (n = 240) in fifteen populations collected from Spain, the Faroe Islands and Finland (**Table 2**). High frequencies (92.4%) of unique nMLGs and eMLGs occurred in the Spanish populations and only four common genotypes were present in

TABLE 3 | Results of analysis of molecular variance analysis for the whole data set, the geographical regions and genetic groups (Bayesian Analysis of Population Structure, $K = 4$) calculated separately using all the isolates ($n = 240$) and using unique MLGs ($n = 103$) in *Epichloë festucae*.

Origin	d.f.	Sum of Squares	Variance components	Variance (%)	*p*
Geographical regions, including all isolates					
Whole data set					
Among regions	2	1326.4	4.115	66.6	<0.001
Among populations within regions	12	184.7	0.451	7.3	<0.001
Within populations	465	749.6	1.612	36.1	<0.001
Faroe Islands					
Among populations	5	49.6	0.388	26.9	<0.001
Within populations	136	143.6	1.056	73.1	<0.001
Finland					
Among populations	5	7.3	0.027	4.5	0.192
Within populations	200	113.6	0.567	95.5	<0.001
Spain					
Among populations	2	127.8	1.366	26.4	<0.001
Within populations	129	492.4	3.817	73.6	<0.001
Georgaphical regions, including unique MLGs					
Among regions	2	350.6	2.646	44.0	<0.001
Within populations	203	761.7	3.752	56.0	<0.001
SSR markers					
Genetic groups, including all isolates					
Among groups	3	1518.9	4.657	74.9	<0.001
Within groups	476	741.8	1.558	25.1	<0.001
Genetic groups, including unique MLGs					
Among groups	3	486.6	3.532	53.3	<0.001
Within groups	202	625.8	3.098	46.7	<0.001

two populations, of which three nMLGs in Mediterranean oak population (SPGD) (**Table 2**). In the Faroe Islands, fifteen (65.2%) nMLGs were unique in the region and eight common nMLGs consist of 78.9% of the isolates ($n = 56$) located in different Faroese populations, three largest clone sizes are 22,11 and 8 isolates/nMLG. In Finland, fifteen (78.9%) nMLGs were unique in the region and four common nMLGs consist of 85.4% of the isolates ($n = 88$) located in different Finnish populations, two largest clone sizes are 64 and 19 isolates/nMLG.

The indices of associations (I_A and r_d) were utilized to estimate the relative importance of reproduction types, i.e., either sexual or asexual reproduction predominated in the regions (Kamvar et al., 2014). Significant linkage disequilibrium was estimated in Finland, the Faroe Islands and Spain and separately in each Spanish population suggesting linked loci and predominance of asexual reproduction in all three regions, although indices of association were clearly lower in Spain than in Finland and the Faroe Islands (**Table 2**).

The frequencies of mating types according to the populations and genetic groups ($K = 4$) are shown in **Figure 1** and **Table 4**. The ratio of mating types *A* and *B* was 0.92, therefore the proportions of each mating type are close to equal among populations in Spain. In SPLV and SPPOR populations most of the isolates were of mating type B, whereas most of SPGD isolates were of mating type *A*. In contrast, majority of the isolates in Finland and the Faroe Islands were of mating type *A*, although both mating types were present in half of the populations in both Finland and Faroe Islands. Mating type genes were not detected in four of the isolates. Based on the genetic groups ($K = 4$) *Fin*-, *Far*-, and *Sp2* -groups shared the mating types A and B, but only mating type A occurred in *Sp1*-group (**Table 4**).

Alkaloid Genotypes and Predicted Alkaloid Production

The alkaloid gene profiles of different aMLGs with mating types, isolate numbers and predicted and confirmed chemotypes are presented in **Table 5** and host information and alkaloid profiles of the *E. festucae* in **Supplementary Table 6**. Overall, 38 multilocus alkaloid genotypes (aMLGs) and 20 unique chemotypes were identified among the Spanish, Faroese and Finnish *E. festucae* isolates based on the seventeen genetic loci or alleles associated to ergot alkaloid (*EAS*), indole-diterpene (*IDT*), pyrrolpyrazine (*PPZ*), and loline (*LOL*) production ($n = 198$; **Figure 1** and **Table 5**). Common to all isolates was the absence of genes required for the biosynthesis of lolines. Most of the isolates (94,4%, $n = 187$) are predicted to produce at least one end product of EAS, PPZ and/or IDT alkaloid class. Most (90.4%, $n = 179$) of the isolates had all the *EAS* genes targeted, and thus, they are likely to have a functional *EAS* pathway and are predicted to produce ergovaline. About one fifth (16.7%, $n = 33$) of the isolates contained all *PER* markers and they are expected to produce peramine, the pyrrolopyrazine-1-one PPZ-1-ones (Berry et al., 2019). Isolates (75.8%, $n = 150$) that lack the reductase domain, are predicted to make pyrrolopyrazine-1, 4-diones (PPZA2). Chemotypically determined ergovaline and peramine production in the same *F. rubra-E. festucae*-symbiosis revealed that 72.7% ($n = 11$) and 100% ($n = 10$) of the isolates corresponded with the predicted alkaloid profiles, respectively (**Table 5**; Vázquez de Aldana et al., 2020). Three isolates contained all functional *IDT* genes and, thus, they are predicted to produce lolitrem B. The earlier IDT pathway intermediates paspaline, terpendoles I and C are predicted to be produced by 1.5% ($n = 3$), 43.9% ($n = 87$), and 8.6% ($n = 17$) of the isolates, respectively.

The numbers of isolates within the aMLGs differed largely from unique genotypes (one isolate/aMLG) to common genotypes (2-71 isolates/aMLG) (**Table 5**). Most of the aMLGs (81.6%) were only located in one region. Altogether, 31.6%, 36.8%, and 50% of the aMLGs were observed in the Faroe Islands, Finland and Spain, respectively.

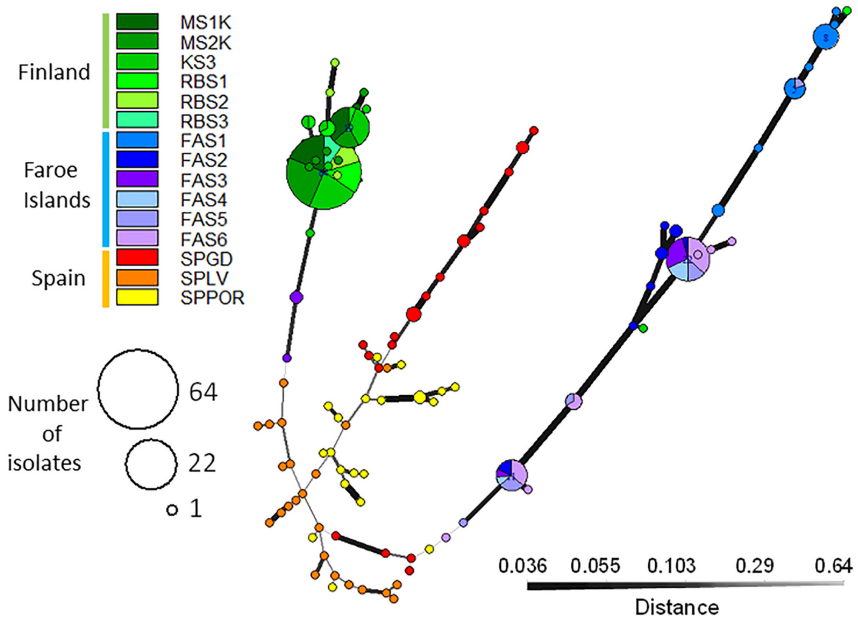

FIGURE 3 | Minimum spanning networks visualize the genotype size (nMLGs) and the genetic distance among *Epichloë festucae* isolates from the populations located in Finland, the Faroe Islands and Spain. Each node represents a unique multilocus genotype and edge widths and shading represents relatedness according to Bruvo's distance, edge length is arbitrary (Kamvar et al., 2014). The largest common nMLG includes 64 Finnish isolates and second largest nMLG 22 Faroese isolates. Single MLGs predominated among Spanish isolates and only three common nMLGs occurred in the region. Faroe Islands: FAS1, FAS2, FAS3, FAS4, FAS5, FAS6; Finland: MS1K, MS2K, KS3, RBS1, RBS2, RBS3; Spain: SPGD, SPLV, SPPOR.

In Spain, 40.4% of the isolates possessed different aMLGs (n = 19) and considerably higher amounts of predicted chemotypes were detected in three Spanish populations compared to northern populations (**Figure 1** and **Table 5**). The observed 13 different chemotypes are predicted to produce different combinations of final products of ergovaline or lolitrem B and their early pathway products and peramine or pyrrolopyrazine-1, 4-diones (**Figure 1** and **Table 5**). All isolates are expected to be toxic to mammals due to presence of all *EAS* and/or *IDT* genes, except one isolate (gt-21) (**Table 5**). In contrast, the frequency of PER and PPZA-2 chemotypes that potentially deter insect feeding differ among the populations. Majority of the SPLV (88.2%) and SPPOR (85.7%) isolates can be expected to produce peramine (PER), which is the case of 37.5% in SPGD isolates (**Table 5**). However, 62.5% of SPGD isolates are expected to produce pyrrolpyrazine-1, 4 diones (PPZA-2), which is the case of one isolate in both SPLV and SPPOR. The most common aMLG, g*t-40* (n = 15), contained about one third (31.9%) of the Spanish isolates and occurred in all populations (SPGD n = 2; SPLV n = 10; SPPOR n = 3) (**Table 5**). *Gt-40* was positive for twelve out of thirteen alkaloid markers and is predicted and confirmed to produce ergovaline and peramine and is predicted to produce terpendoles (**Table 5**). Significant pairwise differentiation levels based on the occurrence of the alkaloid genes were estimated between the Mediterranean oak forest (SPGD) and both dehesa populations SPLV and SPPOR (Phi_{PT} = 0.150, p = 0.005; Phi_{PT} = 0.101, p = 0.037, respectively), but not between the dehesa populations of SPLV and SPPOR (Phi_{PT} = 0.054, p = 0.106) (**Supplementary Table 5C**).

In the Faroe Islands, one fifth (20%) of the isolates possessed different aMLGs (n = 12). Overall, five unique aMLGs and seven common aMLGs with 91.7% of the fungal isolates were present in the region. Several *IDT* genes were absent in the Faroese isolates (**Table 5**). Most (88.3% and 90%) of the isolates are expected to produce ergovaline and pyrrolopyrazine-1, 4-diones (PPZA-2), respectively (**Figure 1** and **Table 5**). The most common aMLG, *gt-27* (n = 30) was observed in half of the Faroese isolates in five populations and tested positive for seven out of thirteen markers including all *EAS* genes and lacked *perAR* marker and all five *IDT* markers (**Table 5**). Second most common Faroese aMLG, *gt-33* (n = 9) contained 15% of Faroese isolates in four populations and tested positive of eight out of thirteen markers including all *EAS* genes and lacked *perAR* marker and all *IDT* genes except *idtG* (**Table 5**). No significant pairwise differentiation levels based on the occurrence of the alkaloid genes were detected among the Faroese populations in alkaloid gene variation ($p > 0.05$) except between FAS1 and FAS5 (0.147, p = 0.039) (**Supplementary Table 5C**).

In Finland, only 15.4% of the isolates possessed different aMLGs (n = 14). Overall, seven unique and seven common aMLGs with 92.3% of the isolates were detected in the region (**Table 5**). Majority (91.2%, and 87.9%) of the isolates contained all the *EAS* and *IDT* early pathway genes (*idtG, idtQ, and idtP*) and they are expected to produce ergovaline and/or terpendole I, respectively (**Figure 1** and **Table 5**). Most (91.2%) of the isolates are predicted to make pyrrolopyrazine-1, 4-diones (PPZA-2) and one isolate peramine (gt-38; **Table 5**). The most common aMLG, *gt-39* (n = 70) was found in 76.9% of Finnish isolates

TABLE 4 | Frequencies of mating types *A* and *B* by populations (*n* = 198), by regions (in bold) and by genetic groups (Bayesian Analysis of Population Structure, *n* = 188) in *Epichloë festucae* in natural *Festuca rubra* populations.

		Mating types			
	No. of isolates	(*A*) *n* (%)	(*B*) *n* (%)	Ratio A:B	ND[a] n (%)
by population					
FAS1	17	17 (100)	–	–	–
FAS2	11	9 (81.8)	2 (18.2)	4.5	–
FAS3	7	7 (100)	–	–	–
FAS4	2	2 (100)	–	–	–
FAS5	8	6 (75)	2 (25)	3.0	–
FAS6	15	14 (93.3)	1 (6.7)	14.0	–
Faroe Islands	**60**	**55 (91.7)**	**5 (8.3)**	**11.0**	–
MS1K	20	19 (95)	–	–	1 (5)
MS2K	21	18 (85.7)	2 (9.5)	9	1 (4.8)
KS3	25	23 (92)	2 (8.0)	11.5	–
RBS1	13	13 (100)	–	–	–
RBS2	8	6 (75)	1 (12.5)	6	1 (12.5)
RBS3	4	4 (100)	–	–	–
Finland	**91**	**83 (91.2)**	**5 (5.5)**	**28.3**	**3 (3.3)**
SPGD	16	12 (75)	4 (25)	3.3	–
SPLV	17	6 (35.3)	11 (64.7)	0.5	–
SPPOR	14	4 (28.6)	9 (64.3)	0.4	1 (7.1)
Spain	**47**	**22 (46.8)**	**24 (51.1)**	**0.9**	**1 (2.1)**
TOTAL	198	160	34	4.7	4
by genetic group					
Far	58	54 (93.1)	4 (6.9)	13.5	–
Fin	84	79 (94)	3 (3.6)	26.3	2 (2.4)
Sp1	9	9 (100)	0	–	–
Sp2	37	12 (32.4)	24 (64.9)	0.5	1 (2.7)
TOTAL	188	154 (81.9)	31 (16.5)	5.0	3 (1.6)

[a]*ND = not detected.*

in all six populations (**Table 5**). The *gt-39* was positive for 11 out of 13 markers, lacked *perAR* marker and *idtJ* gene, and had non-functional *idtF* gene (determined from ten isolates of gt-39). Thus, gt-39 is likely to produce ergovaline due to presence of all five detected EAS genes, pyrrolopyrazine-1, 4-diones (PPZA-2) due to presence of *perA5* and *perAT2* markers and the indole-diterpenes pathway intermediate terpendole I due to presence of *idtG, idtQ* and *idtP* (**Table 5**). No significant pairwise differentiation based on the occurrence of the alkaloid genes was detected among the Finnish populations ($p > 0.05$; **Supplementary Table 5C**).

DISCUSSION

Our results suggest that the postglacial colonization history of the host grass, *F. rubra*, and predominance of asexual reproduction in the heritable symbiotic *E. festucae* largely determines its genetic structure in Europe. During the most recent ice age, the Pleistocene Epoch, arctic ice sheet advanced south covering large parts of Eurasia and North America in the Northern Hemisphere. Of the regions examined in this study, parts of the Iberian peninsula, remained ice-free whereas ice sheet covered much of the Northern Europe. However, some of the grass populations may have survived on sporadic glacial refugia on mountain peaks, nunataks, in the shores of Norway, Kola peninsula and Faroe Islands. Thus, we assume that most genetic variation among and within the examined *F. rubra* populations is best explained by independent long-distance colonization events and genetic adaptation to the local environment (Bazely et al., 1997; Dirihan et al., 2016; Leinonen et al., 2019; von Cräutlein et al., 2019). Although selection can operate on the fungus and host individually or in concert as a phenotypic unit, in *Epichloë-F. rubra* interactions only one fungal genotype is transmitted vertically to seed progeny, promoting stable interaction between the fungal genotype and the host lineage. Similarly to the host grass (von Cräutlein et al., 2019), here we identified three larger regional clusters of *E. festucae* - southern, northeastern, and northwestern European clusters – with genetic diversity reflecting the genetic divergence detected in the host populations. For clarity, we use the same division as in our previous papers describing geographic variation in *Festuca rubra* L. ploidy levels and systemic fungal endophyte frequencies, and the genetic diversity of the host plant (Dirihan et al., 2016; Leinonen et al., 2019; von Cräutlein et al., 2019). Both neutral SSR and adaptive alkaloid gene markers revealed that genetic and genotype diversity of *E. festucae* was highest in Spain, and markedly lower in the Faroe Islands and Finland. These results support our hypothesis that genetic diversity should be highest in ice age refugia and decrease toward the edges of species range.

Potential Forces Driving Distribution History, Genetic Diversity, and Geographic Differentiation of *Epichloë festucae* Populations

Because the occurrence of the host plant is a prerequisite for the dispersal of associated symbiotic microbes, the genetic diversity of microbe should primarily mirror forces driving the postglacial distribution of the host species and secondarily the microbes, or the host and the microbe in concert as a phenotypic unit. During the last ice age, ending about 20,000 years ago, glaciers extended over much of northern Europe and also over much of Canada and some of the northern United States. Presently, *F. rubra* has a broad circumarctic-circumboreal distribution due to its great adaptive ability to colonize new pockets of land exposed from the retreating ice sheet during the postglacial distribution history (Inda et al., 2008; Braun et al., 2020). Taxonomically *F. rubra* is a morphologically variable species complex showing extensive hybridization, polyploidy, as well as phenotypic and genetic variation (Jenkin, 1955; Markgraf-Dannenberg, 1980; Ainscough et al., 1986; Aiken and Fedak, 1992; de la Fuente et al., 2001; Catalan et al., 2004; Catalan, 2006; Soreng et al., 2017). Thus, distinguishing taxonomic entities as species, subspecies and varieties within the complex is challenging (Saikkonen et al., 2019).

Epichloë festucae symbiotic plants can be commonly detected in all three geographic regions examined in this study, but

TABLE 5 | Alkaloid genotypes (aMLGs) of *Epichloë festucae* in the natural host populations of *Festuca rubra* in the Faroe Islands, Finland and Spain.

		No. of isolates within genotype			Ergot alkaloid (*EAS*) genes[a]					Peramine (*PER*) domains[a]			Indole-diterpenes (*IDT*) genes[a]							Alkaloid confirmed[d]	
aMLG	MT ratio A:B	Faroe	Finland	Spain	*dmaW*	*easC*	*easA*	*cloA*	*lpsB*	*perA5*	*perAT2*	*perAR*	*idtG*	*idtQ*	*idtP*[b]	*idtF*	*idtK*	*idtJ*	Predicted chemotype[c]	*ERV*	*PER*[f]
gt-1	B	1			+	−	−	−	+	+	+	−	+	−	nt	−	−	−	PPZA2	nt	nt
gt-2	A	1			+	+	+	+	−	+	+	−	−	−	nt	−	−	−	CC/PPZA2	nt	nt
gt-3	A	1			+	−	−	−	−	−	−	−	+	−	−	−	−	−		nt	nt
gt-4	A	1			+	+	+	+	+	+	+	−	−	−	nt	−	+	−	ERV/PPZA2	nt	nt
gt-5	B	1			−	−	−	−	−	+	+	−	−	−	−	+	+	−	PPZA2	nt	nt
gt-8	A		1		+	+	+	−	+	−	+	−	+	+	nt	ψ[e]	+	−	CC/TDI	nt	nt
gt-9	B		1		+	+	+	+	+	−	+	−	+	+	+	ψ[e]	+	−	ERV/TDI	nt	nt
gt-10	B		1		−	−	−	−	+	−	+	−	−	−	+	−	−	−		nt	nt
gt-11	A		1		+	+	+	+	−	+	+	−	+	−	nt	+	+	−	CC/PPZA2	nt	nt
gt-12	A		1		+	−	−	+	−	−	+	−	+	−	nt	+	+	−		nt	nt
gt-13	nd		1		+	−	−	−	−	−	+	−	+	−	+	+	+	−	PAS	nt	nt
gt-14	A		1		+	+	+	+	−	+	+	−	+	+	nt	−	−	−	PPZA2/TDI	nt	nt
gt-15	A			1	+	+	−	+	−	+	+	+	+	+	+	+	+	+	CC/PER/LTB	nt	nt
gt-16	B			1	+	+	+	+	+	+	+	+	+	+	+	ψ[e]	+	+	ERV/PER/TDI	no	yes
gt-17	B			1	+	+	+	+	+	+	−	+	+	+	nt	−	−	−	ERV/TDI	nt	nt
gt-18	A			1	+	−	+	−	+	+	+	+	+	+	+	+	+	+	PER/LTB	nt	nt
gt-19	B			1	−	−	−	−	−	+	+	+	+	+	+	+	+	−	PER/TDC	nt	nt
gt-20	B			1	+	+	+	+	+	+	+	+	−	+	+	ψ[e]	+	−	ERV/PER	nt	nt
gt-21	B			1	+	+	+	+	−	+	+	+	−	−	nt	−	−	−	CC/PER	no	yes
gt-22	B			1	+	+	+	+	+	+	−	−	+	+	+	+	+	−	ERV/TDC	nt	nt
gt-23	nd			1	+	+	+	+	+	+	+	+	+	−	−	−	−	−	ERV/PER	nt	nt
gt-24	B			1	+	+	+	+	+	+	+	−	+	+	+	+	+	+	ERV/PPZA2LTB	nt	nt
gt-25	A, nd		2		+	−	−	−	−	−	+	−	+	−	+	−	−	−	PAS	nt	nt
gt-26	3:0	3			+	+	+	−	+	−	+	−	−	−	−	−	−	−	CC	nt	nt
gt-27	32:0	30	2		+	+	+	+	+	+	+	−	−	−	−	−	−	−	ERV/PPZA2	yes	no
gt-28	4:0			4	+	+	+	+	+	+	+	+	−	−	−	−	−	−	ERV/PER	yes	yes
gt-29	7:0	6	1		+	+	+	+	+	+	+	−	−	−	−	+	+	−	ERV/PPZA2	yes	no
gt-30	4:0	4			+	+	+	+	+	+	+	−	−	+	−	−	−	−	ERV/PPZA2	nt	nt
gt-31	2:0			2	+	+	+	+	+	+	+	+	−	+	−	−	−	−	ERV/PER	no	yes
gt-32	2:0	2			+	+	+	+	+	+	+	−	−	+	−	+	+	−	ERV/PPZA2	nt	nt
gt-33	8:4	9		3	+	+	+	+	+	+	+	−	+	−	−	−	−	−	ERV/PPZA2	no	no
gt-34	2:0			2	+	+	+	+	+	+	+	−	+	−	nt	−	+	−	ERV/PPZA2	nt	nt
gt-35	7:0		2	5	+	+	+	+	+	+	+	−	+	−	nt	+	+	−	ERV/PPZA2	yes	no

(Continued)

TABLE 5 | (Continued)

		No. of isolates within genotype			Ergot alkaloid (*EAS*) genes[a]				Peramine (*PER*) domains[a]			Indole-diterpenes (*IDT*) genes[a]						Alkaloid confirmed[d]	
aMLG	MT ratio A:B	Faroe	Finland	Spain	*dmaW*	*easC*	*easA*	*cloA*	*lpsB*	*perA5*	*perAT2*	*perAR*	*idtG*	*idtQ*	*idtP*[b]	*idtF*	*idtK*	*idtJ*	Predicted chemotype[c]	*ERV*	*PER*[f]
gt-36	0:2			2	+	+	+	+	+	+	+	+	+	–	+	+	+	–	ERV/PER/PAS	nt	nt
gt-37	1:6	1	6		+	+	+	+	+	+	+	–	+	+	+	–	–	–	ERV/PPZA2/TDI	yes	no
gt-38	1:3	1		3	+	+	+	+	+	+	+	+	+	+	+	–	–	–	ERV/PER/TDI	nt	nt
gt-39	70:1		70	1	+	+	+	+	+	+	+	–	+	+	+	ψ[e]	+	–	ERV/PPZA2/TDI	yes	no
gt-40	4:11			15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	ERV/PER/TDC	yes	yes

[a]*Selected pathway genes of different alkaloids: + presence of gene; -absence of gene.*

[b]*idtP detected in the same F. rubra-E. festucae symbiotum in the set of the samples within genotype (see* ***Supplementary Table 4****, not published data).*

[c]*Predicted chemotypes: CC, chanoclavine; ERV, ergovaline; PER, peramine (equivalent to PPZA1, pyrrolopyrazine-1-ones); PPZA-2, pyrrolopyrazine-1, 4-diones; PAS, paspaline TDI, terpendole I; TDC, terpendole C; LTB, lolitrem B.*

[d]*Ergovaline and peramine production of Festuca rubra – Epichloë festucae symbionts have been determined chemically by Vázquez de Aldana et al. (2020).*

[e]*Expected pseudogene (see* ***Supplementary Table 4****).*

[f]*PER, peramine a pyrrolpyrazine-1-one.*

nt, not tested; nd, not detected.

the frequencies of symbiotic plants vary among regions, and populations and habitats within regions irrespective of phenotypic and genetic variation of the host (Dirihan et al., 2016; von Cräutlein et al., 2019). The highest overall endophyte frequencies were found in Spain, where 69% of plants harbored *E. festucae*. In contrast, 36% and 30% of grasses were *Epichloë* symbiotic in Faroe Islands and northern Finland, respectively (Dirihan et al., 2016). Although the fungus is capable of horizontal transmission by sexual spores, contagious spreading within the host populations and long-distance migration among populations or geographic regions appears to be strongly constrained in the Faroe Islands and Finland where the sexual life cycle appears to be extremely rare (Wäli et al., 2007). The present results support this as mating type representation were skewed toward the presence of MTA. Similar geographic patterns of genetic population structures of *F. rubra* and *E. festucae* suggest that the symbiont has primarily migrated with the host (see von Cräutlein et al., 2019). To date, there has been very little focus on mating type frequency. The recent studies have mainly concentrated on the asexual *Epichloë* species and it appears that only one mating type gene occur in most of the studied species, like *E. festucae* var. *lolii* (Hettiarachchige et al., 2015). In contrast, mating type gene frequency is found in equilibrium in *Epichloë typhina* where sexual stage is active (Bushman et al., 2019).

Here we propose that the classic theory of island biogeography (MacArthur and Wilson, 1967) and the geographic mosaic of coevolution (Thompson, 2005) provide a useful framework to understand distribution, and genetic diversity and geographic differentiation of *E. festucae* populations. Similarly to macro-organisms, a positive species-area relationship has been detected to lead to higher microbial diversity in large and less isolated sampling areas in studies using bacteria diversity in water-filled treeholes (Bell et al., 2005), foliar fungi in birch trees living in fragmented environments (Helander et al., 2007), ectomycorrhizal fungi on "tree islands" (Peay et al., 2007), and soil bacteria and fungi in land-bridge islands as models (Li et al., 2020). Analogously to the prediction that species diversity should reflect "island" size and isolation, we detected highest genetic and genotype diversity of *E. festucae* in Spain which can be treated as a "continent" from where potential *F. rubra* and *E. festucae* colonists dispersed into Faroe Islands and Fennoscandia. Similarly, the reduction of genetic variation detected in *E. festucae* during the postglacial distribution history appears to be formed by local selection pressures imposing the symbiotum across the examined geographic regions.

In Spain, we found the highest genetic diversity that can be explained by the occurrence of populations near glacial refugia and biodiversity center of fine fescues (Saint Yves, 1930) and occasional sexual reproduction. The two genetically distinct groups of *E. festucae* (*Sp1* and *Sp2*) were distributed unevenly in the examined two habitats. The genetically more diverse *Sp2*-group predominated in Mediterranean savannah-like grasslands, dehesa, located higher in altitudes near Salamanca (populations SPLV and SPPOR). *Sp2*-group was detected also in Mediterranean oak forest (population SPGD) to a lesser extent. In contrast, the genetically less diverse *Sp1*-group was prevalent in Mediterranean oak forest and absent in dehesa. The alkaloid

gene assemblage differed also among the habitat types and larger variation of alkaloid chemotypes occurred in dehesa compared to Mediterranean oak forest. Nearly all examined fungal lineages had the potential to produce ergot alkaloids with known anti-vertebrate and anti-invertebrate properties as well as peramine or PPZA-2 with anti-invertebrate properties (Ball et al., 1997; Berry et al., 2019; Caradus and Johnson, 2020; Hudson et al., 2021). More than 60% of the fungal isolates from xerophytic forest grasses had genetic potential to produce PPZA-2 and more than 30% of them also terpendoles. Much larger variation of isolates with both anti-vertebrate and -invertebrate properties occurred in dehesa grassland plants and almost all isolates had genetic potential to produce both ergovaline and peramine whereas only two isolates had potential of PPZA-2. Moreover, low genetic differentiation levels between dehesa populations support the similarities in population genetic structure revealed by both SSR and alkaloid markers, as detected in the studies of Arroyo Garcia et al. (2002), Vázquez de Aldana et al. (2010) suggesting adaptation to similar herbivores selection pressures in dehesa habitat. Much lower genetic differentiation was observed among the host grass populations compared to its fungal symbiont populations in Spain, which maybe due the outcrossing of genetically distant host individuals (von Cräutlein et al., 2019). These results suggest that prevailing selection pressures driving distinct prevalence of genetic structures in two different habitats in Spain is operating either on the fungus or fungus-grass genotype combination rather than on the host grass individually, although some variation is observed within dehesa populations (see also Vázquez de Aldana et al., 2010).

In concordance with the presumption that diversity should decrease with the distance to the source regions, some genetic diversity appears to be lost during the colonization of exposed land following retreating ice sheet in North Europe. However, our previous study on the host grass supports the hypothesis that some of the grass individuals may have survived on nunataks in Faroe Islands (Dirihan et al., 2016; von Cräutlein et al., 2019). The present study on *E. festucae* in the Faroe Islands do not support the same for the symbiotic fungus. Most Faroese isolates fell into locally adapted *Far*-group, had lost several *IDT* genes and were predicted to produce the same alkaloids across islands. Only three non-local SSR isolates were detected, two isolates from the *Fin*-group in FAS3 population and one isolate from *Sp*2-group in FAS6 population. This suggests that the long-distance co-dispersal of microbes with their hosts occur but not as efficiently into the region as its host *F. rubra* which possess a contact zone of various maternal lineages in the Faroe Islands especially since gene flow from the other locations have been found to be more effective in maritime than inland locations as seeds might arrive by floating and by birds (Saikkonen, 2000; Golan and Pringle, 2017; von Cräutlein et al., 2019). The genetic mismatches between the host and the fungal genotypes can affect infection losses of new plant genotypes during the establishment process and consequently reduce the number of novel fungal genotypes in the region (Saikkonen et al., 2010b; von Cräutlein et al., 2019). Thus, relatively large proportions of non-infected *F. rubra* individuals in Faroese populations may reflect the process of infection losses. In addition, similarly to Spanish populations, the Faroese populations were more differentiated from each other revealed by SSR markers than the Finnish ones, although predominantly two relatively large identical genotypes were present in most of the populations indicating fungal gene flow *via* host seeds among the islands. The observed genetic differentiation of the populations may have been caused by varying selection pressures driving populations in different directions, gene flow from other locations and predominance of vertical transmission of *E. festucae* in mainly clonally dispersing *F. rubra* in Faroe Islands (Harberd, 1961; Heide, 1990; Saikkonen et al., 1998, 2002; Zhang et al., 2010; Leinonen et al., 2019).

In the species northernmost distribution range in subarctic Finland, the examined *E. festucae* populations present distinct genetic *Fin*-group with relatively low genetic diversity compared to the Southern Spanish populations. Similarly to its host populations of *F. rubra*, one large genotype were observed also in the fungal populations indicating longevity and expansion of local host-fungus genotypes (von Cräutlein et al., 2019). *F. rubra* clones have been detected to be centuries old and occupying large areas (Harberd, 1961) as selection favors the presence of one dominant genotype in a clonal population (Milgroom, 1996). Only two non-local isolates were found in the region, even though relatively high frequencies of non-local host cpDNA haplotypes were observed in the mostly clonal host grass populations (von Cräutlein et al., 2019), which can be due to reproductive differences between the species (Sullivan and Faeth, 2004). Non-local plants can have reduced probability to flower in northern latitudes preventing vertical transmission of non-local *E. festucae* strains (Leinonen et al., 2019). Moreover, infections can be lost by long-distance seed dispersers during the establishment process because infected plants may have a lower fitness compared to uninfected plants especially in harsh conditions (Leinonen et al., 2019). In contrast to Spanish populations with genetically distinct habitat-specific groups, no differentiation was observed among populations divided in two habitats, meadows and riverbanks, in Finland, which may due to efficient local expansion of dominant host-fungus genotypes.

Alkaloid Production

The alkaloid genotyping has proven to be very powerful to identify potential bioactive alkaloids produced by populations of *Epichloë* (Takach et al., 2012; Charlton et al., 2014; Young et al., 2014; Shymanovich et al., 2017). Genome sequencing of the first two *E. festucae* isolates and other related *Epichloë* species revealed considerable diversity within the genus based on the presence or absence of the alkaloid genes (Schardl et al., 2013a,b,c, 2014; Winter et al., 2018). In the current study, 20 unique chemotypes were predicted from the 198 isolates. These chemotypes ranged from individual alkaloids (e.g., chanoclavine, CC, paspaline, PAS, or the pyrrolopyrazine-1, 4-diones, PZZA-2), to more complex chemotypes representing up to three different classes of alkaloids. Sequencing of genetic loci associated with each alkaloid has revealed repetitive AT-rich transposable elements are associated with alkaloid diversity. In some cases, such as that of the pyrrolopyrazine *perA* gene, transposable elements have disrupted the gene causing the loss of the reductase domain, which results in production of pyrrolopyrazine-1, 4-diones rather than the

expected peramine (Schardl et al., 2013b,c; Berry et al., 2015, 2019). In addition, the ergot alkaloid and indole-diterpene loci are located in what appears to be unstable AT-rich regions of the genome in the subtelomere region. Interestingly, we observed in each Spanish population one isolate able to produce lolitrem B. To our knowledge, the production of lolitrem B is rarely observed in *F. rubra-E. festucae* symbiotum and only mentioned in Young et al. (2009). The ergot alkaloid, ergovaline, and the pyrrolopyrazines, peramine and pyrrolopyrazine-1, 4-diones, were most commonly observed within the populations. However, isolates tested for ergovaline and peramine in a previous study (Vázquez de Aldana et al., 2020) did not consistently detect ergovaline when expected. As explanation, the ergovaline pathway has been reported to be silent, with no gene expression observed (Schardl et al., 2013b,c; Charlton et al., 2014; Young et al., 2015). Sequencing this population of *E. festucae* may provide greater insights into the evolution of these biosynthetic genes. The more limited predicted chemotypes found in Finland and the Faroe Islands versus that of Spain, may be due to selection pressure since the Spanish populations may have an advantage of many different chemotypes.

CONCLUSION

Reproductive strategy of *E. festucae* and mating type distribution likely explain large part of the differences among geographic regions in genetic diversity among the populations within the regions. Low genetic diversity in Finland and Faroe Islands, and lack of differentiation between distinct habitats in Finland, appears to be attributable to the extremely rare production of sexual structures, detectable as symptoms called "choke disease" on the host inflorescences, and dominance of one mating type. For example, we have intensively monitored *F. rubra* populations in northern Finland during the last 20 years but never detected choke disease (Saikkonen et al., 2000, 2010b; Wäli et al., 2007). Furthermore, only a few isolates were heterozygous and/or carrying multiallelic loci suggesting multistrain infections or hybrid origins of the isolates e.g., due to somatic hybridization.

Thus, we assume that postglacial distribution history of the host, founder effect, genetic drift and local adaptation of symbiotum largely explain the detected genetic structure of northern *E. festucae* populations. In contrast, although past studies have detected choke disease in less than 1% of *Epichloë* symbiotic *F. rubra* plants in Spain (Zabalgogeazcoa et al., 1999), the high numbers of unique genotypes and presence of both mating types in the Spanish populations suggests that the recombination may have an important role in shaping the population structure. On the other hand, unique genotypes can be trapped for centuries within very diverse host genotypes and only compatible combinations in the newly recombined seeds have survived. Future studies will reveal whether random distribution, founder effects and genetic drift rather than natural selection explain the detected imbalanced mating type ratio and thereby decreased genetic and chemotypic diversity of *E. festucae* in northern Europe.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in **Supplementary Tables 2, 6**, and the name of the online repository and the accession numbers can be found in **Supplementary Table 3**.

AUTHOR CONTRIBUTIONS

MH and KS designed the collection of the data and performed the sampling. MC, HK, CY, and PL designed the genetic study. MC genotyped the isolates and analyzed the data. CY detected and confirmed the alkaloid profiles. MC and KS wrote the manuscript with contributions by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.756991/full#supplementary-material

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Implications of Seed Vault Storage Strategies for Conservation of Seed Bacterial Microbiomes

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Global seed vaults are important, as they conserve plant genetic resources for future breeding to improve crop yield and quality and to overcome biotic and abiotic stresses. However, little is known about the impact of standard storage procedures, such as seed drying and cold storage on the seed bacterial community, and the ability to recover seed-associated bacteria after storage. In this study, soybean [*Glycine max* (L.) Merr.] seeds were analyzed to characterize changes in the bacterial community composition and culturability under varying storage conditions. The *G. max* bacterial microbiome was analyzed from undried seed, dried seed, and seed stored for 0, 3, 6, and 14 months. Storage temperatures consisted of −20°C, 4°C, and room temperature (RT), with −20°C being commonly used in seed storage vaults globally. The seed microbiome of *G. max* was dominated by *Gammaproteobacteria* under all conditions. Undried seed was dominated by *Pantoea* (33.9%) and *Pseudomonas* (51.1%); however, following drying, the abundance of *Pseudomonas* declined significantly (0.9%), *Pantoea* increased significantly (73.6%), and four genera previously identified including *Pajaroellobacter*, *Nesterenkonia*, env.OPS_17, and *Acidibacter* were undetectable. Subsequent storage at RT, 4, or −20°C maintained high-abundance Genera at the majority of time points, although RT caused greater fluctuations in abundances. For many of the low-abundance Genera, storage at −20°C resulted in their gradual disappearance, whereas storage at 4°C or RT resulted in their more rapid disappearance. The changes in seed bacterial composition were reflected by cultured bacterial taxa obtained from the stored *G. max* seed. The main taxa were largely culturable and had similar relative abundance, while many, but not all, of the low-abundance taxa were also culturable. Overall, these results indicate that the initial seed drying affects the seed bacterial composition, suggesting that microbial isolation prior to seed drying is recommended to conserve these microbes. The standard seed storage condition of −20°C is most suitable for conservation of the bacterial seed microbiome, as this storage temperature slows down the loss of seed bacterial diversity over longer time periods, particularly low-abundance taxa.

Keywords: seed vault, storage strategies, seed bacterial microbiomes, conservation, culturability

INTRODUCTION

Seed vaults play a significant role in facilitating the *ex situ* conservation of germplasm of a range of crop species, their closely associated crop wild relatives (CWRs), and other wild species (Hay and Probert, 2013). Globally, seed vaults preserve plant genetic diversity for research and plant-breeding activities for improving food and nutritional security (Asdal and Guarino, 2018). International standards are adapted by seed vaults for long-term seed storage. According to the standard method, seeds are first kept under drying conditions at 10–15% relative humidity and 10–15°C to achieve a seed moisture content of 3–7% followed by their storage at or below −18°C. This method has been identified to work well for seeds of many plant species known as orthodox seeds (Cochrane et al., 2007).

Crop seeds are known to transmit a plant-specific core microbiota (Berg and Raaijmakers, 2018). The seed-associated microbes are reported to have plant genotype specificity and can vertically transmit from one generation to the next plant generation. Horizontal transfer of the microbes can occur *via* their uptake from the surrounding environment (Johnston-Monje et al., 2016; Shade et al., 2017; Adam et al., 2018; Nelson, 2018). The seed microbiome is known to harbor a wide range of microbial species (Johnston-Monje et al., 2016; Shade et al., 2017; Adam et al., 2018). The seed-borne microbes can colonize the emerging seedlings before the intake of microbes from the surrounding environment and can promote germination and early plant vigor and survival (Truyens et al., 2015). However, how seed-associated microbes influence the different growth stages during seed germination and later plant growth and provide biotic–abiotic stress resistance still need to be investigated (Adam et al., 2018; Berg and Raaijmakers, 2018).

Different seed-associated microbes were identified to protect crops against various biotic–abiotic stresses and enhance plant growth (Links et al., 2014; Mousa et al., 2016; Gdanetz and Trail, 2017; Shahzad et al., 2018; Li et al., 2020, 2021; Hone et al., 2021). In last two decades, the use of growth-promoting bacteria in agriculture has increased significantly to reduce the use of chemical fertilizers and enhance plant nutrient uptake (Rascovan et al., 2016). It is suggested that embracing greater use of beneficial microorganisms can improve crop yield and encourage biology-based agriculture (Schmidt et al., 2015).

Soybean [*Glycine max* (L.) Merr.] is one of the most important crops and a major source of animal feed and vegetable oil worldwide (Sugiyama et al., 2015). Due to a high protein (40–42%) and oil content (18–22%), soybean is also used for aquaculture feed and production of biofuel and as a source of protein for the human diet (Pagano and Miransari, 2016). Soybeans can play an important role in matching the food demands of the growing population by 2050, although the estimated yield increase of only 1.3% per year is not satisfactory (Pagano and Miransari, 2016). Some bacterial genera including *Pseudomonas*, *Bacillus*, *Bradyrhizobium*, *Streptomyces*, *Rahnella*, and *Azospirillum* and fungi, e.g., *Piriformospora* and *Trichoderma*, have proven to be promising in plant growth promotion in soybean production (Tsavkelova et al., 2006; Ramírez and Kloepper, 2010; Schmidt et al., 2015; Bakhshandeh et al., 2020). Notably, many crop, plant, and vegetable seeds were also reported to be inhabited by some of these bacterial genera (Johnston-Monje et al., 2016; Liu et al., 2017; Adam et al., 2018; Berg and Raaijmakers, 2018; Khalaf and Raizada, 2018; López et al., 2018; Wassermann et al., 2019a; Abdullaeva et al., 2021; Hone et al., 2021). However, despite the enormous potential of seed microbiomes to promote plant growth and sustainable agricultural practices, the impact of current international seed storage strategies on the seed microbial diversity and composition has not yet been evaluated (Berg and Raaijmakers, 2018).

Therefore, in the present study, amplicon sequencing of the V4 region of 16S rRNA was used to examine the impact of standard storage methods on the diversity and composition of the *G. max* seed bacterial microbiome and bacteria isolated from these seeds. The aim was to determine the effectiveness of current storage methods in maintaining the original bacterial composition and viability of seed-associated bacteria over time, thus providing an experimental basis for our understanding of the implication of seed vault storage strategies in conservation of seed bacterial microbiome.

MATERIALS AND METHODS

Soybean Seed Samples

Soybean seeds (*G. max*-Burrinjuck) used in this study were sourced from Australian Grains Genebank, Horsham, Australia. About 1 kg of seed was placed in a cloth bag and stored in a drying chamber at 15°C and 15% relative humidity for about 1 month. This is the standard drying protocol used prior to storage of seed in the seed vault (FAO, 2014). At the end of the drying phase, about 100 g of seed were weighed and transferred into heat-sealed aluminum bags. One bag of undried seed was also prepared to use it as control for the drying process at 0 time point. The seed bags were then transferred after 2 days to the laboratory in AgriBio, Bundoora, Victoria, Australia. Seed bags containing dried seed were then stored at −20°C (±2°C), at 4°C (±2°C), and at room temperature (RT), 22°C (±2°C), for 3, 6, and 14 months. Seed bags for RT were kept in an airtight plastic container at RT for 3, 6, and 14 months. For further study, germinated seedlings were selected for profiling the *G. max* microbiome to focus on the seed-borne microbes that can colonize seedlings during germination, with the hypothesis that these microbes have a function in this process, thus focusing only on the viable microbial communities that remain after storage.

Seed Germination

For germination, *G. max* seeds for each time point and storage temperature were washed 10 times with an excess amount of sterile distilled water. The seeds were transferred into sterile petri dishes (12-cm diameter) by placing them between pre-water soaked Whatman™ filter paper (two sheets underneath and one on top) and then sealed with Parafilm™ and kept in darkness for 24–32 h at RT. Then, the top layer of filter paper was removed, and the plates were resealed with Parafilm™ followed by a further 8–10 days of incubation on a lab-benchtop

under ambient light conditions. Non-germinated seeds were discarded immediately to avoid any antagonistic fungal outgrowth from these seeds. If needed, water was sprayed on seedlings during the incubation under sterile conditions. The average germination rate for the *G. max* seeds remained between 55 and 60% during the study. Seedlings were harvested for microbiome profiling and microbial isolation once the cotyledons reached an unfolded growth stage (**Supplementary Figure S1**).

DNA Extraction, 16S Amplicon Library Construction, and Sequencing

For seed microbiome profiling, 15–20 seedlings were selected for DNA extraction. Whole seedlings (root, shoot, and cotyledon) were cut into pieces of approximately 0.5–1 cm using a sterile scalpel blade, collected in 1.2-ml QIAGEN collection tubes, and snap-frozen in liquid nitrogen and stored at −80°C until processed for DNA extraction. DNA extraction was performed using the MagAttract® 96 DNA plant kit using a Biomek FX^P Lab Automation Workstation coupled to a Synergy 2 multi-mode reader controlled by Biomek software version 4.1 and Gen 5 (2.08) software (Biotek Instruments, United States) with slight changes in manufacturer's guidelines.

Amplicon libraries for Illumina sequencing were prepared using barcoded primer 5151f-806r, specific to V3–V4 regions of the bacterial 16s rRNA gene. Amplification of the host chloroplast and mitochondrial 16s DNA was blocked by adding peptide nucleic acids, pPNA and mPNA, respectively, to the PCR mix (Lundberg et al., 2013). PCR for 16s rRNA gene amplification was performed in a total volume of 25 µl [Kapa HiFi Hotstart 2× ReadyMix DNA polymerase (Kapa Biosystems Ltd., London, United Kingdom), 50 µM of pPNA and mPNA mix, 5 µM of each primer, PCR-grade water, and 5 µl of template DNA] under the following cycling conditions: 94°C for 3 min, 30 cycles of 94°C for 15 s, 75°C for 10 s, 55°C for 10 s, 72°C for 45 s, and a final elongation at 72°C for 10 min using a thermal cycler (Agilent SureCycler 8,800, Agilent Technologies, United States). Libraries were further purified using AMPure XP beads (LABPLAN, Naas, Ireland). Dual indices and Illumina sequencing adapters from the Illumina Nextera XT index kits v2 B and C (Illumina, San Diego, United States) were added to the target amplicons in a second PCR step using Kapa HotStart HiFi 2× ReadyMix DNA polymerase (Kapa Biosystems Ltd., London, United Kingdom). Cycle conditions were 95°C (3 min), then 10 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, then a final extension of 72°C for 5 min followed by library cleanup using AMPure XP beads.

The barcoded libraries were quantified on a Nanodrop™ 1000 spectrophotometer and pooled together in an equimolar concentration. Library pools were further quantified for concentration and size using QuantiFluor® dsDNA assay (Promega Corporation, United States) and Tape station 2,200 High Sensitivity D1000 kit (Agilent Technologies, United States), respectively. Paired-end sequencing was performed on Miseq v3 (2 × 300 bp v3 chemistry cartridge). All Illumina sequences have been submitted to the NCBI Sequence Read Archive (SRA accession PRJNA766782).

Data Analysis

The raw Illumina® paired-end reads were quality filtered and merged into a single read using PEAR with default parameters (Zhang et al., 2014). Afterward, sequencing data analysis was performed using QIIME 22020.11.1 (Bolyen et al., 2019). The primers from single-end reads were then removed using cutadapt plugin with the following parameters; error rate-0.2, flags; adapter-wildcards, read-wildcards, and discard-untrimmed (Martin, 2011). The single-end reads were then trimmed to a read length of 253 bp and then dereplicated and filtered to remove chimeras. A feature table was then constructed containing the amplicon sequencing variants (ASVs) and representative sequences using the default algorithm in DADA2 (Callahan et al., 2016). The ASVs were then aligned with mafft (Katoh et al., 2002; *via* q2-alignment) and used to construct a phylogeny tree with fasttree2 (Price et al., 2010; *via* q2-phylogeny). The taxonomic classification of ASVs was performed using a naive Bayes taxonomy classifier (Bokulich et al., 2018) trained on the silva-138 release (V4 region-16s rRNA gene; Quast et al., 2013). Host-associated mitochondria and chloroplast reads and low-abundance features (<10 counts and present in at least two samples) were discarded from the data using the filter-features plugin. Alpha diversity (Observed OTUs) and beta diversity (Jaccard distance) were explored by running the core-metrics script in QIIME2 by rarefying feature tables to a read count of 6,000 sequences. The taxa classified up to the genus level were then exported and used to perform the presence/Absence test in Genedata Expressionist® Analyst™ v.10.0 using the default parameters to identify the shared features between undried seed and dried seed stored for 0, 3, 6, and 14 months at RT, 4, and −20°C (Genedata, Basel, Switzerland). Statistical analyses of the 16s rRNA gene data were performed using scripts in QIIME2 2020.11.1. Alpha diversity was tested for significant differences using the Kruskal–Wallis pairwise test and Beta diversity using Analysis of Similarity (ANOSIM) test. To determine the changes in bacterial abundance after seed drying, the QIIME2 feature table was exported in biom format and one-way ANOVA test was performed on individual ASVs (>0.1% *in planta*) using OriginPro 2019 (v9.6.0.172).

Microbial Isolation, DNA Extraction, 16S Amplicon Library Construction, and Sequencing

For all storage temperatures, seedlings in triplicates were harvested by collecting the shoot and root tissues and discarding the seed coat. The plant tissues were cut into small pieces (0.5–1 cm) and homogenized using a sterile pestle or two cycles of a Qiagen TissueLyser II for 1 min at 30 Hertz in 400–500 µl of 1× PBS buffer followed by centrifugation at 4,000 rpm for 1 min. Serial dilutions were prepared (10^{-1}–10^{-4}), and 20-µl aliquots, including undiluted macerate, were plated onto Reasoner's 2A agar (R2A; Oxoid, United Kingdom), and the plates were incubated at RT for up to 10 days. The bacterial growth was observed both in undiluted and diluted plates, though only undiluted plates were selected for further study to capture a snapshot of the original viable bacterial community

composition that was culturable, as it is likely to contain the most diversity. The DNA was extracted by scraping all microbial colonies off the plate using a sterile plastic loop into an Eppendorf tube®. The colonies were then resuspended in 1× PBS buffer and spun at 10,000 rpm for 5 min. According to the manufacturer's guidelines, the supernatant was discarded, followed by DNA extraction using Promega™ Wizard™ Genomic DNA Purification Kit (United States). The gDNA's optical density measurements were performed in a Quantus™ Fluorometer (Promega Corporation, Madison, WI, United States), 16S rRNA bacterial genes were amplified, and libraries were prepared and sequenced using steps mentioned in Section DNA Extraction, 16S Amplicon Library Construction, and Sequencing.

Data Analysis of Cultured Microbial Libraries

The raw paired-end reads were processed and analyzed as mentioned in Section Data Analysis, except the features were only filtered based on frequency (<10 counts), and the sequences were rarefied to the read counts of 16,529 sequences.

RESULTS

16S Amplicon Sequencing

After aligning paired-end reads, removal of low-frequency features, singletons, and chimeric and plant sequences, a total of 17,038,933 sequences were assigned to 361 Amplicon Sequence Variants (ASVs) for microbiome profiling (*in planta*) and 4,020,424 sequences were assigned to 155 ASVs for microbial isolation (on plates). After rarefaction and collapsing biological replicates, the ASV table was assigned to 89 genera for microbiome profiling (**Supplementary Table S1**) and 26 genera for microbial isolation (**Supplementary Table S2**).

G. max Seed Microbiome Profiling (*In planta*)

At the class level, the bacterial communities were mainly dominated by the presence of *Gammaproteobacteria* (20.1–97.0%), *Bacilli* (1.8–21.9%), *Alphaproteobacteria* (0.1–22.1%), *Actinobacteria* (0.2–15.2%), and *Bacteroidia* (0.01–20.6%). Notably, after 3 months at RT, the relative abundance was almost equally dominated by *Alphaproteobacteria* (22.1%), *Gammaproteobacteria* (20.1%), *Bacilli* (21.9%), *Bacteroidia* (20.6%), and *Actinobacteria* (15.2%). Additionally, low-abundance bacterial classes (<0.1%) such as *Polyangia* (0.0038%) and *Acidobacteriae* (0.00083%) designated as "Others" were observed in undried seed and seed stored for 3 months at −20°C, respectively (**Figure 1A, Supplementary Table S1**).

At the genus level, 25 bacterial genera were identified with >0.1% relative abundance. The bacterial communities of the stored *G. max* seed mainly consisted of genera including *Pantoea* (1.8–91.2%), *Pseudomonas* (0.31–51.1%), *Bacillus* (1.4–14.9%), *Sphingomonas* (0.04–17.8%), *Curtobacterium* (0.17–13.7%), *Paenibacillus* (0.15–7.2%), *Mucilaginibacter* (0.0–17.8%), *Novosphingobium* (0.001–6.2%), and *Massilia* (0.01–4.9%) (**Figure 1B**). In addition to the community structure changes, the relative abundance of bacterial genera varied across all time points and storage temperatures. Interestingly, high variations in bacterial abundance were observed after 3 months in seed stored at RT. In particular, majority of the ASVs mainly belonged to the genera *Sphingomonas* (20.7%), *Mucilaginibacter* (17.8%), *Bacillus* (14.9%), and *Curtobacterium* (13.7%), which were represented at much higher levels than at other time points (**Figure 1B**). Notably, the relative abundance of *Pantoea* (1.9%) was the lowest after 3 months at RT compared to all other time points. The bacterial genera with >10% relative abundance *in planta* such as *Pantoea*, *Pseudomonas*, *Mucilaginibacter*, *Bacillus*, and *Curtobacterium* responded differently to drying treatment and storage temperature. It was observed that the relative abundance of *Pantoea* increased under cold storage, while *Pseudomonas* oscillates in abundance across all temperatures (**Figure 1B, Supplementary Figure S2A**). The bacterial genera with <10 and > 1% relative abundance *in planta* such as *Paenibacillus*, *Novosphingobium*, *Massilia*, *Microbacteriaceae*, *Spirosoma*, *Microbacterium*, *Siphonobacter*, Unidentified group of *Comamonadaceae*, and *Erwiniaceae* were increased in abundance at RT especially after 3 and 6 months of storage (**Figure 1B, Supplementary Figure S2B**). On the other hand, the bacterial genera with <1% such as *Methylobacterium*, *Rhizobium*, *Burkholderia*, *Ralstonia*, *Hymenobacter*, *Rummeliibacillus*, *Roseomonas*, Unidentified group of *Sphingomonadaceae*, *Yersiniaceae*, and *Planococcaceae* showed more variations in their abundance at 4°C and RT (**Figure 1B, Supplementary Figure S2C**).

Composition Differences in the Seed Microbial Communities After Different Lengths of Storage

Alpha diversity and beta diversity were used to assess the changes in bacterial diversity and composition in undried seed, dried seed, and seed stored for 0, 3, 6, and 14 months at −20°C, 4°C, and RT. Based on alpha diversity, when compared to the undried seed, the number of observed features significantly ($p < 0.05$) declined in dried seed stored at −20°C and 4°C in all time points (3, 6, and 14 months) and in dried seed stored at RT for 6 and 14 months. In contrast, no significant differences were observed between undried seed and dried seed (0 month) and between undried seed and dried seed stored at RT for 3 months. Additionally, when differences were compared for each storage temperature, the number of observed features significantly ($p < 0.05$) declined after 3 months and 6, 3, and 14 months, in all time points at −20°C, 4°C, and RT, respectively (**Figure 2A, Supplementary Table S3**). No significant differences were observed for seed stored for 3 and 14 months at −20 and 4°C, respectively.

Based on the Jaccard dissimilarity metrics, the bacterial composition significantly ($p < 0.05$) varied under all conditions (**Supplementary Table S3**). These differences were also evident in the Principal Coordinates Analysis (PCoA) of the bacterial communities, where undried seed, dried seed (0 month), and stored seed (3, 6, and 14 months) formed a separate cluster

(**Figure 2B**). Interestingly, the undried seed, dried seed (0 month), and seed stored for 6 months formed a close cluster compared to seed stored for 3 and 14 months. The statistical analysis based on the presence/absence test was performed in Genedata Expressionist® Analyst™, and the number of shared genera was calculated between undried seed and dried seed (0 month) and between undried seed and stored seed (3, 6, and 14 months). There were 68.8% genera (33) shared between undried seed and dried seed (0 month). While after 3 months of storage, there were 56.3% genera (27) shared between undried seed and seed stored at RT, followed by 54.2% genera (26) with seed stored at 4°C and 47.9% genera (23) with seed stored

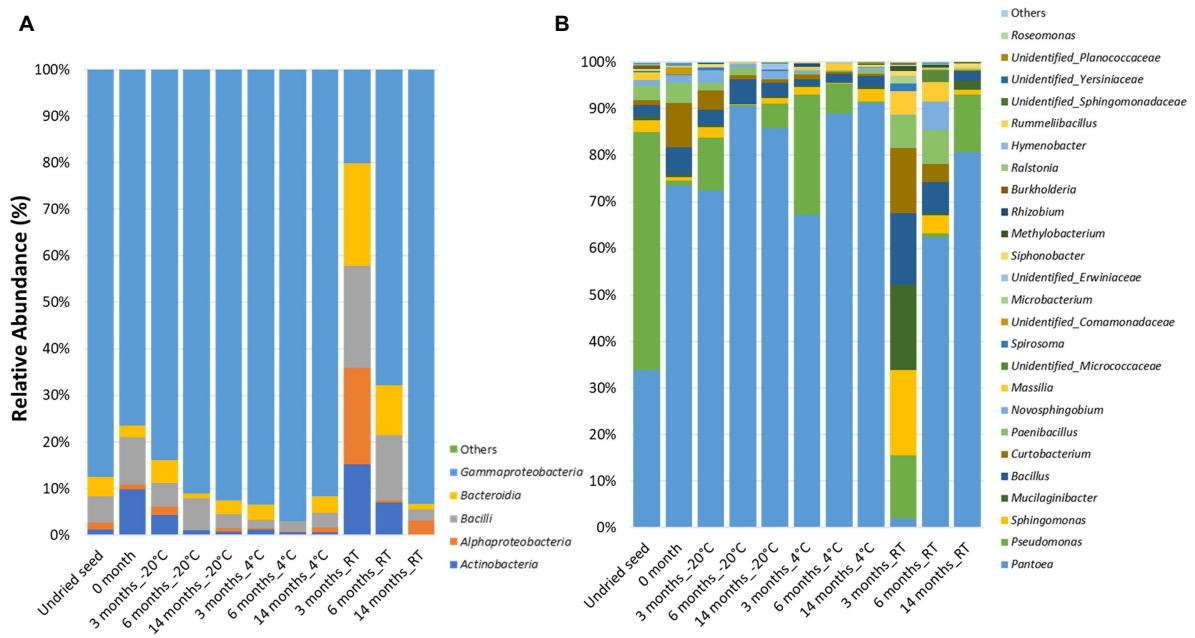

FIGURE 1 | Relative abundance of the bacterial taxa *in planta* **(A)** at class level and **(B)** at genus level in undried seed and dried seed stored at different time points (0, 3, 6, and 14 months) at −20°C, 4°C, and room temperature (RT). The bacterial taxa occurring with <0.1% are shown as "Others."

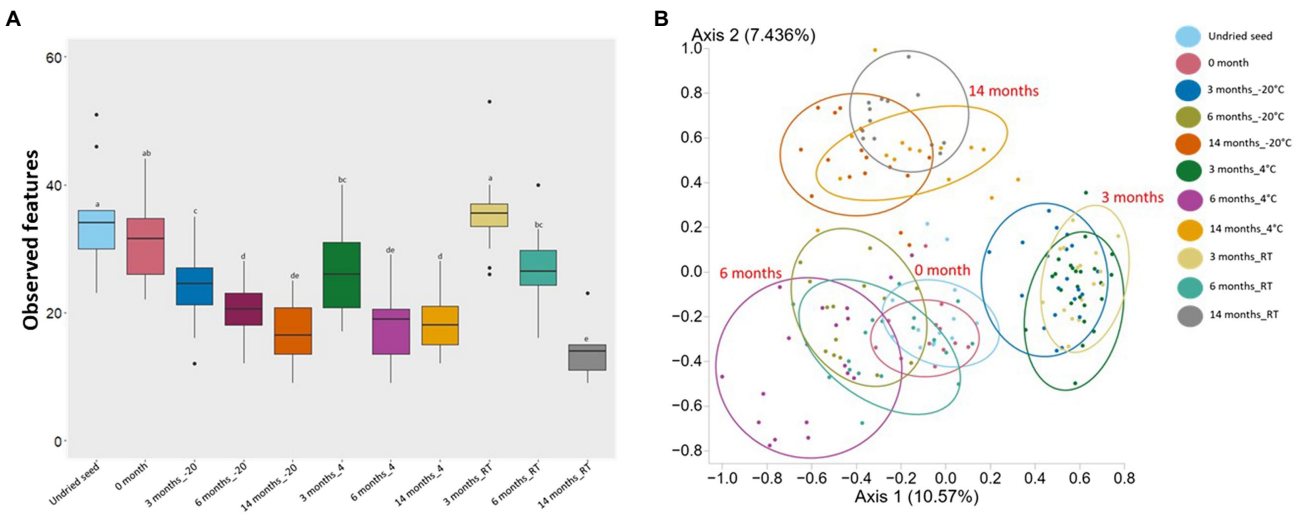

FIGURE 2 | Alpha diversity (Observed features) and Beta diversity analysis (Jaccard dissimilarity) of the *G. max* seed (*in planta*). **(A)** Box-and-whisker plots showing the number of features observed under all conditions. Significant differences ($p \leq 0.05$) were assessed by ANOSIM pairwise test and are indicated by different lowercase letters (**Supplementary Table S3**). **(B)** Principal Coordinates Analysis (PCoA) plot showing the distances in the bacterial communities between undried seed and dried seed (0, 3, 6, and 14 months) stored at −20°C (±2°C), 4°C (±2°C), and room temperature (RT; 22 ± 2°C). Significant differences in bacterial composition were tested using the ANOSIM pairwise test (**Supplementary Table S3**).

at −20°C. Remarkably, after 6 months of storage, there were 72.9% genera (35 genera) shared between undried seed and seed stored at RT followed by 64.6% (31) with seed stored at 4°C and 54.2% genera (26) with seed stored at −20°C. Next, after 14 months of storage, there were 45.8% genera (22) shared between undried seed and seed stored at 4°C followed by 43.8% genera (21) seed stored at −20°C and 35.4% genera (17) seed stored at RT (**Supplementary Figure S3**).

Culturability of the *G. max* Seed Bacterial Microbiome (On Plates)

At the class level, the bacterial communities were mainly dominated by the presence of *Gammaproteobacteria* (0.7–91.1%), *Alphaproteobacteria* (0.3–42.4%), *Bacilli* (1.2–18.1%), *Actinobacteria* (0.3–31%), and *Bacteroidia* (0.0–7.8%). In contrast to the microbiome profiling (*in planta*), after 3 months at RT (on plates), the relative abundance of bacterial classes was dominated by *Alphaproteobacteria* (42.4%), followed by *Actinobacteria* (31%), *Bacilli* (18.1%), *Bacteroidia* (7.8%), and *Gammaproteobacteria* (0.7%) (**Figure 3A**).

At the genus level, 18 bacterial genera were identified with >0.1% relative abundance. The bacterial communities on plates mainly consisted of genera *Pantoea* (0.2–87.3%), followed by *Pseudomonas* (0.0–40.7%), *Curtobacterium* (0.3–30.4%), *Rhizobium* (0.0–26.1%), *Bacillus* (0.01–17.9%), *Paenibacillus* (0.2–11.2%), *Novosphingobium* (0.0–9.3%), *Sphingomonas* (0.004–6.7%), *Siphonobacter* (0.0–4.8%), and *Mucilaginibacter* (0.0–3.0%). The relative abundance of these bacterial genera varied across all the time points and storage temperatures (**Figure 3B**). Similar to the microbiome profiling (*in planta*), a dramatic change in the relative abundance of bacterial genera was observed after 3 months at RT (on plates). The distribution of abundance of these was different from that of the "*in planta*" data, with the most abundant bacteria belonging to *Curtobacterium* (30.4%), *Rhizobium* (26.1%), *Bacillus* (17.9%), *Novosphingobium* (9.3%), *Sphingomonas* (6.7%), *Siphonobacter* (4.8%), and *Mucilaginibacter* (3.0%). However, the relative abundance of *Pantoea* (0.2%) was lesser than the other time points, similar to *in planta* data (**Figure 3B**).

When compared to the 16S rRNA gene sequencing data (*in planta*), not all the bacterial taxa (at genus level) were culturable. The overall culturability of the bacterial genera was more consistent at −20°C storage compared to the seed stored at 4°C and RT (**Figure 4A**). Many of the bacterial genera present with >0.1% relative abundance in undried seed (*in planta*) were culturable, with −20°C providing a more stable recovery compared to 4°C and RT after 14 months of storage (**Figure 4B**).

In total, 48 genera were associated with undried seed (*in planta*), of which 27 genera were culturable under the conditions of this experiment. There were 16 genera present with >0.1% relative abundance in undried seed (**Supplementary Table S1**), of which 13 genera were culturable with the exceptions being *Burkholderia* (0.6%), *Hymenobacter* (0.2%), and *Unidentified_Yersiniaceae* (0.1%) (**Figure 5A**). There were only five bacterial genera including *Pantoea*, *Sphingomonas*, *Bacillus*, *Curtobacterium*, and *Paenibacillus* detected on plates under all conditions. Notably, these were also some of the abundant genera *in planta*. While the presence of other bacterial genera on plates varied across all time points and storage temperatures (**Figure 5A**).

Of note, the abundance of *Massilia* declined when stored at −20°C *in planta* and was also not detected on plates from

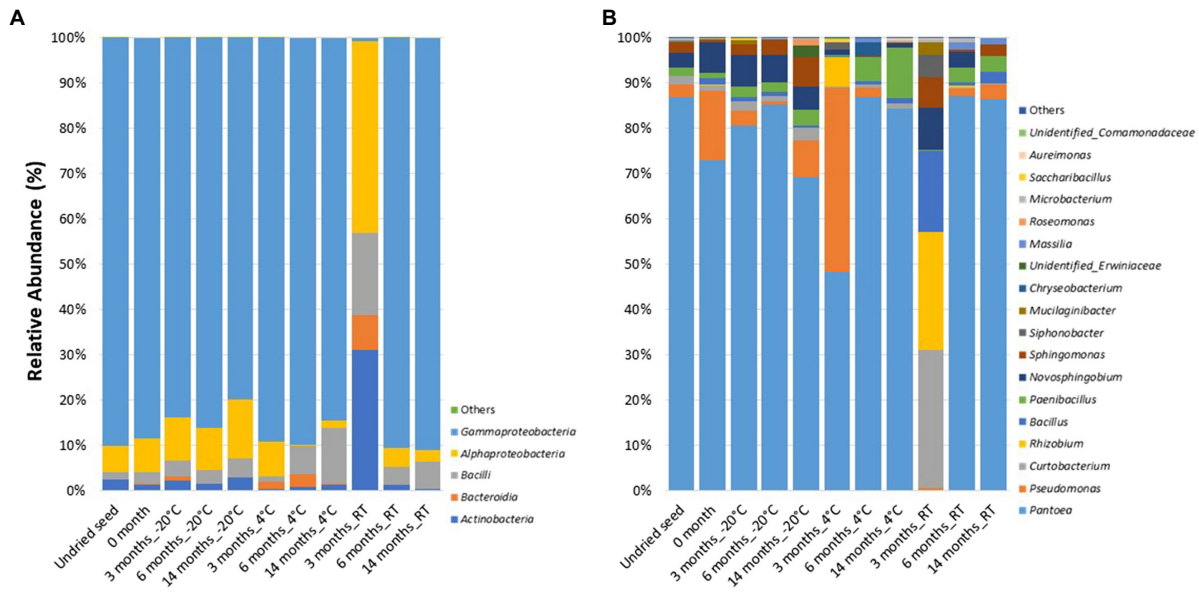

FIGURE 3 | Relative abundance of bacterial taxa on plates **(A)** at class level and **(B)** at genus level in undried seed and dried seed stored at different time points (0, 3, 6, and 14 months) at −20°C, 4°C, and room temperature (RT). The bacterial taxa occurring with less than 0.1% are shown as "Others."

these seeds, showing its sensitivity to cold storage conditions (**Figure 5A, Supplementary Figure S4**). It was observed that some of the low-abundance bacterial genera (<0.1%) were also culturable including some unidentified bacteria (**Figure 5B, Supplementary Table S2**). On the other hand, some genera that were below the level of detection in undried seed and dried seed (0 month) *in planta* including *Advenella*, *Aureimonas*, *Chryseobacterium*, *Uncultured_Spirosomaceae* and *Saccharibacillus* were detected on plates from varying storage temperatures (**Figure 5B**).

Effect of Drying on the *G. max* Seed Microbiota

The drying treatment of *G. max* seed at 15°C and 15% relative humidity for 1 month was found to alter the relative abundance

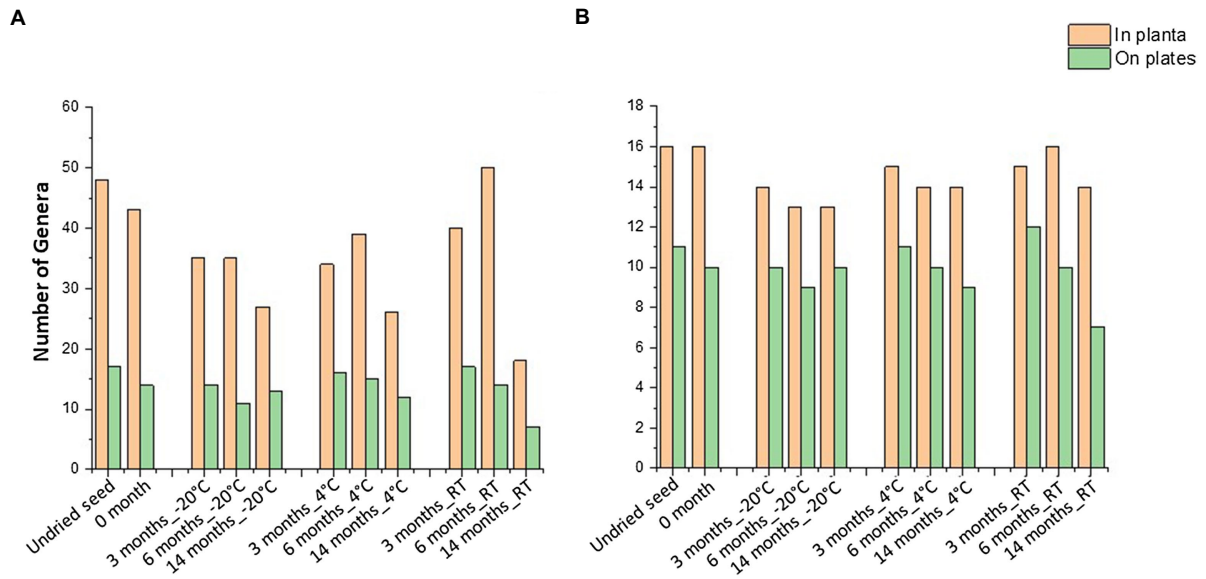

FIGURE 4 | Culturable seed bacterial microbiome. **(A)** Number of genera observed *in planta* and on plates. **(B)** Number of genera with >0.1% abundance in undried seed and their culturability over time at different storage temperatures. All the seed samples belong to the undried seed, dried seed (0 month), and seed stored for 3, 6, and 14 months at −20°C, 4°C, and room temperature (RT).

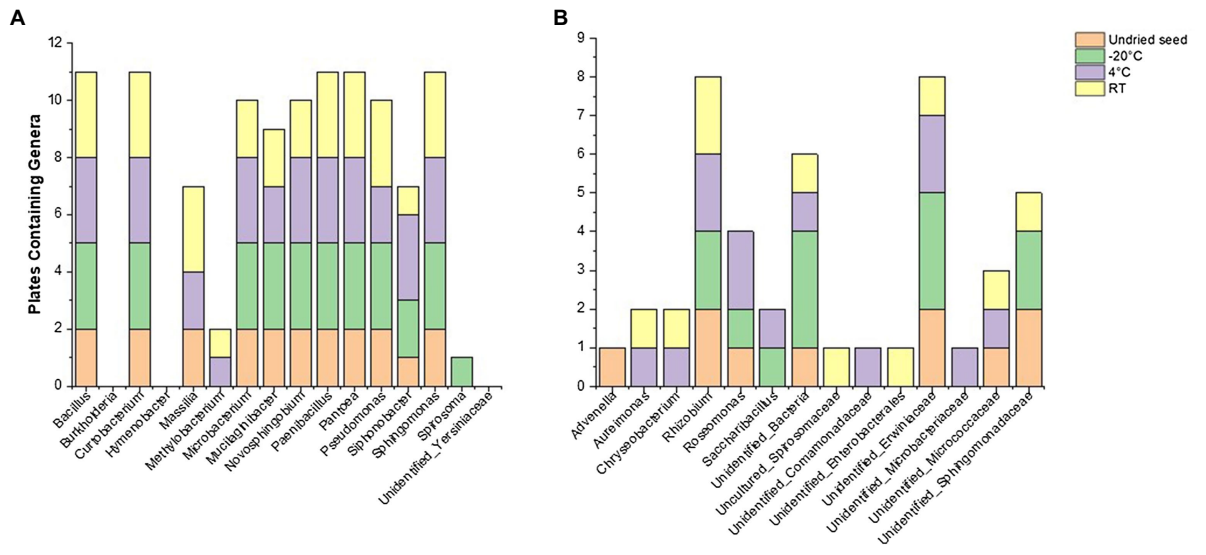

FIGURE 5 | Culturability of bacterial genera that were associated with undried seed (*in planta*) across different time points (0, 3, 6, and 14 months) when stored at −20°C, 4°C, and room temperature (RT). **(A)** Genera with >0.1% relative abundance and **(B)** <0.1% relative abundance in undried seed (*in planta*).

of bacterial genera. The seed microbiome profiling (*in planta*) showed a significant change in the abundance of bacterial genera after drying including *Pseudomonas* (51.1 to 0.9%), *Pantoea* (33.9 to 73.6%), *Curtobacterium* (1.0 to 9.5%), *Sphingomonas* (2.5 to 0.7%), *Massilia* (1.6 to 0.005%), *Methylobacterium* (0.3 to 0.09%), and *Unidentified_ Erwiniaceae* (0 to 0.03%) (**Figure 6A, Supplementary Table S5**). There were four low-abundance genera including *Pajaroellobacter*, *Nesterenkonia*, env.OPS_17, and *Acidibacter* that completely disappeared after seed drying (**Supplementary Table S1**).

In contrast, no significant differences were observed for bacterial abundance on plates except for *Unidentified_ Erwiniaceae* (0.1 to 0.004%) (**Figure 6B, Supplementary Table S5**). The culturing assays, however, showed a different pattern for *Pseudomonas* (2 to 15%) and *Pantoea* (86.9 to 73.0%). It was observed that one replicate plate for dried seed (0 month) was equally dominated by *Pantoea* (47.0%) and *Pseudomonas* (45.7%), contributing to an increased abundance of *Pseudomonas* (15.3%) and decreased abundance of *Pantoea* (73.0%) on plates post drying, indicating that the microbial diversity could vary from seed to seed to some extent (**Figure 6B, Supplementary Table S4**).

DISCUSSION

Seed banks have been created to preserve plant and crop genetic diversity for future use. However, the value of current seed storage techniques in conserving seed-borne microbial diversity has not been investigated (Berg and Raaijmakers, 2018). In this study, we demonstrated that drying *G. max* seed before storage changes the abundance and composition of the seed microbiota. Moreover, we found that the different bacterial communities respond differently to the seed drying and storage temperature. Additionally, the seed bacterial composition changed more dramatically under RT storage than the cold storage (−20 and 4°C). The culturability of seed-associated microbes was found to be largely driven by abundance. Seed storage at −20°C provided a long-term stable recovery of the culturable microbes under the conditions of this experiment.

G. max Seed Microbiome Composition

Soybean seeds are known to lose viability and vigor under high temperature and relative humidity conditions (Shelar et al., 2008). Nevertheless, in this study, the impact of such environmental factors was reduced due to the optimal and stable conditions during a 14-month storage period. In general, the *G. max* seed microbiome consisted of bacterial classes such as *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacilli*, *Bacteroidia*, and Actinobacteria. Previous studies also observed these bacterial classes for the seed of red sage (Chen et al., 2018), bean (Klaedtke et al., 2016), ryegrass (Tannenbaum et al., 2020), rice (Nakaew and Sungthong, 2018), native alpine plants (Wassermann et al., 2019a), and *Brassicaceae* family plants (Barret et al., 2015). It has been demonstrated that the microbes colonizing seedling during germination can confer important functional traits to the plant such as nutrient availability (Torres-Cortés et al., 2018). Representatives of the majority of the detected bacterial genera including *Pantoea*, *Pseudomonas*, *Bacillus*, *Sphingomonas*, *Curtobacterium*, *Paenibacillus*, *Mucilaginibacter*, *Novosphingobium*, and *Massilia* are known to have a beneficial impact on plants. For instance, *Bacillus* strains isolated from soybean root nodules have been observed to promote soybean plant growth when

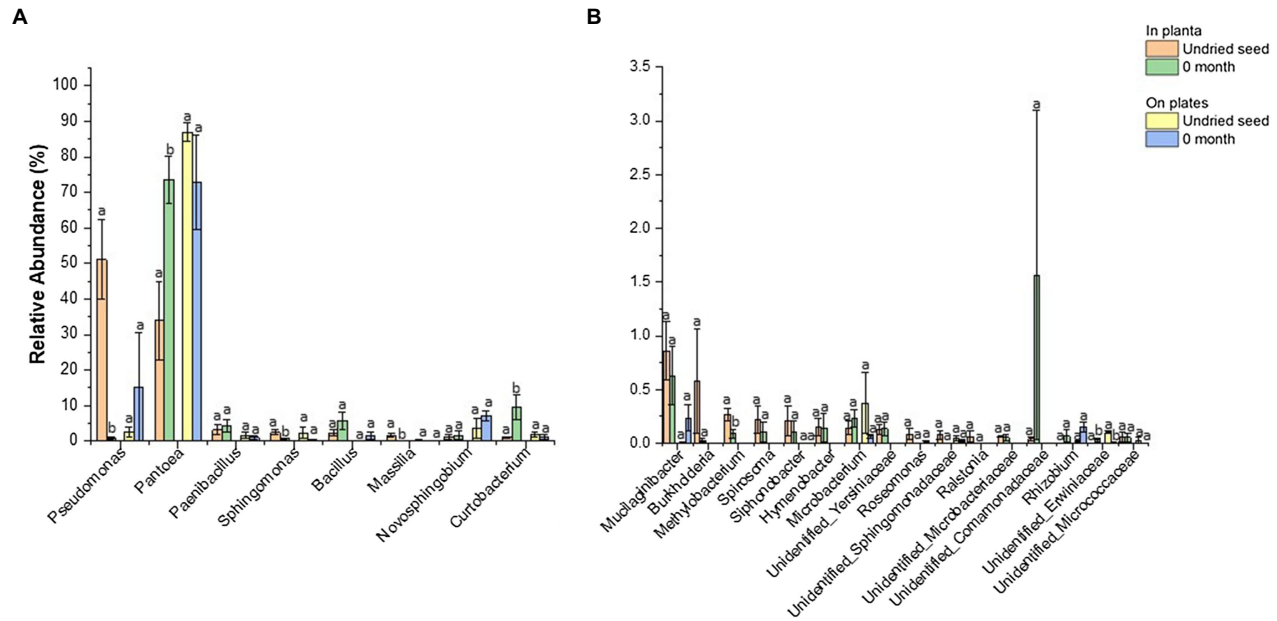

FIGURE 6 | Comparison of relative abundance of bacterial genera detected in undried seed and dried seed (0 month) *in planta* and on plates dataset. The abundance threshold was set based on *in planta* dataset (**A**) >1% and (**B**) <1% and >0.1% for statistical analysis. Significant differences ($p \leq 0.05$) were determined by one-way ANOVA test and are indicated by different lowercase letters. Error bars show SE of mean (SEM).

co-inoculated with *Bradyrhizobium japonicum* under nitrogen-free conditions (Bai et al., 2003). Also, some *Pseudomonas* species isolated from the soybean rhizosphere inhibited the growth of soilborne pathogenic fungi (Susilowati et al., 2011). However, other species of *Pseudomonas* are well known for their pathogenicity (Xin et al., 2018; Solanki et al., 2019). The endophytic bacteria from the soybean root nodules were also identified to contain plant growth-promoting traits and antagonistic properties against pathogenic fungi (*Phytophthora sojae*; Zhao et al., 2018).

Effect of Seed Drying on *G. max* Seed Microbiome Composition

Seed banks globally follow seed drying treatment prior to low-temperature storage to increase seed longevity (Hay and Probert, 2013). While this treatment is valuable for increasing seed longevity in storage, there is little evidence about the effect of seed drying on seed microbiome conservation. In this study, both the seed drying and storage temperature were identified to affect the seed bacterial composition. Some of the abundant bacteria, including *Pseudomonas*, *Sphingomonas*, *Massilia*, *Curtobacterium*, and *Methylobacterium* declined significantly after seed drying treatment. This change in composition corresponded to a significant increase in abundance of *Pantoea* from an average of 33.9% to more than 73%, with four bacterial genera remaining undetected. It was recognized that temperature, humidity, water activity, and grain moisture could affect the seed microbial community (Schmidt et al., 2018). It must be stated that the bacterial communities can respond differently to the water stress caused during the seed drying process (Esbelin et al., 2018). The exclusive increase in the abundance of *Pantoea* after seed drying may indicate their ability to tolerate the stress caused due to water loss. *Pantoea* spp. along with *E. sakazakii*, *E. vulneris*, and *K. oxytoca* were reported to persist over 2 years when individual bacterial strains were subjected to desiccated storage. This ability was attributed to the formation of an extracellular polysaccharide that can facilitate the survival of bacterial strains during an extended desiccation period (Lehner et al., 2005; Barron and Forsythe, 2007). Notably, the results obtained with culturing assays of microbes isolated from undried seed and dried seed (0 month) were in general agreement with results obtained by culture-independent 16S rRNA gene sequencing data obtained *in planta*. However, an opposite pattern was observed for the relative abundance pattern of *Pantoea* and *Pseudomonas* between the culturing assay and the *in planta* assay. Interestingly, it was identified that the differences in bacterial abundance among seed samples were responsible for this variation. This was also in line with previous studies that have identified that relative abundance of bacterial inhabitants of seed can vary significantly between seed samples of the same plant species (Barret et al., 2015; Klaedtke et al., 2016; Rybakova et al., 2017; Rezki et al., 2018; Torres-Cortés et al., 2018). It has been reported that other than plant genotype, abiotic factors such as storage, harvesting methods, and field management practices were identified as possible drivers of such variations in bacterial abundance among seed samples (Barret et al., 2015).

Effect of Storage Temperature on *G. max* Seed Microbiome Composition

In the present study, *Gammaproteobacteria* dominated the *G. max* seed bacterial microbiome under all conditions. Notably, this was due to an increased abundance of *Pantoea* after seed drying compared to other abundant genera including *Pseudomonas*, *Sphingomonas*, *Mucilaginibacter*, *Bacillus*, and *Curtobacterium*. Endophytic strains of *Pantoea* isolated from the surface-sterilized leaves of *Alhagi sparsifolia Shap*. and wheat roots have been shown to improve plant growth under drying conditions (Chen et al., 2017; Cherif-Silini et al., 2019). A strain of *Pantoea dispersa* (Selvakumar et al., 2008) isolated from a sub-alpine soil was able to grow under different temperature conditions ranging from 4 to 42°C. Notably, more variations in bacterial abundance were observed in seed stored at RT. For instance, after 3 months of storage at RT, the abundance of genera including *Sphingomonas*, *Mucilaginibacter*, *Bacillus*, and *Curtobacterium* collectively accounted for about 67% of the total bacterial abundance, which was higher than that at other time points. Interestingly, this change corresponded with a significant decrease in *Pantoea* from an average of 62.6% in undried seed and dried seed (0 month) and seed stored for 6 and 14 months to only 1.8% after 3 months at RT. The bacterial strains belonging to the genera *Sphingomonas*, *Mucilaginibacter*, *Curtobacterium*, and *Bacillus* were identified to be more tolerant to drying conditions (Mannisto et al., 2010; Vardharajula et al., 2011; Chimwamurombe et al., 2016; Molina-Romero et al., 2017). Thus, it is highly plausible that these bacterial strains might be benefited by the sudden environmental changes created by seed drying and packaging. It must be stated that the majority of these genera are either aerobic or facultative anaerobes. It is highly possible that a reduction in available oxygen level due to an airtight heat-sealed packaging along with the drying conditions promoted favorable conditions for these genera. Moreover, this study showed that such changes in abundance were only for a limited period and the bacterial communities were more similar to the pre-drying conditions after 6 months in all storage temperatures. Šťovíček et al. (2017) in their study showed that certain anaerobic taxa in soil dominated after a rainfall, but after 5–7 days, the microbial composition returned to the pre-rainfall conditions. A similar trend was also reported by Supramaniam et al. (2016) who reported a shift in soil bacterial composition initially after a short variation in temperature and water content, though the bacterial composition was more similar to the control after 4 weeks. A similar trend was also observed for the cultured bacterial taxa isolated from the stored *G. max* seed after 3 months at RT, though the dominating bacterial genera varied compared to the *in planta* data. The differences in the bacterial abundance among individual seed samples were identified to contributing to these variations as observed for the seed drying in the above section (*Effect of Seed Drying on G. max Seed Microbiome Composition*). Notably, such variations in the abundance of bacterial genera at RT were not reflected in seed under cold storage.

We have shown that the seed bacterial diversity and composition co-vary with time and storage temperature. Results indicated that the seed can be stored at RT for 6 months without losing

much diversity and original bacterial composition. While the bacterial diversity and composition reduced rapidly after 6 months. The disappearance of the lower-abundance bacterial genera was observed as the major reason for these diversity losses during storage. Seed storage at −20°C was identified as showing a gradual disappearance of the lower-abundance genera compared to more rapid losses at 4°C and RT. Soybean seed is known to go through various biochemical changes, such as decreases in fat, water-soluble nitrogen, sugars, nitrogen solubility index, trypsin inhibitor activity, available lysin, pigment, and lipoxygenase activity of seed and increases in seed browning, free fatty acid content, and peroxidase value when stored under ambient conditions (Narayan et al., 1988; Sharma et al., 2013). For instance, the increased level of free fatty acid in soybean seed invaded by *Aspergillus ruber* resulted in loss of seed viability (Dhingra et al., 2001). In our study, the significant decline in bacterial diversity and composition in *G. max* seed, specifically the loss of lower-abundance genera at RT and 4°C, might be linked to an increased level of free fatty acid. It has been identified that free fatty acid can kill bacteria by inhibiting enzyme activity, disrupting nutrient uptake, and lysing bacterial cells directly or indirectly (e.g., toxic peroxidation and autoxidation products; Desbois and Smith, 2010).

Effect of Storage Temperature on the Culturability of *G. max* Seed Microbiome

Seed-associated microbes have the potential to promote plant growth and to provide sustainable ways to protect crops against various biotic and abiotic stresses in the form of seed treatments. Seed banks can play an important role in conservation of the beneficial seed-associated microbes. However, there is a need to design international conservation strategies for seed banks to protect seed microbes so that their untapped benefits for plant, human, and environment can be further explored in the future (Berg and Raaijmakers, 2018). Culturing of the seed microbes independently of stored seed has been suggested as a necessary step to ensure that the beneficial microbes remain available to use them for enhancing crop productivity (Rascovan et al., 2016; Sarhan et al., 2019). To understand the importance of independent microbe culturing, we decided to examine the impact of seed storage temperature on the culturability of microbes. Overall, the results obtained with culturing assay of the stored *G. max* seed agreed with results obtained by *in planta* 16S rRNA gene sequencing data. In the current study, the isolated bacteria mainly belonged to the *Gammaproteobacteria* and *Alphaproteobacteria*, with *Pantoea*, *Sphingomonas*, *Bacillus*, *Curtobacterium*, *Rhizobium*, and *Paenibacillus* being some of the dominant bacterial genera isolated from *G. max* seed throughout the study. Notably, most of these genera have been characterized with a range of beneficial features such as the ability to fix nitrogen, indole-3-acetic acid production, and antagonistic abilities against various bacteria and fungi (Silini-Cherif et al., 2012; Hansen et al., 2017; Goyal et al., 2019; Liu et al., 2019). Data indicated that the genera present with more than 0.1% relative abundance in undried seed remained culturable after 14 months of storage under all conditions. Many of the bacterial genera identified by 16S rRNA gene sequencing data *in planta* were culturable; however, not all of them were identified in culturing assays. These results were consistent with previous findings, where majority of the microbes identified by sequencing were not detected using classical culturing approach (Sylla et al., 2013; Schmidt et al., 2014; Qaisrani et al., 2019; Solanki et al., 2019). Notably, the cold storage temperatures, especially −20°C storage provided a stable recovery for the bacterial genera present with greater than 0.1%, while the bacterial viability was adversely affected in seed stored at RT. Cabello-Olmo et al. (2020) investigated the effect of storage temperature and packaging on bacteria and yeast viability in a plant-based fermented food and indicated that the microbial content seemed to be better preserved at −20 and 4°C compared to storage at 37°C. We also observed that the genera *Massilia* was sensitive to cold storage. Especially at −20°C storage, their relative abundance gradually declined *in planta* and was poorly represented in the culturing assay. A significant reduction in the abundance of *Massilia* was also observed in apples after they were stored for 6 months in a commercial cold storage. The cold sensitivity of *Massilia* was suspected as the main reason for the significant decline in abundance (Wassermann et al., 2019b).

In conclusion, our study demonstrated that standard storage methods can be used for conservation of seed-associated bacterial microbiome, especially for high-abundance genera. Given that seed drying significantly impacts the composition of *G. max* seed microbiome, we suggest that a fresh bacterial isolation can help to conserve the original bacterial composition. Moreover, −20°C storage has been identified as a better alternative to RT and 4°C, as the overall bacterial diversity losses including lower-abundance genera were reduced, and culturability rate was high in −20°C storage. A better understanding about the effect of the standard storage methods on the seed microbiome composition of different plant species including their wild relatives can assist in designing new international conservation strategies for seed microbiomes.

DATA AVAILABILITY STATEMENT

The raw sequence files supporting the findings of this article are available in the NCBI Sequence Read Archive (SRA) under the BioProject ID PRJNA766782.

AUTHOR CONTRIBUTIONS

TS conceptualized the study. AC prepared the article. AC, TS, JE, and RM designed the experiment. SN provided the soybean seed and helped with the seed drying. AC and JK contributed to the laboratory work. TS and RM reviewed and edited the article. TS, JE, and RM supervised the study. GS contributed to the funding acquisition. All authors have read and agreed to the submitted version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.784796/full#supplementary-material

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Seed Endophytic Bacteria of Pearl Millet (*Pennisetum glaucum* L.) Promote Seedling Development and Defend Against a Fungal Phytopathogen

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Seed endophytic bacteria (SEB) are primary symbionts that play crucial roles in plant growth and development. The present study reports the isolation of seven culturable SEB including *Kosakonia cowanii* (KAS1), *Bacillus subtilis* (KAS2), *Bacillus tequilensis* (KAS3), *Pantoea stewartii* (KAS4), *Paenibacillus dendritiformis* (KAS5), *Pseudomonas aeruginosa* (KAS6), and *Bacillus velezensis* (KAS7) in pearl millet seeds. All the isolates were characterized for their plant growth promoting activities. Most of the SEB also inhibited the growth of tested fungal phytopathogens in dual plate culture. Removal of these SEB from seeds compromised the growth and development of seedlings, however, re-inoculation with the SEB (*Kosakonia cowanii*, *Pantoea stewartii*, and *Pseudomonas aeruginosa*) restored the growth and development of seedlings significantly. Fluorescence microscopy showed inter and intracellular colonization of SEB in root parenchyma and root hair cells. Lipopeptides were extracted from all three *Bacillus* spp. which showed strong antifungal activity against tested fungal pathogens. Antifungal lipopeptide genes were also screened in *Bacillus* spp. After lipopeptide treatment, live-dead staining with fluorescence microscopy along with bright-field and scanning electron microscopy (SEM) revealed structural deformation and cell death in *Fusarium* mycelia and spores. Furthermore, the development of pores in the membrane and leakages of protoplasmic substances from cells and ultimately death of hyphae and spores were also confirmed. In microcosm assays, treatment of seeds with *Bacillus subtilis* or application of its lipopeptide alone significantly protected seedlings from *Fusarium* sp. infection.

Keywords: pearl millet, seed endophytic bacteria, plant growth promotion, biocontrol, lipopeptides, live-dead staining

INTRODUCTION

Plants have evolved with continuous interaction with diverse microorganisms. Many of these microorganisms actively colonize into the endospheric compartment of plants as endophytes and provide benefits to plants (Glassner et al., 2018; White et al., 2019). Endophytes, mostly bacteria and fungi, are frequently reported from all parts of the plant including root, stem, leaves, fruits, and seeds. Endophytes play an essential role in every stage of plant development and adaptation to various ecological conditions (Márquez et al., 2007; Llorens et al., 2019). In the recent past, many crop seeds including maize, wheat, rice, millets, cotton, etc., were reported to host endophytic bacteria (Gond et al., 2015; Herrera et al., 2016; Irizarry and White, 2017; Verma et al., 2017; Verma and White, 2018). Seed endophytic bacteria (SEB) are believed to have more influence on the development of plant because they can be transmitted to the next generation and become the first colonizers of roots and shoots of the seedlings after germination (Johnston-Monje and Raizada, 2011; Verma et al., 2019). Due to positional advantage, SEB may influence plant growth and fitness starting from seed germination to seedling formation and over time continue to influence plant development. SEB have been reported to increase the process of germination (Pitzschke, 2016), and plant growth by producing auxin, ethylene, mobilizing various nutrients (N, P, K, etc.) and producing siderophores (Ruiza et al., 2011; Verma and White, 2018; Maheshwari et al., 2019; Soldan et al., 2019; Kumar et al., 2020; Chang et al., 2021). Endophytes protect developing seedlings from soil pathogens by producing antimicrobial compounds; for example, *Bacillus* spp. produce antifungal lipopeptides including iturins, fengycins, surfactins, and bacillomycin whereas pseudomonads are known to produce antimicrobial metabolites like HCN, pyrrolnitrin and phenazine (Malfanova et al., 2012; Gond et al., 2015; Li et al., 2015; Verma and White, 2018). Endophytic bacteria also increase plant fitness indirectly by inducing or modulating plant gene expression related to growth development and defense (Gond et al., 2015; Mousa et al., 2016; Irizarry and White, 2018; Khalaf and Raizada, 2018). In comparison to other plant-associated microbes, SEB are more competent in benefiting hosts, but very little has been explored regarding their mechanisms of colonization during a seedling's formation, or their functional roles in seedling development and protection. To the best of our knowledge, this is the first report which describes the role of pearl millet's seed inhabiting bacteria on seedling establishment and protection. Pearl millet (*Pennisetum glaucum* L.) is an important, annual, small grain, warm season crop belonging to the family Poaceae, widely cultivated and consumed in tropical and subtropical countries of the world (Kumar et al., 2016). Pearl millet is a highly nutritious crop. Seeds contain high amounts of proteins, minerals (iron, zinc, sodium, phosphorus, and magnesium), vitamin B complexes, and high fiber (Kumar et al., 2016). In the present study, we hypothesized that seeds of pearl millet might be inhabited by endophytic bacteria that play a crucial role during the early development of seedlings, and also protect them from fungal pathogens. In this report, seven endophytic bacteria were isolated from pearl millet seeds and all the isolates were evaluated for their plant growth promoting and biocontrol activities. We found that the removal of bacteria from the seeds compromised the seedling's development, and when we re-inoculated with the same bacteria, seedling development was restored. Colonization of SEB in root tissues was observed by fluorescent microscopy. In the seedling protection assay in microcosm, we found that treatments with *Bacillus subtilis* and its lipopeptides inhibited the growth of *Fusarium* and protected the seedlings from its infection. Using bright field, fluorescent and, scanning electron microscopy (SEM), we examined the lipopeptide effects on fungal hyphae and spores.

MATERIALS AND METHODS

Plant Material

Pearl millet seeds were procured from B&B Organics Company, Tamil Nadu, India, and stored at 4°C in the refrigerator. Seeds were collected by B&B Organics Company from an agricultural field in Theni, Tamil Nadu (10.0104°N, 77.4768°E).

Surface Sterilization and Disinfection of Seeds

Pearl millet seeds were surface sterilized by soaking seeds in 4% NaOCl solution for 10, 20, and 40 min. with constant shaking and after that, seeds were washed with sterile distilled water then transferred to 70% ethanol for one min. Then the seeds were washed several times with sterile distilled water. To check the efficacy of surface sterilization, 100 μl water from the last wash was transferred onto nutrient agar media. 10 min −4% NaOCl treatment was found efficient for surface sterilization. For complete disinfection of seeds, 10 min surface-sterilized seeds were dipped in streptomycin sulfate solution (100 μg ml^{-1}) for different time periods including 6, 8, and 12 h; after that, seeds were washed thoroughly with sterile distilled water.

Isolation, Molecular Identification and Phylogenetic Analysis of Seed Endophytic Bacteria Isolated From Pearl Millet Seeds

Around 100 surface-sterilized seeds of different time intervals (10, 20, and 40 min) were plotted onto the nutrient agar plates (8–10 seeds per plate) and incubated in a BOD incubator for 3–5 days at 27 ± 2°C. Bacterial colonies emerging around the seeds were sub-cultured. Based on growth pattern and color, bacterial isolates were selected and purified. All the purified isolates were preserved in 20% glycerol at −20°C freezer in the Department of Botany, BHU, Varanasi.

For molecular identification, all the pure bacterial isolates were grown in a nutrient broth with constant shaking with 200 rpm on a rotary shaker at 27 ± 2°C. Day-old bacterial cultures were used for genomic DNA extraction. For that, 1.5 ml bacterial cultures were centrifuged at 10,000 rpm for 2 min at 4°C and bacterial pellets were washed with de-ionized water to remove any metabolites. Bacterial genomic DNA was extracted by using Wizard® Genomic DNA purification kit (Promega,

United States) and the 16S rDNA conserved sequences were amplified using 16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SR (5′-CTACGGCTACCTTGTTACGA-3′) primers. The PCR amplification was carried out by using T100 Thermal Cycler (Bio-Rad); the total volume of PCR reaction mixtures was 25 μl containing 12.5 μl of 2X PCR master mix (Promega, United States), 2 μl of 10 μM concentration of each primer, 5 μl DNA template (40 ng μl^{-1}) and 3.5 μl nuclease free water. The following conditions were used for PCR amplification; initial denaturation 95°C for 5 min., followed by 35 cycles of denaturation step at 95°C for 1 min., annealing step at 55°C for 1 min. and extension step at 72°C for 1.5 min; final extension was at 72°C for 10 min. After PCR amplification, PCR products were visualized on 1% agarose gel with 1X TAE buffer. Purification and sequencing of amplified PCR products were done at AgriGenome Labs Pvt. Ltd. (Cochin, Kerala). The sequences were identified using BLASTn and closest matches were found by comparing the sequences with those in the NCBI GenBank database. Based on 16s rDNA sequences of SEB of pearl millet, the closest bacterial sequences were downloaded from the NCBI. All the nucleotide sequences were aligned by Clustal W and then the phylogenetic tree was constructed by the neighbor-joining (NJ) method using MEGA 11 software (Tamura et al., 2021). A bootstrap analysis was carried out with 1,000 repeats using the same software to test the phylogenetic tree's reliability.

Characterization of Seed Endophytic Bacteria for Plant Growth Promoting and Enzymatic Activities

Overnight bacterial cultures were streaked onto Pikovskaya agar media (Pikovskaya, 1948) and Aleksandrow agar media (Aleksandrov et al., 1967) and incubated for four days; and clear zone around the colonies was taken as confirmation of phosphate and potassium solubilization, respectively. Auxin (IAA) production was assessed by the method given by Gordon and Weber (1951). For that, all the bacterial isolates were grown in nutrient broth (5 g peptone, 1.5 g yeast extract, 1.5 g beef extract, 5 g sodium chloride, 1,000 ml distilled water) with or without tryptophan (100 $\mu g\ ml^{-1}$). Four-day-old bacterial broth cultures were centrifuged at 4,500 rpm for 5 min. then 1 ml of culture supernatant and 2 ml of freshly prepared Salkowski reagent (1 ml of 0.5 mol l^{-1} $FeCl_3$ was added to 50 ml sterile distilled water then finally mixed with 30 ml of H_2SO_4) were mixed together and incubated for 30 min., after which optical density (absorbance) was assessed at 530 nm using a spectrophotometer (Modal U-2900, Hitachi, Japan). IAA produced by endophytic bacteria was measured comparing with a standard curve as described elsewhere (Verma and White, 2018). Siderophore production test of bacterial isolates was performed by using chrome azurol S (CAS) agar plates (Schwyn and Neilands, 1987). Catalase activity was evaluated by using the tube slant method (MacFaddin, 2000). For that, 1 ml of 3% H_2O_2 was directly poured onto overnight grown bacterial cultures on nutrient agar slants and was placed over a dark background to observe bubble formation. For amylase activity, overnight bacterial cultures were streaked onto GYP agar plates (1 g glucose, 0.1 g yeast extract, 0.5 g peptone, 1.5% agar, 1,000 ml distilled water) supplemented with 1% starch (Hankin and Anagnostakis, 1975) and incubated for 5 days at 27 ± 2°C; after that, all plates were stained with iodine solution (1% iodine was mixed with 2% potassium iodide); the un-stained area around the bacterial isolate was measured. For cellulase activity, overnight bacterial cultures were streaked onto yeast extract peptone agar (yeast extract 0.1 g, peptone 0.5 g, agar 1.5%, distilled water 1,000 ml) containing 0.5% CMC (Na-carboxymethyl cellulose) (Teather and Wood, 1982). Five-day-old culture plates were stained with 0.2% (w/v) congo-red solution for 20 min and de-stained for 20 min with 1 M NaCl; the un-stained zone around the colony was measured. Pectinase activity of bacterial isolates was evaluated by the protocol described by Aguilar and Huitrón (1990). Bacterial isolates were inoculated onto pectin agar (5 g pectin, 1.5% agar, 1,000 ml distilled water) plates; after 5 days of incubation, plates were flooded with 1% (w/v) CTAB aqueous solution for 20 min. and cleared zones around colonies were observed to confirm pectinase activity. For protease activity, bacterial isolates were transferred onto GYP agar plates containing 0.4% gelatin (Sunitha et al., 2013) and after 5 days of incubation, saturated aqueous ammonium sulfate was flooded onto plates. The cleared zone around bacterial growth was measured. For chitinase activity, colloidal chitin was prepared by the method described by Hsu and Lockwood (1975). Chitin medium was prepared by adding 1% (w/v) colloidal chitin with 0.5% peptone, 0.1% yeast extract, and 1.5% agar in 80 ml sterile water and the final volume was maintained 100 ml. Overnight grown bacterial isolates were streaked on chitin plates and incubated for 5 days to check for clearing zones around colonies as positive indication of chitinase activity.

Standardization of Growth Media for Re-inoculation Experiments

Several media including 1.5% agar, sand, filter paper, potting mix in magenta boxes including mixed substrates (cocopeat: perlite: sand) in two different ratio (2:1:1 and 2:2:1) were prepared. After that, various treatments including (1) completely disinfected seeds were (surface sterilized with 4% NaOCl and then treated with streptomycin sulfate-100 $\mu g\ ml^{-1}$ for 8 h) then re-inoculated with selected SEB (10^6–10^8 cell ml^{-1}) as treatments; (2) only surface-sterilized seeds as positive control, and (3) one set of completely disinfected seeds as negative control were transferred onto above mentioned growth media and incubated for 7–10 days at 27°C. All experiments were done in triplicate. The best medium was selected for further study.

Re-inoculation Experiment With Selected Plant Growth Promoting Seed Endophytic Bacteria in Magenta Box

A potting mix containing cocopeat, perlite and sand in 2:2:1 ratio was found best out of all standardized media and selected for the re-inoculation experiment with SEB having plant growth promoting activities.

The experiment was set up as: (a) surface sterilized seeds with 4% NaOCl only (positive control-surface sterilized seeds),

(b) surface sterilized seeds with 4% NaOCl + 100 μg ml^{-1} streptomycin sulfate (negative control-disinfected seeds) and (c) KAS1 (surface sterilized seeds with 4% NaOCl + 100 μg ml^{-1} streptomycin sulfate then treated with KAS1- *Kosakonia cowanii*) (d) KAS4 (surface sterilized seeds with 4% NaOCl + 100 μg ml^{-1} streptomycin sulfate then treated with KAS4–*Pantoea sterwarti*), and (e) KAS6 (surface sterilized seeds with 4% NaOCl + 100 μg ml^{-1} streptomycin sulfate then treated with KAS6–*Pseudomonas aeruginosa*). Each treatment was done in triplicate.

For re-inoculation of SEB, disinfected seeds (100–120) were treated with 5 ml suspensions of selected SEB (10^6–10^8 cell ml^{-1}) separately for 2 h. Around 100 seeds were transferred into three magenta boxes containing potting mix for each treatment (25–30 seeds in each magenta box). All the magenta boxes were transferred in the plant growth chamber in controlled conditions (photoperiod 12 h, 80–90% humidity, and temp 27 ± 2°C). After 8 days, various growth parameters including root-shoot length, fresh weight, and photosynthetic pigments in leaves of seedlings were measured. Bacteria were re-isolated from roots of bacterial treated and control seedlings to prove that isolated bacteria from roots were the same or different than bacteria used during treatments with seeds. For that, roots were cut by sterile scalpel and transferred onto nutrient agar plates with the help of sterilized forceps and incubated in a BOD incubator for 2 days; afterward bacterial growth around roots was sub-cultured and identified by the molecular method as described earlier.

Estimation of Photosynthetic Pigments

For quantification of photosynthetic pigments, 100 mg of leaf tissues from all treated seedlings were taken in a mortar (contained 15 ml of 80% acetone) and homogenized with a pestle; homogenized mixtures were filtered through a filter (0.45 μm) and absorbance was taken at 663, 645 and 440.5 nm by using UV/Visible spectrophotometer (Modal U-2900, Hitachi, Japan). The photosynthetic pigments including Chl a, b, and carotenoids were calculated by method given by Smith and Benitez (1955) using the following formulas:

Chlorophyll a mg g^{-1} fresh leaf tissue = 12.7 (OD) 663–2.69 (OD) 645 × (v/w × 1000),

Chlorophyll b mg g^{-1} fresh leaf tissue = 22.9 (OD) 645–4.68 (OD) 663 × (v/w × 1000),

Total carotenoids mg g^{-1} fresh leaf tissue = 46.95 (OD) 440.5–0.268 × chlorophyll (a + b).

Microscopic Visualization of Seed Endophytic Bacteria Colonization on/in Root by Using Fluorescent Microscope

SYTO-9 fluorescent dye (Thermo Fisher Scientific, United States) was used for staining both live and dead bacteria which were present onto root parenchyma, root hairs, and inside the root tissues of seedlings. SYTO-9 stain was mixed in sterilized de-ionized water to make the final concentration up to 50 μM and incubated for 30 min. in dark condition at 27°C. Roots from both control and treated seedlings were collected from magenta boxes and cleaned with sterile water. 1–2 cm length size of root was cut by sterilized scalpel and was transferred on the slide then 20 μl (50 μM) of SYTO 9 solution was poured on the root surfaces and incubated for 5 min. in dark conditions after which roots were examined under a fluorescent microscope (Nikon, Japan).

Antifungal Activity of Endophytic Bacterial Isolates

All the bacterial isolates from pearl millet seeds were screened for antagonistic activity against selected fungal phytopathogens including *Fusarium* sp., *Curvularia* sp., *Alternaria* sp., *Rhizoctonia solani*, *Epicoccum sorghinum*, and *Exserohilum rostratum* onto potato dextrose agar media using dual culture technique. After 5 days of incubation in a BOD incubator at 27°C, fungal growth inhibition (%) due to antagonistic bacteria was calculated by the formula described by Whipps (1997):

% inhibition of fungal growth = R1–R2/R1 × 100,

where R1 is the growth of pathogenic fungi on the control plate and R2 is the growth of pathogenic fungi toward the antagonistic bacterial isolates on test plates.

Screening of Lipopeptide Genes in Bacterial Isolates

All the bacterial isolates were screened for selected lipopeptide genes including surfactin, bacillomycin D, iturin A, and fengycin using primers mentioned in **Supplementary Table 3**. The PCR amplification reaction was set up as initial denaturation (5 min at 95°C), followed by 35 cycles of denaturation (45 s at 95°C), annealing (1 min for 55°C), extension (1 min for 72°C), and final extension for 10 min at 72°C. Amplified PCR products were sent for purification and gene-specific DNA sequencing; obtained DNA sequences were identified using the BLASTn program to confirm lipopeptide genes present in respective bacterial isolates.

Lipopeptide Extraction and Antifungal Disc Diffusion Assay

Based on the presence of lipopeptide genes in bacterial isolates, three bacterial isolates (*Bacillus subtilis*-KAS-2, *Bacillus tequilensis*-KAS3, and *Bacillus velezensis*-KAS7) were grown in liquid culture for lipopeptide production and extraction. Using the method described by Gond et al. (2015), lipopeptide was extracted from bacterial isolates. For that, selected bacterial isolates (KAS2, KAS3, and KAS7) were grown in nutrient broth with constant shaking with 200 rpm on a rotary shaker at 27°C. After 4 days of incubation, bacterial cultures were centrifuged at 4,500 rpm (15 min at 4°C); culture supernatants were collected and acidified (up to pH 2°C) with concentrated HCl and were incubated overnight at 4°C. Acidified culture supernatants were centrifuged at 9,000 rpm (15 min at 4°C). The pellets were collected and dissolved in methanol and then filtered through a 0.22 μm filter membrane to remove bacterial cells debris. Methanol filtrate was dried through a vacuum evaporator and stored at 4°C. Using disc diffusion assays, the antifungal activity of lipopeptide was checked against fungal phytopathogens including *Fusarium* sp., *Curvularia* sp., *Alternaria* sp., *Rhizoctonia solani*, *Epicoccum sorghinum*, and *Exserohilum rostratum*. For that, a sterile paper disc was loaded with 20 μl (200 μg) of methanolic solution of lipopeptides and

the control disc contained only 20 μl methanol. Loaded discs were transferred onto potato dextrose agar (PDA) plates with a centrally placed small disc of fungal mycelia and incubated for 4 days. Stereo microscopy (Magnus, India) was used to observe the zone of inhibition between lipopeptide-loaded disc and fungal pathogens.

Effects of Different Concentrations of Lipopeptides on *Fusarium* sp. Growth and Evaluation of Minimum Inhibitory Concentration

Different concentrations (0.25, 0.50, 1, 2, and 3 mg ml^{-l}) of lipopeptide extracts (from *Bacillus subtilis*) were prepared in PDA media. A 5 mm diameter fungal mycelial disc was placed in the center onto PDA plate and incubated in a BOD incubator for 4 days. Growth inhibition of the fungus was measured using the following formula (Borah et al., 2016):

Mycelial growth inhibition = 100–(Diameter of mycelium growth in lipopeptide medium/Diameter of mycelium growth on control medium plate × 100).

For calculation of MIC, several concentrations (0.25, 0.50, 1, 2, 3, 4, 5, 6, 7, and 8 mg ml^{-1}) of lipopeptide extract (from *Bacillus subtilis*) were prepared in potato dextrose broth (PDB) medium in test tubes having 10 ml each concentration. 50 μl of fungal spore suspension (10^3–10^4 spores ml^{-1}) were transferred to each test tube and incubated for 7 days. After 7 days of incubation, fungal growth was checked for MIC.

Microscopic Analysis of the Effect of *Bacillus subtilis* and Its Lipopeptides on *Fusarium* Hyphal and Spore Structure

Effects of treatment with both *Bacillus subtilis* and its lipopeptide extract on *Fusarium* hyphal and spore structures were examined under bright field, fluorescent, and SEM. Lactophenol cotton blue (Himedia) was used for observation of fungal mycelia and spores under light microscopy (Olympus, India). Fluorescent stains such as SYTO-9 (Thermo Fisher Scientific, United States), propidium iodide (Sigma-Aldrich), DAPI (Sigma-Aldrich), and calcofluor white (Sigma-Aldrich) were used to study the fungal mycelia and spores under fluorescent microscopy (Nikon, Japan). For SEM, sample preparation was done using the method described by Wu et al. (2019). A portion of fungal mycelia was placed onto the slide and fixed with 2.5% glutaraldehyde. After 2 days of incubation, it was rinsed with phosphate buffer (10 mM) and dehydrated with increasing concentrations of ethanol. Dehydrated fungal samples were examined under SEM (EVO 18, Carl Zeiss, Germany) in the Department of Geology, BHU, Varanasi.

Preparation of Fluorescent Stains

Twenty-five micromolar concentration of SYTO 9, propidium iodide (PI), DAPI and calcofluor white (CFW) were prepared separately from their respective stock solutions. A combination of SYTO 9-PI, DAPI-PI, and CFW-PI were prepared in a 1:1 ratio and 20 μl from all combination of stains were used separately for fungal staining. After putting the combination of stains on mycelial pieces on a slide, samples were incubated for 5 min. in a dark room at room temperature before being examined under a fluorescent microscope. All selected fungal tissues were observed using fluorescent microscopy (Nikon DAPI-FITC-TRITC filter combinations).

Seedling Protection Assay in Microcosms

In seedling protection assays, surface-sterilized seeds (4% NaOCl–10 min) were treated with *Bacillus subtilis* (10^5–10^7 cells ml^{-1}) and its lipopeptides (200 μg ml^{-1}) separately then inoculated with *Fusarium* sp. spores (10^3–10^4 spores ml^{-1}). Seeds only inoculated with fungal spores were set up as control. From all treatments, 20–25 seeds were transferred into each magenta box, containing sterile cocopeat, perlite and sand in a 2:2:1 ratio, then magenta boxes were placed into a growth chamber. Each treatment was done in triplicate.

Statistical Analysis

Microsoft excel was used for the preparation of bar diagrams and measurement of standard errors. SPSS-16 program was used for one-way ANOVA followed by *post hoc* Duncan analysis to evaluate the significant difference in means of root-shoot length, fresh weights and photosynthetic pigments among and between the treatments and controls.

RESULTS

Seed Surface Sterilization, Disinfection, Isolation and Identification of Seed Endophytic Bacteria

Surface sterilization with 4% sodium hypochlorite for 10 min. followed by 1 min. in 70% ethanol was found effective since no bacteria emerged from the last wash solution of the treatment. A total of seven types of endophytic bacteria were isolated from pearl millet seeds on nutrient agar media. Five bacterial isolates (KAS1, KAS2, KAS3, KAS4, and KAS5), three (KAS4, KAS5, and KAS7), and two (KAS6 and KAS7) were isolated from 10, 20, and 40 min. surface-sterilized seeds with sodium hypochlorite, respectively. KAS1 and KAS4 were found to be the most common isolates with 65 and 21% colonization in seeds (**Table 1**). No bacteria observed around completely disinfected (treated with sodium hypochlorite-10 min + 100 μg ml^{-1} streptomycin −8 h) seeds on the nutrient agar plate. Completely disinfected seeds were treated as endophytes-free and were further used for the re-inoculation experiment.

The bacterial isolates were identified as KAS1, KAS2, KAS3, KAS4, KAS5, KAS6, and KAS7 (**Table 1**). The phylogenetic analysis confirmed close relationship of bacterial isolates of pearl millet seed with the corresponding species recovered from the NCBI database (**Supplementary Figure 1**).

Plant Growth Promoting and Enzymatic Activities of Seed Endophytic Bacteria

Bacterial isolates (KAS1, KAS4, and KAS6) were found to produce IAA (auxin) in greater amounts as compared to KAS2, KAS3, KAS5, and KAS7 isolates in nutrient broth (both with

TABLE 1 | List of molecularly identified endophytic bacteria using 16S rDNA sequencing with their closet matches, percentage similarity and accession no. of isolates.

Bacterial isolates and% CF	Closet matches	Similarity (%)	GenBank accession no. of bacterial isolates
KAS1 (65.55)	*Kosakonia cowanii*	99.65	MN134077
KAS2 (6.66)	*Bacillus subtilis*	100	MN367975
KAS3 (6.66)	*Bacillus tequilensis*	100	MN134078
KAS4 (21.11)	*Pantoea stewartii*	100	MN134079
KAS5 (3.33)	*Paenibacillus dendritiformis*	99.66	MN134080
KAS6 (3.33)	*Pseudomonas aeruginosa*	99.89	MN134081
KAS7 (1.11)	*Bacillus velezensis*	100	MN134082

% CF–Colonization Frequency of bacterial isolates in seeds.

TABLE 2 | Auxin (IAA) production, phosphate solubilization, potassium solubilization, and siderophore production activities of seed endophytic bacteria.

Bacterial isolates	Auxin production (μg ml^{-1} ± SE)		Phosphate soulubilization	Potassium soulubilization	Siderophore
	Without Trp	With Trp			
KAS1	7.2 ± 0.06	9.0 ± 0.09	+++	++	–
KAS2	3.2 ± 0.08	4.0 ± 0.10	+	–	++
KAS3	2.2 ± 0.11	3.3 ± 0.11	+	–	–
KAS4	7.7 ± 0.26	13.9 ± 0.36	+++	++	+
KAS5	2.3 ± 0.08	3.2 ± 0.13	–	–	–
KAS6	4.9 ± 0.18	6.2 ± 012	++	–	–
KAS7	2.0 ± 0.13	3.5 ± 0.10	–	–	+++

Where, (-, no activity; +, <5 mm clear zone; ++, 5–10 mm clear zone; +++, more than 10 mm clear zone around endophytic bacterial isolates); KAS1, Kosakonia cowanii, KAS2, Bacillus subtilis, KAS3, Bacillus tequilensis, KAS4, Pantoea stewartii, KAS5, Paenibacillus dendritiformis, KAS6, Pseudomonas aeroginosa, KAS7, Bacillus velezensis, (Trp, Tryptophan).

or without tryptophan supplementation) (**Table 2**). Bacterial isolates, KAS1 and KAS4, showed very good phosphate and potassium solubilization activities while three bacterial isolates: KAS2, KAS4, and KAS7, showed siderophore production activity (**Table 2**). *Bacillus* strains: KAS2, KAS3, and KAS7, were found to be more active in amylase and cellulase production activities. Out of seven strains, only KAS2 showed pectinase activity and KAS5 showed chitinase activity (**Supplementary Table 2**). Except for KAS5, all the isolates: KAS1, KAS2, KAS3, KAS4, KAS6, and KAS7, showed catalase activity (**Supplementary Figure 2**). Except for KAS1, all isolates: KAS2, KAS3, KAS4, KAS5, KAS6, and KAS7, showed protease activity (**Supplementary Table 2**).

Standardization of Growth Media for Re-inoculation Experiments

Out of several media (including 1.5% agar, filter paper, river sand, a potting mix including ratio cocopeat: perlite: sand−2:1:1 and ratio −2:2:1) which was used for initial standardization, a potting mix containing cocopeat, perlite and sand with 2:2:1 ratio showed best result of seed germination and seedling development compare to others, hence this medium was further selected for re-inoculation experiments.

Removal and Re-inoculation of Seed Endophytic Bacteria on Seedling Growth and Development

Based on plant growth promoting activities, the best three SEB, including KAS1, KAS4, and KAS6, were used for re-inoculation experiments with disinfected seeds. All the bacterial isolates including KAS1, KAS4, and KAS6 significantly increased the root-shoot length and fresh weight of seedlings as compared to the negative control (without bacteria) (**Figures 1**, **2**); further, re-inoculation with bacteria improved chlorophylls a and b, and carotenoids contents in the leaves of seedlings (**Figure 3**). Out of the three, KAS4 was found to be best in stimulating seedling development in terms of root-shoot length and photosynthetic pigments compare to negative controls (**Figures 1–3**). We repeated the experiment twice (trials 1 and 2) and found similar trends in the results. We also re-isolated bacteria from the roots of treated seedlings and confirmed their identities (**Supplementary Table 1**).

Microscopic Visualization of Roots for Colonization of Seed Endophytic Bacteria

Colonization of bacteria was observed on the root surface, root hairs and, inter- and intra-cellular spaces of root parenchyma cells of positive controls and bacterial treated seedlings which were visible with SYTO 9 stain under fluorescent microscopy, however, no bacteria were found on the root surface, and root hairs of negative control seedlings (**Figure 4**). Multiple roots from each sample were visualized and we found more or less similar colonization patterns.

Antifungal Activity of Endophytic Bacterial Isolates

Out of seven bacterial isolates tested, five bacterial isolates:KAS2, KAS3, KAS4, KAS6, and KAS7, showed significant antifungal

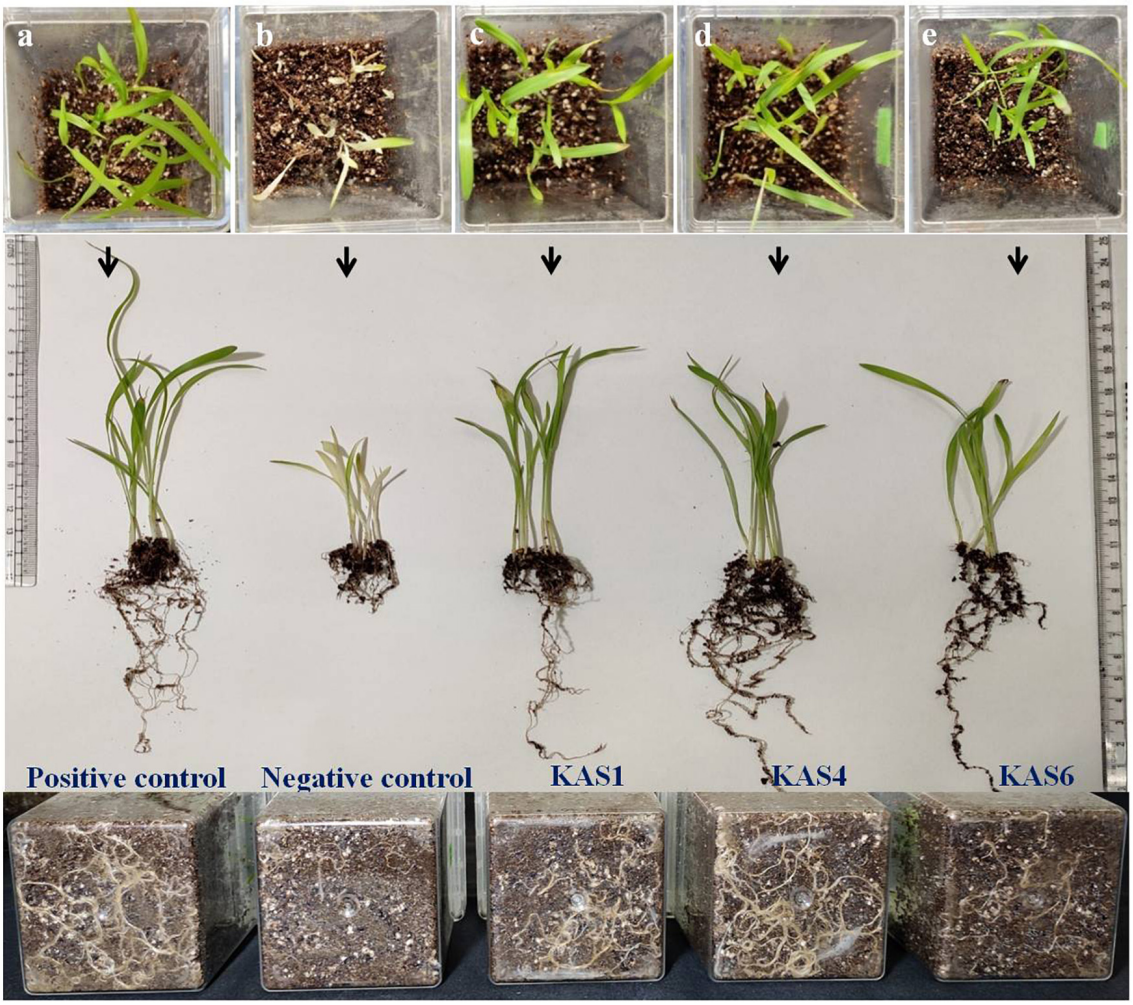

FIGURE 1 | Effect of disinfection and re-inoculation of seed endophytic bacteria (SEB) on seedling growth and root-shoot length (seedlings were 8 days old). **(a)** Positive control is where seeds were treated only with NaOCl; **(b)** negative control is where seeds were treated with NaOCl + antibiotic; **(c–e)** KAS1, KAS4, and KAS6 are where seeds initially treated NaOCl + antibiotic were inoculated with strains KAS1 (*Kosakonia cowanii*), KAS4 (*Pantoea stewartii*), and KAS6 (*Pseudomonas aeruginosa*), respectively. In the first lane: seedlings grown in magenta boxes viewed from the top; second lane: seedlings taken out from the boxes and viewed; and third lane: viewed from the back of magenta boxes showing root growth.

activity against all the tested fungal pathogens including *Fusarium* sp., *Curvularia* sp., *Alternaria* sp., *Rhizoctonia solani*, *Epicoccum sorghinum*, and *Exserohilum rostratum* (**Table 3**).

Screening of Lipopeptide Genes and Antifungal Activity of Lipopeptide

Three *Bacillus* spp. including KAS2, KAS3, and KAS7, which were most active against fungal phytopathogens were also found to have at least one antifungal lipopeptide gene (**Supplementary Table 4**). Gene-specific sequencing (lipopeptide genes) also confirmed the lipopeptide genes present in specific *Bacillus* spp. (**Supplementary Table 4**). Furthermore, in disc diffusion assays, lipopeptide extracted from KAS2, KAS3, and KAS7, showed good antifungal activity against pathogenic fungi, including *Fusarium* sp., *Curvularia* sp., *Alternaria* sp., *Rhizoctonia solani*, *Epicoccum sorghinum*, and *Exserohilum rostratum* (**Supplementary Figure 3**).

Effects of Different Concentrations of Lipopeptide on *Fusarium* sp. and Minimum Inhibitory Concentration

To check the effective concentration of lipopeptide for antifungal activity against *Fusarium* sp., the fungus was challenged with different concentrations of lipopeptide prepared in PDA as well as PDB. With increasing concentrations of lipopeptides, growth of *Fusarium* was found reduced and stopped in broth (PDB) and PDA plates (**Figure 5**). After 4 days of incubation, more than 50% of fungal growth was inhibited at 2 mg ml^{-1} on PDA media. No fungal growth was observed in PDB at 7 mg ml^{-1} concentration of lipopeptide after 7 days of incubation. Hence, 7 mg ml^{-1}

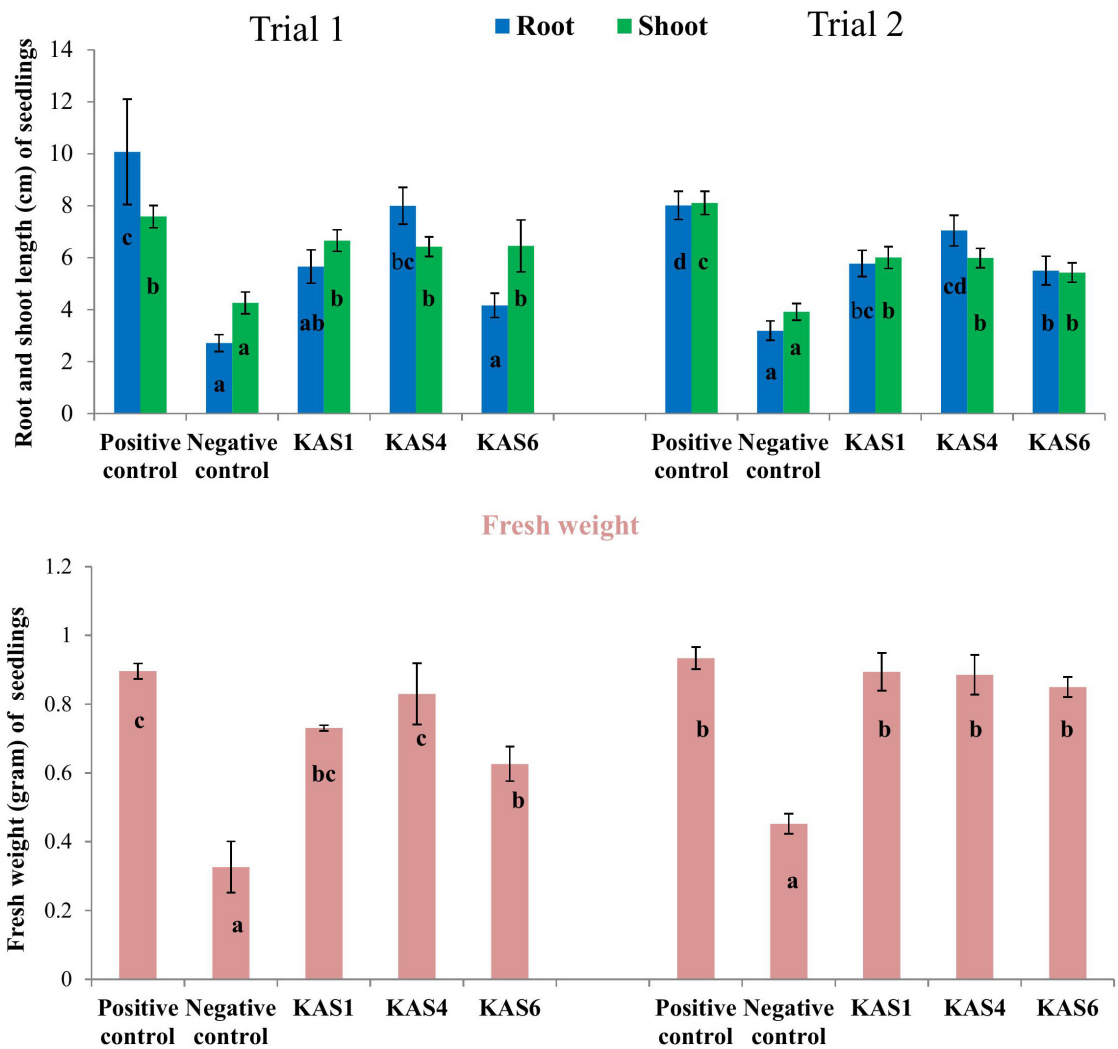

FIGURE 2 | Effect of removal and re-inoculation of SEB on the root-shoot length and fresh weight of seedlings. Positive control: where seeds were treated only with NaOCl; Negative control: where seeds were treated with NaOCl + antibiotic; KAS1, KAS4, and KAS6: where seeds initially treated NaOCl + antibiotic then inoculated with strains KAS1, KAS4, and KAS6. Different letters represent the significant differences among the means of treatments ($N = 30$, $P \leq 0.05$). Experiment was repeated in two trials **(**Trial 1 and Trial 2**)**.

concentration of lipopeptide was recorded as MIC of lipopeptide against *Fusarium* sp. (**Figure 5**).

Microscopic Analysis of Effects of *Bacillus subtilis* and Its Lipopeptides on *Fusarium* Mycelia and Spores

Bacillus subtilis and its lipopeptide extract showed significant inhibitory effect on the tested fungal pathogen; it retarded the growth and also caused deformation in hyphal and spore structures of the fungus. Microscopic analysis with bright field, fluorescence, and SEM of bacterial and lipopeptide treated *Fusarium* revealed the development of abnormal structures in fungal hyphae and spores including a ball like swelling and bulging. Furthermore, disintegration and lysis of spore/cells were also observed. However, smooth and normal structures of hyphae and spores (without any deformation) were observed in untreated (control) (**Figures 6–8**). In propidium iodide (PI) staining (which stains only dead cells), significant numbers of dead cells of *Fusarium* hyphae and their spores were observed in *Bacillus* and lipopeptide treatments, however, no or very few damaged or dead cells were observed in controls. Lactophenol cotton blue (LCB), SYTO-9, CFW, and DAPI stained both live and dead cells. PI in combination with other stains (SYTO-9, DAPI, and CFW) helped in improving visualization and differentiation between the dead and living structures of fungal hyphae and spores. Live-dead staining with combination of PI and other stains (SYTO-9, DAPI, and CFW) clearly showed that the burst and dead parts of hyphae and spores were only stained with PI, while CFW and other stains, stained all parts of hyphae and spores (**Figures 6**, **7**). CFW also differentiated between dead and live cells by staining the chitin present in the cell wall of

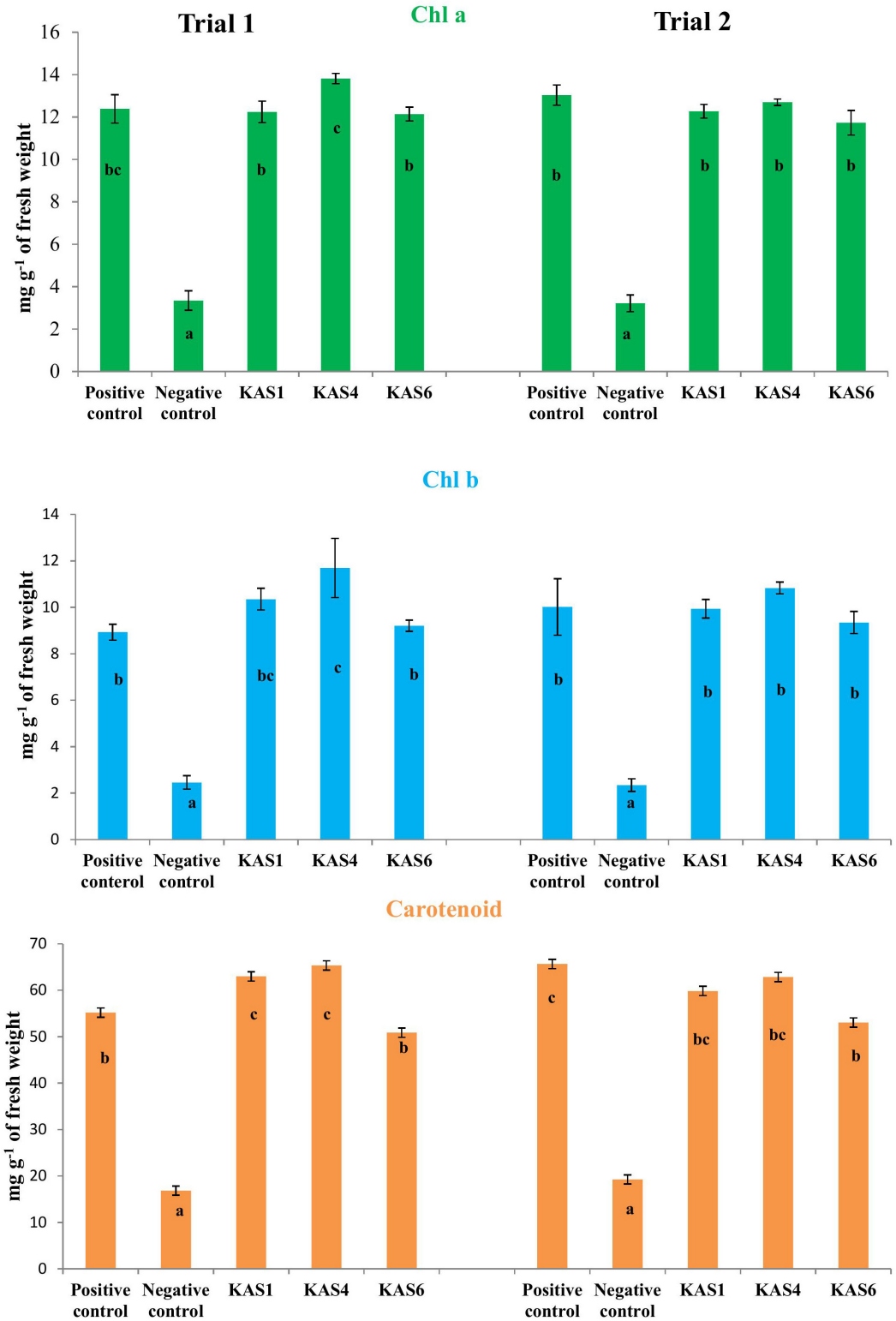

FIGURE 3 | Effect of removal and re-inoculation of SEB on the content of photosynthetic pigments of leaf tissues from 8 day old seedlings. First lane- Chlorophyll a, second lane- chlorophyll b and third lane- carotenoids. Positive control: seeds were treated only with NaOCl; negative control: seeds were treated with NaOCl + antibiotic; KAS1, KAS4, and KAS6: seeds initially treated with NaOCl + antibiotic and then inoculated with strains KAS1 (*Kosakonia cowanii*), KAS4 (*Pantoea stewartii*), and KAS6 (*Pseudomonas aeruginosa*). Different letters represent the significant differences among the means of treatments. ($P \leq 005$). Experiment was repeated in two trials **(**Trial 1 and Trial 2**)**.

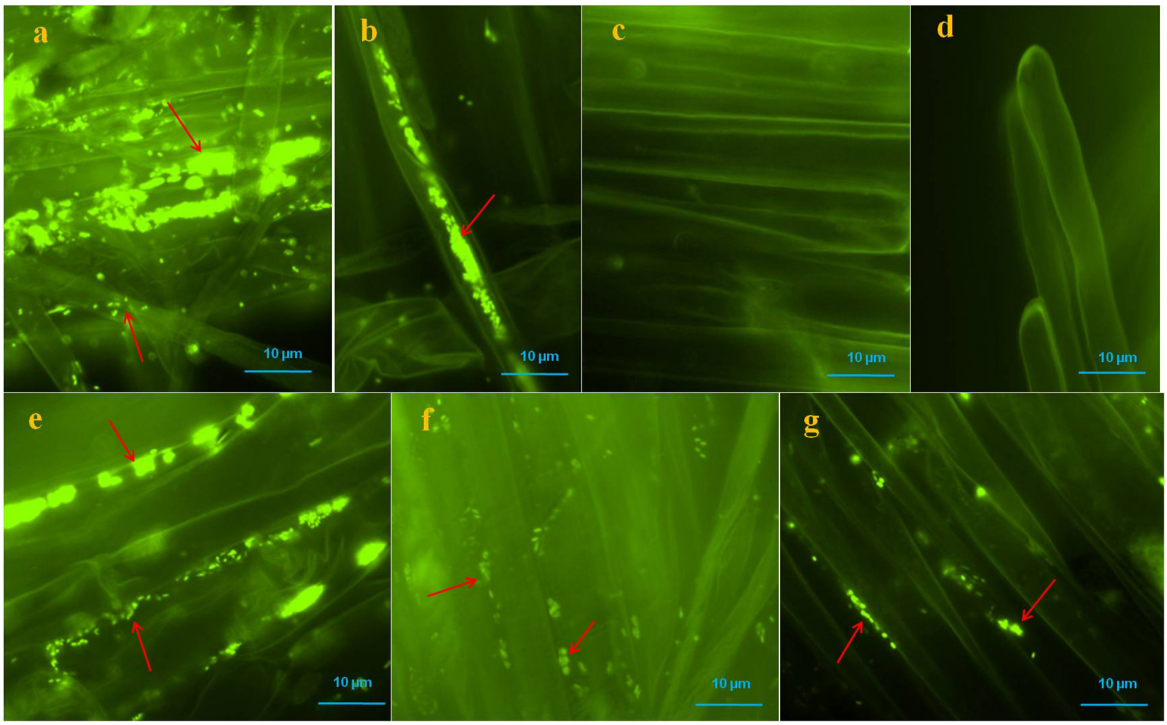

FIGURE 4 | Microscopic visualization of bacteria on or in roots of pearl millet seedlings after SYTO-9 staining under fluorescence microscope. Where, in **(a,b)** several bacteria were observed on the root surfaces and inside of the root parenchyma and hair cells of positive control seedlings (arrows); **(c,d)** no bacteria were observed onto root surfaces and root hairs of negative control seedlings; **(e–g)** KAS1 (*Kosakonia cowanii*), KAS4 (*Pantoea stewartii*), and KAS6 (*Pseudomonas aeruginosa*) were observed on surfaces of root and in intercellular spaces of root parenchyma cells, respectively (arrows).

TABLE 3 | Antagonistic activity (as% inhibition) of endophytic bacteria against selected fungal phytopathogens in dual plate culture.

Bacterial isolates	*Fusarium* sp.	*Rhizoctonia solani*	*Alternaria* sp.	*Curvularia* sp.	*Eppicocum sorghinum*	*Exserohilum rostratum*
KAS1	66.7	26.1	65	0	21.4	20
KAS2	50	39.1	70	65	50	56
KAS3	43.33	37	65	40	46.4	56
KAS4	60	23.9	65	28	42.8	66
KAS5	25	4.3	0	0	0	0
KAS6	80	50	70	80	71.4	80
KAS7	50	43.5	65	70	65	66

Where, KAS1, Kosakonia cowanii, KAS2, Bacillus subtilis, KAS3, Bacillus tequilensis, KAS4, Pantoea stewartii, KAS5, Paenibacillus dendritiformis, KAS6, Pseudomonas aeroginosa, KAS7, Bacillus velezensis.

hyphae and spores. Live fungal mycelial cells and spores were clearly visible from tips and margins (intact cell walls) because CFW stained the chitin present in the cell wall and spores while burst/ruptured or damaged cells were not clearly visible (blurred) at tips and margins of spores and mycelial cells (**Figures 6**, **7**).

Seedling Protection From *Fusarium* Infection

Seeds treated with the bacterium *Bacillus subtilis* and its lipopeptides grew as healthy seedlings with few or no infections when challenged with *Fusarium* spores, but untreated control seedlings were heavily infected and almost all seedlings were collapsed within 8–10 days of infection due to the pathogen (**Figure 9**). Microscopy of the roots also showed that untreated control seedling roots were extensively colonized by the fungus, whereas very little or no infection was observed in treatments.

DISCUSSION

Surface Sterilization, Isolation, and Removal of Seed Endophytic Bacteria From Pearl Millet Seeds

All plants and their tissues contain multiple microbes internally that play crucial roles in plant development and protection against diseases. Past studies have shown that seed-associated microbes, including bacteria and fungi, improve seed germination and seedling establishment (Puente et al., 2009;

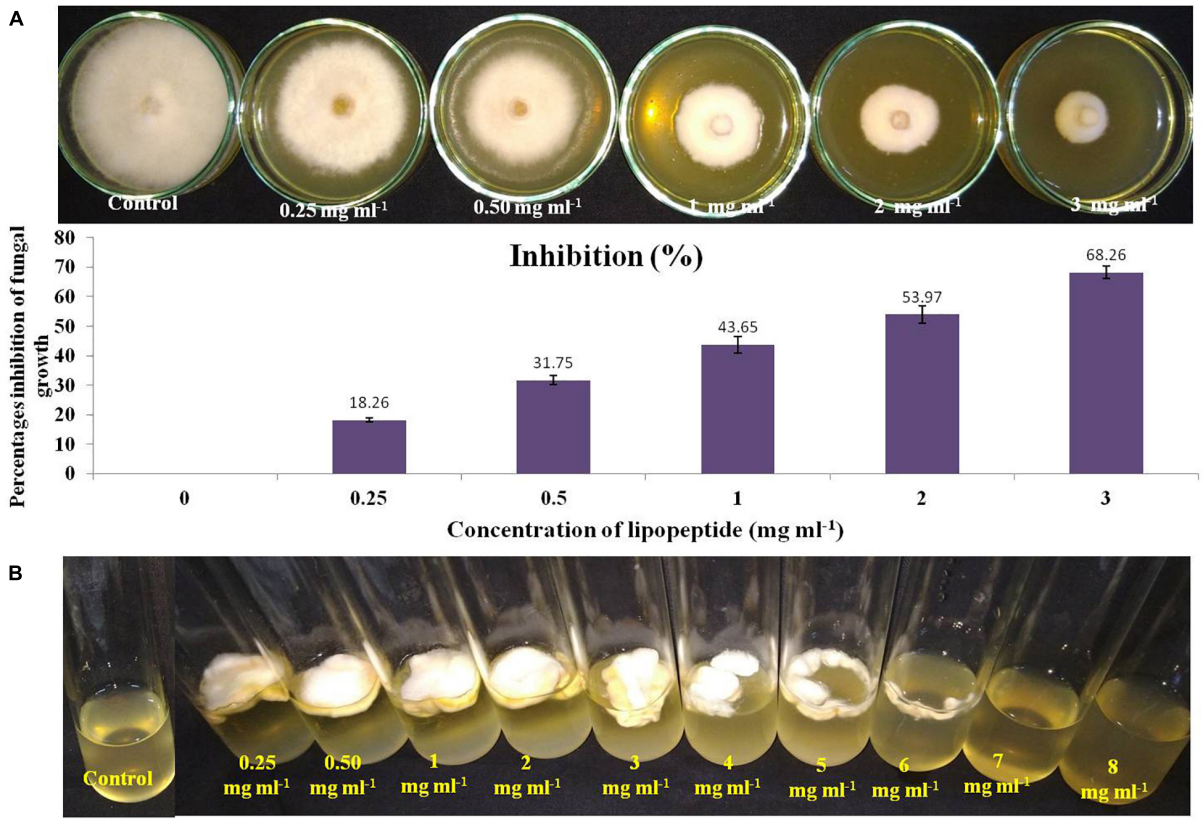

FIGURE 5 | Measurement of minimum inhibitory concentration (MIC) of lipopeptide against *Fusarium* sp. **(A)**. Effects of different concentration of lipopeptides on PDA plate (A-first lane) and their percentages inhibition (A-second lane), **(B)**. Effects of different concentration of lipopeptides in PDB, (Control A- without lipopeptide, Control B- without lipopeptide and fungus).

Rasmussen et al., 2015; Cope-Selby et al., 2017; Irizarry and White, 2017; Verma et al., 2017). Many crop seeds, including maize, rice, wheat, and cotton, have been reported to harbor bacterial seed endophytes that have seedling growth modulation activity (Gond et al., 2015; Pitzschke, 2016; Irizarry and White, 2017; Verma et al., 2017). In the present study, we found that a 10 min. sodium hypochlorite treatment followed by 70% ethanol for 1 min. was efficient to completely remove surface microflora since no growth of bacteria was observed from the last wash solution on NA plates (Kumar et al., 2020). As we increased the sterilization time from 10 to 20 and 40 min., numbers of recovery of isolates were reduced, and seeds were also found to have reduced germination frequency, which could be due to over-sterilization. Overall, seven endophytic bacteria, including KAS1, KAS2, KAS3, KAS4, KAS5, KAS6, and KAS7, were isolated from the surface-sterilized seeds and identified by using 16S rDNA sequencing (**Table 1**). In this study, KAS1 was found to be the most dominant isolate since it was most frequently isolated from seeds of millet followed by KAS4 (**Table 1**). Recently, a study reported the occurrence of *Kosakonia cowanii* as the most common SEB in seeds of *Lactuca serriola* (Jeong et al., 2021). *Pantoea* and *Bacillus* spp. were also recovered as seed endophytes in previous studies (Pitzschke, 2016; Irizarry and White, 2017; Verma and White, 2018). We successfully disinfected seeds (free from SEB) by further treating NaOCl-surface sterilized seeds with streptomycin sulfate (100 μg ml^{-1}) for 8 h and disinfection was confirmed by non-emergence of bacteria from the seeds onto NA plates. Application of antibiotics with standardized concentrations and time has been suggested in the past for curing the seeds for re-inoculation experiments (Puente et al., 2009; Verma et al., 2017). However, the possibilities of non-culturable bacteria present inside the seeds have not been ruled out (Hardoim et al., 2012; Truyens et al., 2015; Chimwamurombe et al., 2016; Pei et al., 2017).

Crucial Role of Seed Endophytic Bacteria in Seedling Growth and Development

Seedling growth and development was found suppressed in terms of root-shoot length and fresh biomass when seeds were cured using antibiotic (negative control) in comparison to seeds carrying their natural SEB (positive control) (**Figures 1**, **2**). We also found that seedlings developed from cleaned seeds started showing bleaching effects within 4–5 days of their growth which was further evidenced by reduced photosynthetic pigments in comparison to the SEB-positive controls (**Figure 3**). This demonstrates the importance of the seed-vectored endophytic bacteria during the early stages of seedling development.

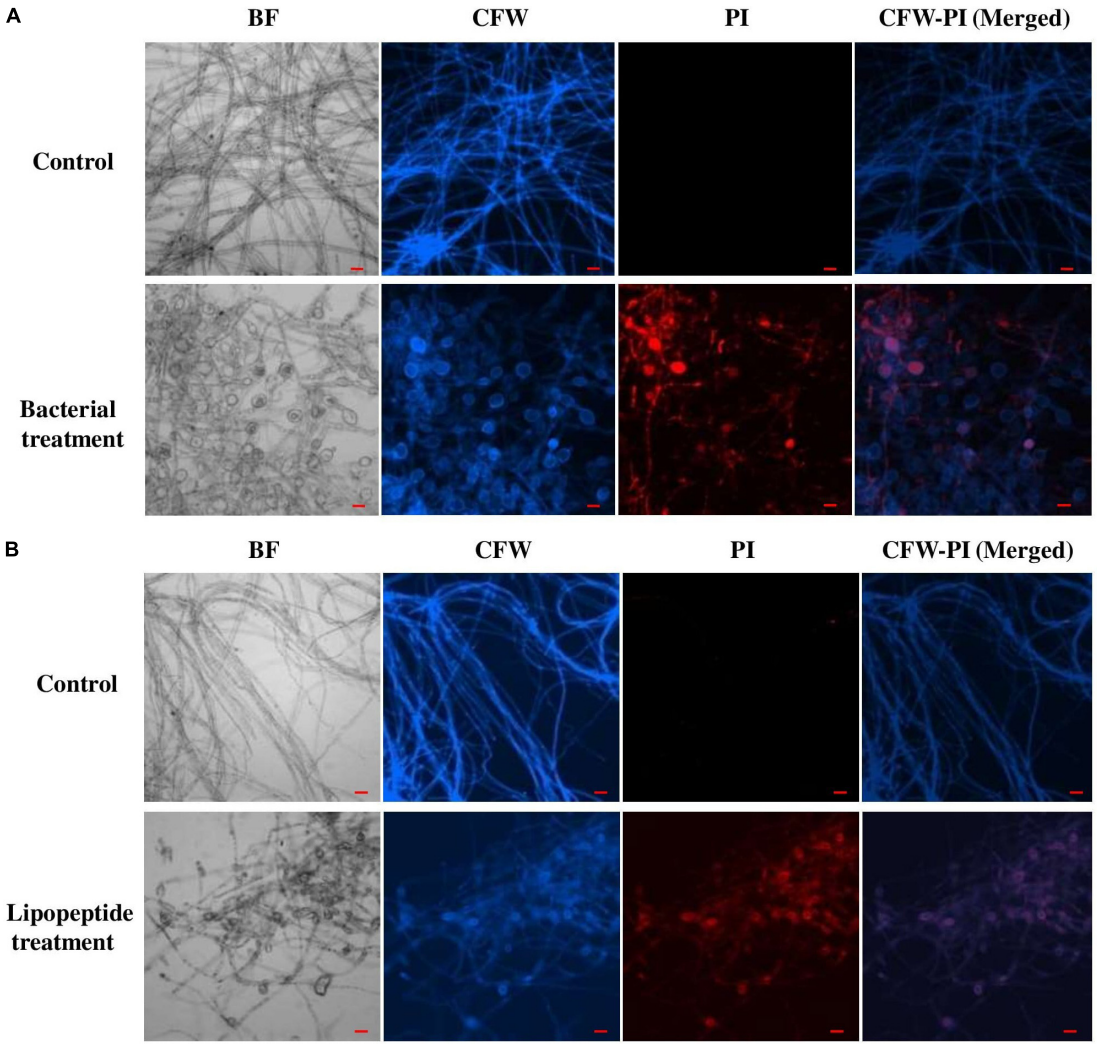

FIGURE 6 | Effect of bacterial (*Bacillus subtilis*) **(A)** and lipopeptides **(B)** on *Fusarium* hyphae was observed under bright field and fluorescent microscopy (CFW and PI). Bacterial treatment was done by co-cultivation of *Fusarium* with *Bacillus subtilis* in mixed media of nutrient and potato dextrose agar. Lipopeptide treatment was done by disc diffusion (each disc contain 20 μl of 10 μg μl^{-1} methanolic solution of lipopeptide), hyphae growing near the disc was taken for microscopy. In control-A fungus was not challenged with bacteria and control B- disc was only loaded with 20 μl of methanol without lipopeptide (Scale bar = 20 μm).

Research in the past has shown that the removal of bacteria from seeds negatively affects germination and seedling development (Puente et al., 2009; Holland, 2016; Verma and White, 2018). Puente et al. (2009) in their study found that elimination of bacteria from seeds using antibiotics significantly suppressed seedling development in cardon cactus plants. Holland (2016) reported that the removal of bacteria from several crop seeds including soybean, rice, and kidney beans significantly reduced germination and further seedling development.

When we re-inoculated the disinfected seeds with their own best plant-growth promoting SEB (**Table 2**), including KAS1, KAS4, and KAS6, seedlings showed restored growth and development in terms of root-shoot lengths and fresh weights (**Figures 1**, **2**), and improved photosynthetic pigments, such as chlorophyll a, b, and carotenoids in their leaves (**Figure 3**). We found similar effects in two experiment trials. Seed endophytes are primary symbionts that may play an essential role in germination and seedling development (Puente et al., 2009; Herrera et al., 2016; Chen et al., 2017; Ridout et al., 2019; Kumar et al., 2020). In this study, all seven isolated SEB showed IAA production, five: KAS1, KAS2, KAS3, KAS4, and KAS6, showed P solubilization and two: KAS1 and KAS 4, showed K solubilization activities (**Table 2**), and this could be a reason behind the positive effect of SEB on seedling growth restoration (Etesami et al., 2017; Afzal et al., 2019; Abdelaal et al., 2021). SEB KAS2, KAS4, and KAS7, also showed siderophore production activity. Siderophores are iron-chelating agents that have a greater affinity for ferric ions and convert insoluble ferric form of iron to soluble ferrous form which are easily accessible to the plant. Siderophore producing bacteria also reduce the growth of pathogenic fungi by reducing the iron availability in soil (Afzal et al., 2019; Duponnois and L'Hoir, 2021; Kumar et al., 2021). In both trials,

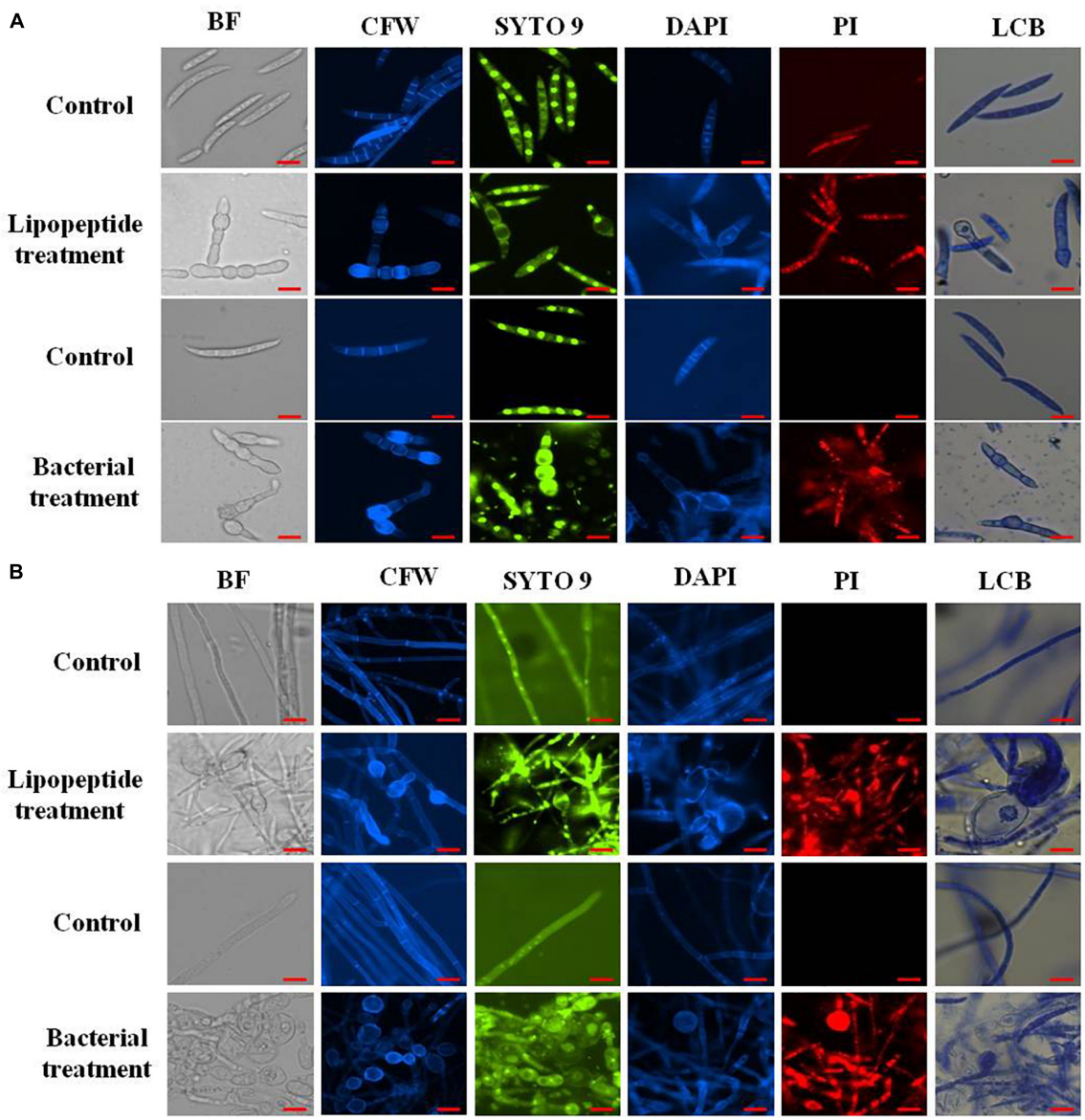

FIGURE 7 | Closer view of effect of bacterial (*Bacillus subtilis*) and lipopeptides on fungal spores **(A)** and hyphae **(B)** of *Fusarium* sp. under bright field, fluorescent microscopy (CFW, Syto-9, DAPI, and PI) and lactophenol cotton blue (LCB) light microscopy. Bacterial treatment was done by co-cultivation of *Fusarium* with *Bacillus subtilis* in mixed media of nutrient and potato dextrose agar/broth. Lipopeptide treatment was done by disc diffusion (each disc contain 20 μl of 10 μg μl^{-1} methanolic solution of lipopeptide), hyphae and spores growing near the disc was taken for microscopy. In control-A fungus was not challenged with bacteria and control B- disc was only loaded with 20 μl of methanol without lipopeptide (Scale bar = 10 μm).

we found that isolates KAS1, and KAS4 significantly increased the root-shoot lengths, biomass, and photosynthetic pigments in seedlings, and both isolates were the highest producers of IAA and the best P solubilizers (**Table 2**). *Kosakonia cowanii* and *Pantoea* species have been reported as seed vectored bacteria in many crop seeds with plant-growth promoting activities (Chimwamurombe et al., 2016; Verma et al., 2017; Jeong et al., 2021). The profound effect of KAS1 and KAS4 treatment on root architecture development of seedlings may be because of their IAA activities. IAA is known to increase root architecture in plants and endophyte-mediated IAA effects on root development have been described in the past (Maggini et al., 2019; Verma et al., 2021). A study of the interaction of *Arabidopsis* seedlings with *Pseudomonas* significantly improved the root structure, and further study suggested that bacterium-mediated auxin signaling is important for better development of root system architecture (Zamioudis et al., 2015). Endophytes may also modulate the endogenous level of auxin in their host plants (Wang et al., 2015; Maggini et al., 2019). Recently, Chang et al. (2021) reported that root hair elongation may be modulated by ethylene secreted by SEB that become intracellular in root cells; these intracellular microbes were also found to secrete nitric oxide. Both ethylene

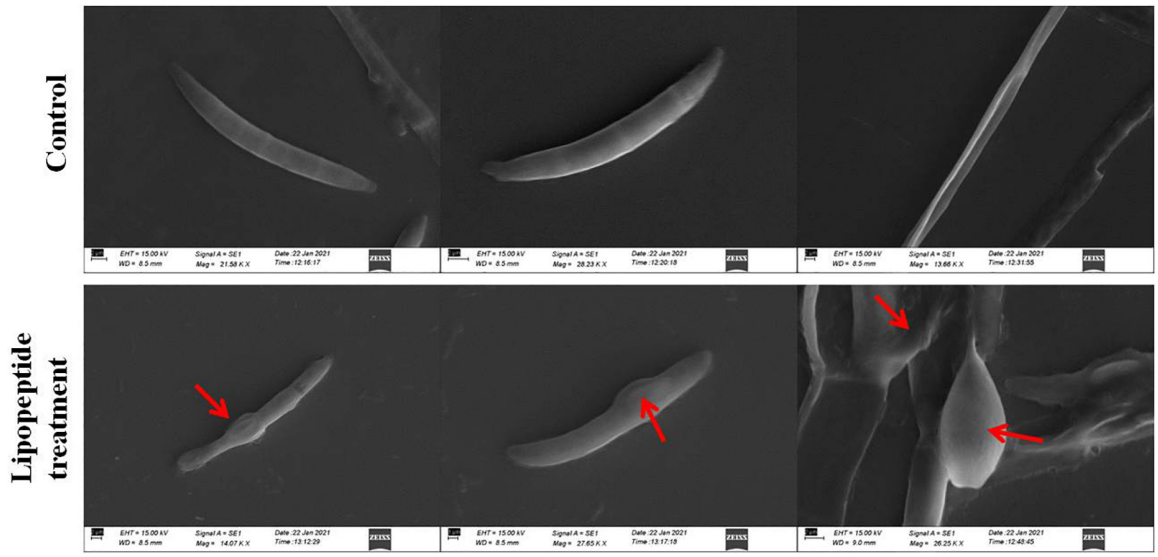

FIGURE 8 | Effects of lipopeptide on fungal hyphae and spores were observed under scanning electron microscopy (SEM). Lipopeptide treatment was done as described in **Figures 6**, **7**. In control spores and hyphae were found intact and normal in structure whereas in treatment, deformation, swelling and rupturing of spores and hyphae were observed (arrows).

and nitric oxide together could function with auxin to modulate development in seedlings. Regardless of the exact mechanism, SEB KAS1, KAS4, and KAS6 of pearl millet were shown to have an important role in seedling development.

Colonization of Seed Endophytic Bacteria Into Root Tissue of Seedlings and Proposed Nutrient Mobilization

In our microscopy study of treated seedling roots, we observed that KAS1, KAS4, and KAS6 colonized into inter and intracellular spaces of root parenchyma and root hairs cells (**Figures 4e–g**). In positive controls (with all possible SEB), SEB colonized inside root hairs and on and in root parenchyma cells (**Figures 4a,b**). No bacteria were found in the roots of negative control seedlings (**Figures 4c,d**). We also re-isolated the bacteria from the roots of treated seedlings and confirmed that they were identical to the inoculated bacteria by 16S rDNA sequencing (**Supplementary Table 1**). Colonization and growth of seed inhabiting bacteria onto developing roots and rhizosphere confirmed the importance of SEB in developing rhizospheric microbiota (López-López et al., 2010; Truyens et al., 2015; Verma et al., 2019). Successful colonization of SEB onto root surfaces and rhizospheres may help developing seedlings in nutrient acquisition in two ways; first, they may mineralize/solubilize nutrients in the rhizosphere, and these will be easily available to root hairs; secondly, within root parenchyma and root hair cells, nutrients may be extracted from bacteria oxidatively through the rhizophagy cycle (Paungfoo-Lonhienne et al., 2010; White et al., 2018). It has been shown that the secretion of ethylene by bacteria, triggers release of superoxide by the root cell, and it is hypothesized that superoxide produced by root cells acts on bacteria in extraction of nutrients from cell walls and cytoplasm (White et al., 2018; Chang et al., 2021; Verma et al., 2021). In this study, most of the isolates showed at least one extracellular enzyme production activity including cellulase, pectinase and amylase. Hydrolytic enzymes like cellulase, pectinase and amylase might play important role in endophytic colonization and establishment of bacteria in plant tissues (Dogan and Taskin, 2021).

Role of Seed Endophytic Bacteria in Seedling Protection Against Fungal Disease

Bacillus ssp. (*B. subtilis*, *B. tequilensis*, and *B. velezensis*) and *Pseudomonas aeruginosa* showed strong antifungal activity against fungal phytopathogens including *Fusarium* sp., *Curvularia* sp., *Alternaria* sp., *Rhizoctonia solani*, *Epicoccum sorghinum*, and *Exserohilum rostratum* (**Table 3**). Fungal pathogens including *Fusarium* spp., *Curvularia* spp., *Alternaria* spp., *Rhizoctonia solani*, and *Exserohilum rostratum* have been reported to decrease crop yield by causing several diseases such as leaf blight, root rot, stalk rot, leaf spot, seedling fall, and grain mold in millet crops (Wilson, 2000; Das, 2017). Several plant-associated bacteria have been reported to show antifungal activity by producing antifungal metabolites, volatile gases, cell wall degrading enzymes, and various types of lipopeptides (Ongena and Jacques, 2008; Gond et al., 2015; Mousa et al., 2016; Jadhav et al., 2017; Sekar et al., 2018; Zhang et al., 2020). SEB KAS2, KAS3, KAS4, KAS5, KAS6, and KAS7 showed protease and KAS5 showed chitinase activity. *Bacillus* spp. are known to have lipopeptide genes and express a variety of antifungal lipopeptides (Ongena and Jacques, 2008). In disc diffusion assays, extracted lipopeptides significantly inhibited the growth of tested fungal phytopathogens (**Supplementary Figure 3**). Amplification of lipopeptide genes by PCR confirmed the

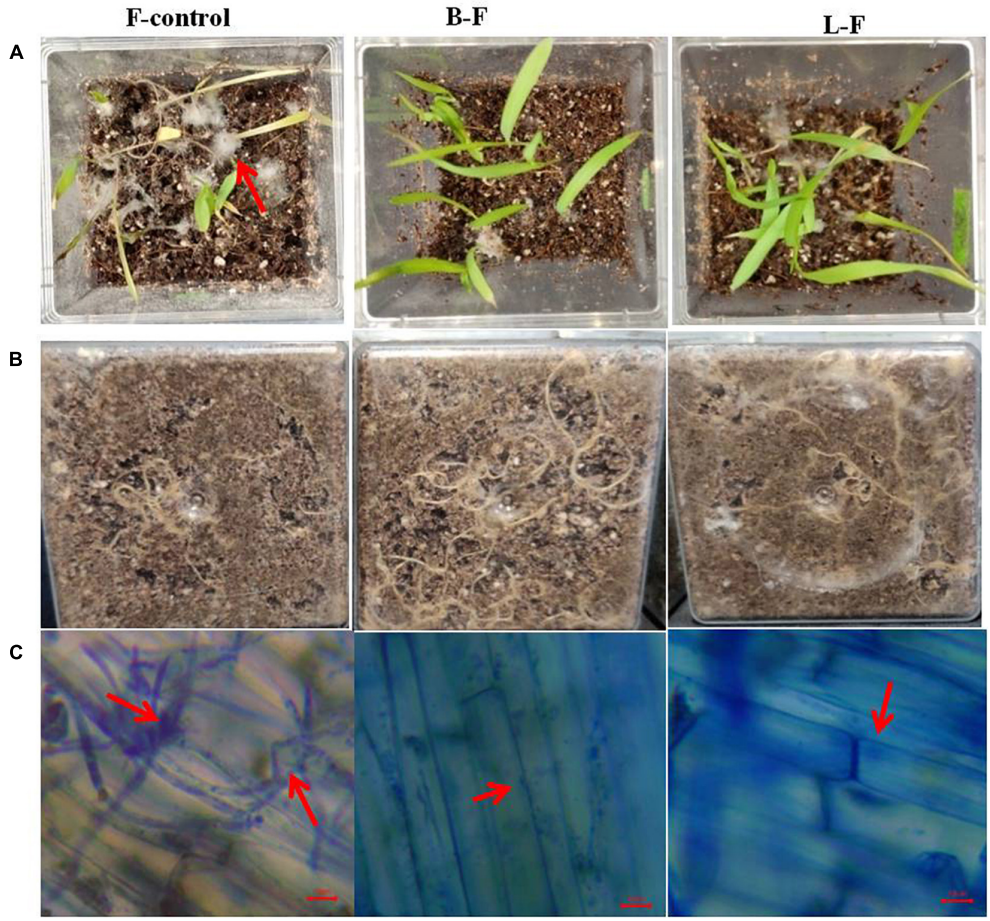

FIGURE 9 | Effect of bacterium and its lipopeptide treatments in protection of seedlings from *Fusarium* sp. infection inmicrocosm assay in magenta boxes. Where, surface sterilized seeds were inoculated with fungal spores only (column 1: F), *Bacillus subtilis* and fungal spores (column 2: B-F) and lipopeptide and fungal spores (column 3:L-F). Lane **(A)** are the images of 8 day old seedlings from the top of the magenta boxes, lane **(B)** image from the bottom of the same magenta boxes showing root growth, and lane **(C)**- microscopic images of root surface from all corresponding treatment showing heavy infection of fungal pathogen in control in comparison to treatments (arrows).

presence of surfactin (Sfp) in *Bacillus subtilis*, surfactin (Sfp) and fengycin (FenD) in *Bacillus tequilensis*, iturin A (ItuD) in *Bacillus velezensis*, and these may be the reason for antifungal activity of the bacteria (**Supplementary Table 4**). Furthermore, extracted lipopeptides from *Bacillus subtilis* with 2 mg ml^{-1} concentration in PDA, inhibited around 54% of the growth of *Fusarium* sp., and completely checked (MIC) the growth of the fungus at 7 mg ml^{-1} (**Figures 5A,B**). In a study on maize seed-associated bacteria, Gond et al. (2015) reported that lipopeptides produced by *Bacillus* spp. inhibited *Fusarium moniliforme* growth and stimulated the expression of host defense genes, including PR1 and PR4, which are inhibitory to fungal phytopathogens.

Mechanism of Antifungal Activity: Microscopic Analysis

Microscopic study revealed that *Bacillus subtilis* and its lipopeptides inhibited the growth of fungal hyphae and spores. Fungi challenged with the bacterium and its lipopeptide showed deformation, swelling, and bulging in hyphae and spores which are clearly visible in bright field (BF), SYTO 9, DAPI, LCB, and calcofluor white (CFW) staining in **Figures 6**, **7**. Live-dead staining with PI alone, and in combination with CFW, indicated that spore and hyphal cells that were swelled/bulged/burst were dead (**Figures 6**, **7**). The swelled hyphal and spore cells further started showing rupturing and leaking out their protoplasm that can be seen in **Figure 7**. SEM images also confirmed the swelling and bulging/rupturing in spores and hyphae due to lipopeptide treatment (**Figure 8**). Recently few studies suggested that *Bacillus* lipopeptides inhibit the fungal pathogen by damaging cell walls and cell membranes (Cawoy et al., 2015; Toral et al., 2018). In one study, the lipopeptide surfactin was reported to increase swelling and cytoplasmic leakage and cell death in hyphae of *Magnaporthe grisea* by creating pores in the cell membranes (Wu et al., 2019). Ball-like swellings and vacuolization in hyphae were observed in lipopeptide treated hyphae of *Fusarium moniliforme*, *Botrytis cinerea*, and *Fusarium verticillioides* (Gond et al., 2015; Blacutt et al., 2016; Toral et al., 2018). Lipopeptides create

pores in fungal hyphae by depolarizing membranes, inhibiting chitin and glucan synthases, and inducing apoptosis in fungal cells by affecting mitochondrial functions (Kurtz and Douglas, 1997; Qi et al., 2010; Fernández de Ullivarri et al., 2020). Using CFW staining, we observed that burst or swelled portions of the hyphae or spores had blurred images as compared to controls. This may be due to the loss of chitin and glucans from the cell walls (**Figure 7**). Thus, lipopeptides could be responsible for the damage that results in swelling and pore formation in fungal hyphae and spores. In a microcosm assay for seedling protection, we found that treatment with both *Bacillus subtilis* and its lipopeptide significantly protected seedlings from *Fusarium* infection. Endophytic *Bacillus* spp. have been also reported to induce the expression of defense-related genes of their host plants (Gond et al., 2015; Irizarry and White, 2018).

CONCLUSION

The present study shows that pearl millet seeds carry bacterial endophytes which are important for seedling development, establishment, and protection from fungal disease. SEB colonize onto the root surfaces and into root endosphere, mobilize nutrients during germination and growth, and produce antifungal compounds which reduce pathogen infection. This study raises several questions, including: (1) Is there an indirect signaling role of seed endophytes in modulating the expression of developmental and defense genes during seedling development? (2) How do multiple endophytes in seeds interact with each other? (3) What happens in interactions under changing environmental conditions? (4) How may SEB be better utilized in developing microbial products for a sustainable agricultural system?

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SV and KK proposed the idea. KK and Anubha performed and designed the experiments. KK and SV wrote the manuscript and prepared the images. GP, AV, and JW contributed to the final editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Fungal Seed Endophyte FZT214 Improves *Dysphania ambrosioides* Cd Tolerance Throughout Different Developmental Stages

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Phytoremediation is a promising remediation method of heavy metal (HM)–contaminated soils. However, lower HM tolerance of metal accumulator inhibits its practical application and effects. The current study was aimed to illustrate the role of fungal seed endophyte (FZT214) in improving *Dysphania ambrosioides* Cd tolerance during different developmental stages under various Cd stresses (5, 15, 30 mg kg^{-1}) by pot experiments. The results showed that FZT214 significantly ($p < 0.05$) improved the host plant's growth at the flowering and fruiting stage in most of the treatment, while at the growing stage the increase was less ($p > 0.05$). The seed yield was also improved ($p < 0.05$) in the FZT214-inoculated plants (E+) and induced early flowering was observed. Moreover, the inoculation also positively affected total chlorophyll content, antioxidant process, and lipid peroxidation in most of the treatments throughout three developmental stages. Not all but in most cases, IAA and GA were more in E+ plants while JA was more in the E− plants (non-inoculated plants) during three developmental stages. The results suggested that the colonization of FZT214 to the *D. ambrosioides* might trigger multiple and comprehensive protective strategies against Cd stress, which mainly include activation of the dilution effects, induced biochemical changes to overcome damage from Cd toxicity, and alteration of the endogenous phytohormones. FZT214 can find competent application in the future to improve the growth of other crop plants.

Keywords: seed endophytes, heavy metal tolerance, plant developmental stages, synthetic seeds, plant microbe interactions, fungal endophyte

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INTRODUCTION

The existence of potentially toxic elements (PTEs) in the surrounding environment causes numerous ecological consequences (Chen et al., 2014). These elements often persist in the environment for a long period due to their non-degradable nature and are difficult to be broken into less toxic forms. Cadmium (Cd) is recognized as a PTE that is transferred into the surrounding

Abbreviations: ABA, abscisic acid; BAF, bioaccumulation factor; DTNB, dithionitrobenzoic acid; DTPA-TEA, diethylenetriaminepentaacetic acid–triethanolamine; E+, inoculated plants; E−, non-inoculated plants; GA, gibberellic acid; GSH, glutathione; T-GSH, total glutathione; GSSG, oxidized glutathione; HM, heavy metal; IAA, indole-3-acetic acid; JA, jasmonic acid; MDA, malondialdehyde; PTEs, potentially toxic elements; TF, translocation factor.

ecosystem either naturally or through anthropogenic activities such as discharge of industrial wastes, mining wastes, smelting, uses of sewage sludge for agricultural purpose, etc. (Gallego et al., 2012; Robson et al., 2014). Yunnan Province, China, is full of valuable metal resources such as lead (Pb), zinc (Zn), Cd, and copper (Cu) (Yanqun et al., 2004), and some of these metal mines are as big as 26.053 million tons (Bai et al., 1985) and mining has been carried out for more than 300 years (Parmar et al., 2018). Cd is a non-biodegradable, highly toxic, and persistent metal pollutant, which causes numerous diseases, for example, kidney disorders, neurotoxicity, and osteoporosis (Park et al., 2012). Although Cd is a non-essential element that causes toxicity in crop plants (Kuriakose and Prasad, 2008), it can be translocated to vegetative parts including seeds (Sharma et al., 2006). Cd has great mobility and water solubility; its transport to the growing plant from the contaminated soil depends on bioavailability and several cation transporter elements such as calcium (Ca), iron (Fe), and Zn (Aravind and Prasad, 2005; Chirila and Carazeanu, 2008; He et al., 2019). Subsequently, from the contaminated plants, Cd enters the food chain and induces toxic symptoms on all living organisms and human beings. Besides, it is carcinogenic to humans even at low concentrations (Khan et al., 2015). Chlorosis of leaf, necrosis of leaf and root, reduced growth, genomic DNA damage, initiation of cell death, photosynthetic inhibition, oxidative stress, and lipid membrane damage are some of the major symptoms of Cd toxicity (Hernandez and Cooke, 1997; Kabata-Pendias and Pendias, 2001; Clemens, 2006; Groppa et al., 2007; Xu et al., 2013; Lomaglio et al., 2015; Rizwan et al., 2016). Cd was listed at the seventh position for the observed toxicity by the Agency for Toxic Substances and Disease Registry in 1997 (Liao and Freedman, 1998). According to the "National Soil Pollution Survey Bulletin" release in 2014 by the Chinese Ministry, the extreme level of Cd reached 7.0% (Liu et al., 2019). Therefore, the remediation of Cd-contaminated soil is of utmost importance to maintain the ecological balance and provide safe food to mankind.

For the remediation of the PTEs in the soil, several physical and chemical methods can be employed like soil flushing and stabilization by a suitable sorbent etc., but these methods come with some limitations such as high cost and labor (Laghlimi et al., 2015; Parmar and Singh, 2015). Phytoremediation using metal accumulators is one of the low-cost and environment-friendly methods for the extraction or stabilization of toxic metals from contaminated soils. Metal phytotoxicity is evident and well known in the contaminated soils, but often plants growing in contaminated soil having a long history of PTEs become tolerant and accumulate high amounts of PTEs. But how these plants can survive excessive PTEs is a matter of investigation and scientific importance.

Dysphania ambrosioides (L.) is an invasive plant in China that was also known as *Chenopodium ambrosioides* in the past and reported as a Pb hyperaccumulator (Wu et al., 2004). It is a dominant plant in the Pb–Zn contaminated mining areas in Huize County, Yunnan Province, Southwest China (Li et al., 2012, 2016). Our previous studies have revealed that *D. ambrosioides* growing in Pb–Zn contaminated locations were colonized with a high diversity of bacterial and fungal endophytes (Li et al., 2016; Parmar et al., 2018; Sun et al., 2019). Some of these endophytes demonstrated Pb, Zn, and Cd tolerance properties and enhanced host plant growth and influenced its metal accumulation (Li et al., 2016; Sun et al., 2019). The possible mechanism of the endophyte-induced stress tolerance to the host plant growing in soil containing high amounts of metal involves metal detoxification, altering metal distribution in plant cells and positively affecting the antioxidative system (Wang et al., 2016). Several recent studies have reported that the plant-associated symbiotic microbes can affect translocation and accumulation of Cd, improve Cd tolerance, and can enhance the host plant growth under Cd stress conditions (Wang et al., 2016, 2019; Zhang et al., 2019; Li et al., 2020; Yung et al., 2021). Apart from the microbial assemblage associated with the foliar and belowground plant tissues, a portion of the microbial community is conserved into the seeds for the next generation (Walitang et al., 2019). Vertically transmitted seed endophytes are the first symbiotic microbes that colonize the young seedling and subsequently determine the fate of the plant (Li et al., 2019). The high association with the seed endophytes and their potential functional role in increasing host plant tolerance against abiotic stress, such as salt and metal stress (Walitang et al., 2018; Li et al., 2019), make these endophytes the most suitable candidate for the microbial-engineered plant with improved beneficial traits. For example, growth-promoting characteristics conferred by a seed endophyte will be automatically transferred to the subsequent plant generation through the seeds.

In our previous studies, the fungal endophyte FZT214 isolated from the seeds of *D. ambrosioides* showed better Pb, Zn, and Cd tolerance, and can improve host plants' seed germination and seedling growth under Cd stress. However, the mechanism is unknown. Thus, the present study was designed to understand the way seed endophyte FZT214 improves host plant (*D. ambrosioides*) Cd tolerance at different developmental stages, which includes host plant growth promotion, important stress-related biochemical factors, and endogenous changes to the phytohormones levels.

MATERIALS AND METHODS

The Fungal Seed Endophyte FZT214

The fungal endophyte FZT214 was isolated from the seeds of *D. ambrosioides* and was identified to be *Epicoccum nigrum* based on morphology characteristics and molecular analysis (GenBank accession number MN847628.1).

Pot Experiments

For the pot experiments, the seeds of *D. ambrosioides* were surface sterilized by sequentially dipping in 75% ethanol for 2 min, followed by 5% sodium hypochlorite for 2 min and finally 3–5 times rinsed with sterile distilled water (Li et al., 2012). The isolate FZT214 was cultured on PDA plates at 25°C for 4–7 days, and the mycelia were collected and cut into small fragments and suspended in autoclaved distilled water to obtain the mycelia suspension. Then, the surface-sterilized seeds were imbibed in the mycelia suspension for 1 h at 28°C. The control

seeds were soaked into an equal volume of autoclaved mycelia suspension and kept under the same conditions. Subsequently, these seeds were germinated on sterile-water-moistened filter paper in autoclaved Petri dishes under aseptic conditions in a growth chamber (26 ± 1 / 18 ± 1°C, 16/8 h light/dark cycle, 60% relative humidity) for 21 days. The germinated seedlings, equal in size, were transferred to plastic pots (three seedlings per pot) containing sterilized soil substrate (30% perlite:70% peat moss, vol/vol). The soil substrate was supplied with the overages of $CdCl_2 \cdot 2.5H_2O$ to the final concentration of 0, 5, 15, and 30 mg kg^{-1} Cd. The pots were arranged randomly and kept under artificial plant light (16:8 h light/dark cycle). The plants were watered with autoclaved water every 2–3 days according to the requirement and supplied with a nutrient solution once a week. The inoculated plants (E+) were sprayed with the mycelia suspension of FZT214 at 30, 60, 90, and 120 days of the transplant while the control/non-inoculated plants (E−) were sprayed with the autoclaved mycelia suspension of FZT214 in equal amount. There were eight treatments: four Cd concentrations in combination with E+ and E− plants and three developmental stages. Each treatment involved three replicate pots and each pot has three plants. In addition, for each treatment, two pots were kept to recover mature seeds. Thus, the full experiment consists of 88 (72+16) pots.

Plant Growth Attributes and Cd Content Analysis

The plants were harvested at 40 days (growing stage or pre-flowering stage), 105 days (flowering stage), and 130 days (fruiting stage). After harvest, the fresh leaves were collected from each treatment plant, immediately frozen in liquid nitrogen, stored at −80°C, and used for biochemical and phytohormone analysis within 2 weeks. The plants were separated into the shoot (all aboveground parts) and roots (all belowground parts) and washed under tap water to remove adhered soil particles, and then rinsed with deionized water. Finally, the shoot and root lengths were measured. Thereafter, the plants were put into paper bags and oven dried at 50–60°C to constant weight, then the dry biomass and Cd concentration were tested. The rhizospheric soil from each treatment was collected, air dried, and used for Cd analysis. Two pots of each treatment were kept under the same growing conditions for seed maturation. Approximately after 6 months, the seeds were collected, and dry weight was recorded.

For the Cd content analysis, the dried plant samples were crushed to fine powders with a mortar and pestle, and 0.2 g roots/shoots powders was digested with 5 ml HNO_3 (65% w/w) at 110°C for 2 h, then cooled and added with 1 ml H_2O_2 (30% w/w) and heated for 1 h. Finally, the digests were diluted to 50 ml with triple deionized water in a volumetric flask (Shen et al., 2013). The total Cd concentration in the soil was determined by digesting 0.5 g fine soil powder with 4 ml HCl–HNO_3 (3:1, v/v) mixture at 80°C for 30 min, then 100°C for 30 min, and finally 120°C for 1 h. Thereafter, cooled and 1 ml $HClO_4$ was added to continue digestion at 100°C for 20 min, followed by 120°C for 1 h. Finally, the digests were diluted to 50 ml with triple deionized water in a volumetric flask. The concentrations of bio-available Cd in soils were extracted by diethylenetriaminepentaacetic acid–triethanolamine (DTPA-TEA) (Huang et al., 2006). All the samples were prepared in triplicates. The concentrations of Cd in plant parts and soil digests were determined by flame atomic absorption spectrometry (Li et al., 2014). The mean and SD of the HM concentrations were calculated using triplicates, with each replicate consisting of mixed plant parts and soil samples from the individual pot.

Estimation of Total Chlorophyll

Just before the harvesting of the plants at each growth stage, the total chlorophyll was measured with a chlorophyll meter (SPAD 502 plus; Konica Minolta, Inc., Tokyo, Japan). Each individual recorded value of the chlorophyll was average of the 10 readings from the youngest fully developed leaves of the same plant.

Bioaccumulation and Translocation Analysis

Bioaccumulation factor (BAF) and translocation factor (TF) are used in the monitoring of plant's phytoremediation efficiency (Testiati et al., 2013). To assess Cd translocation ability from root to shoot of plants, TF was estimated as per Khan et al. (2015).

$$\mathrm{TF} = \frac{\text{Metal concentration in the aboveground plant parts } \left(\mathrm{mg\ kg^{-1}}\right)}{\text{Metal concentration in the belowground plant parts } \left(\mathrm{mg\ kg^{-1}}\right)}$$

BAF was calculated as the ratio of Cd accumulation in the shoots to the Cd accumulation in the soil. In short, the BAF explains the potential of plants to absorb metal from the soil and subsequently and translocate it to aboveground tissues. The BAF was estimated as defined by Sharma et al. (2019).

$$\mathrm{BAF} = \frac{\text{Metal concentration in the aboveground plant } \left(\mathrm{mg\ kg^{-1}}\right)}{\text{Metal concentration in the soil } \left(\mathrm{mg\ kg^{-1}}\right)}$$

Lipid Peroxidation Analysis

The lipid peroxidation was measured by evaluating the malondialdehyde (MDA) using commercial chemical assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. The test was performed in triplicates. The extent of lipid peroxidation was expressed in terms of nanomoles of malondialdehyde (MDA) formation per gram of leaf tissue. Briefly, the frozen leaf tissue sample was homogenized with a mortar and pestle in a chilled phosphate buffer (50 mM, pH 7.2). The tissue homogenate was centrifuged at 3,500 rpm for 10 min at 4°C. After centrifugation, the resulting supernatant was collected and used to measure MDA concentration using a spectrophotometer (MAPADA UV-1800 PC).

Determination of Glutathione

Glutathione a tripeptide compound having important functions in the antioxidant processes involved in the plant defense mechanism and also a metal chelator (Guo et al., 2012). The reduced glutathione (GSH) was determined using the total glutathione (T-GSH) and oxidized glutathione (GSSG) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The methods were principally based on the previously described enzymatic recycling method evolving cyclic reaction of DTNB (Rahman et al., 2006). Briefly, frozen leaves were homogenized in extraction buffer (1:4 ratio, wt/vol). Afterward, the homogenate was centrifuged at 3,500 rpm for 10 min at 4°C, the supernatant was collected, and stored at 4°C (−20°C if required to keep overnight) until further analysis according to the manufacturer's protocol. The absorbance of the assay mixture was measured twice at 412 nm, first at 30 s and second at 10 min 30 s of the reaction initiation. T-GSH and GSSG content were determined using given formulas. The GSH content was calculated by subtracting the GSSG content from the T-GSH content according to the formula provided in the kit, expressed in micromoles per gram fresh leaf weight.

Determination of the Plant Hormones

Frozen leaves (about 200 mg fresh weight) were crushed in liquid nitrogen with a mortar and pestle then extracted with 10 ml of chilled ethanol. The extracts were vortexed. Subsequently, the extracts were centrifuged at 4,000 rpm for 15 min at 4°C. The supernatant was collected and used for the determination of jasmonic acid (JA), gibberellic acid (GA), and indole-3-acetic acid (IAA). The ELISA-based phytohormone kits from MLBIO Biotechnology Co., Ltd., Shanghai[1] were used for the assay according to the manufacturer's instructions. The color change of the reaction mixture was measured spectrophotometrically at a wavelength of 450 nm. The concentrations of JA, GA, and IAA in the extracts were determined by comparing the O.D. of the samples to the standard curve plotted with the provided standards. The test was performed in triplicate; each replicate consisted of the leaves of all three plants of a single pot pooled together as one sample.

To determine IAA, GA, and JA in crude secondary metabolites of FZT214, the endophytic isolate was cultured in potato dextrose broth for 21 days at 28 ± 2°C in a BOD incubator. Thereafter, the broth culture was filtered and extracted thrice in equal volume ethyl acetate. The extracted metabolites were concentrated in a vacuum rotary evaporator and dissolved in methanol. Subsequently, this crude secondary metabolite of FZT214 was tested for phytohormones as described previously.

Statistical Analysis

The results are presented as the mean of the replicates and SD for each treatment, calculated using Excel 2007. One-way ANOVA, Duncan test ($p < 0.05$) was performed using SPSS 16.0 to determine the differences in mean values between the different treatments of inoculated and non-inoculated plants, and p-value was set at < 0.05. The figures were generated through Origin V8.0724 software.

[1]www.mlbio.cn

RESULTS

Effects of FZT214 on the Growth of *D. ambrosioides*

Pot experiments indicated that seed endophyte FZT214 supported the host plant's growth throughout different developmental stages under variable Cd stress (**Figure 1**).

Aboveground Growth

The shoot length significantly improved ($p < 0.05$) at the flowering and fruiting stage, while at the growing stage there was an increase but not significant ($p > 0.05$). However, the shoot length decreased with the increase in Cd concentration in the amended soil substrate for both inoculated (E+) and non-inoculated plants (E−) (**Figure 1**). At all three stages, the dry biomass of E+ plant shoot was more than that of E− plant shoot irrespective of the Cd concentrations. The difference was significant ($p < 0.05$) at the flowering and fruiting stages, while it was non-significant ($p > 0.05$) at the growing stage (**Figure 1**).

Belowground Growth

During the growing stage, the root length of E+ plants was more than that of E− plants at all Cd concentrations except 5 mg kg^{-1}. The difference was significant ($p < 0.05$) at 30 mg kg^{-1} Cd. Also during the flowering stage, the root length of E+ plants was more than that of E− plants at all Cd concentrations; the difference was significant ($p < 0.05$) at 5, 15, and 30 mg kg^{-1} Cd while non-significant ($p > 0.05$) at 0 mg kg^{-1} Cd. However, during the fruiting stage, the root length of E− plants was more than that of E+ plants at all Cd concentrations except at 30 mg kg^{-1}, and the difference was significant ($p < 0.05$) except at 30 mg kg^{-1}. The dry biomass of E+ plant root was more than that of E- plants at different developmental stages and Cd concentrations. The difference was significant ($p < 0.05$) at the flowering and fruiting stages, while it was non-significant ($p > 0.05$) at the growing stage.

Seed Production

The seed production decreased both in E+ and E− plants as the Cd increase in the soil, except at 15 mg kg^{-1} Cd for E+ plants. Overall, seed production was significantly ($p < 0.05$) higher in E+ plants than that of E− plants (**Figure 2**).

Effects of FZT214 on Cd Accumulation, Uptake, and Translocation

Cd uptake and accumulation in aboveground and belowground parts of E+ and E− plants at different Cd concentrations are presented in **Table 1**. It was found that Cd concentrations decreased in aboveground parts of E+ plants as compared with E− plants except at 30 mg kg^{-1} Cd at the growing stage and 5 mg kg^{-1} Cd at the fruiting stage. Similarly, Cd concentrations also decreased in belowground parts of E+ plants as compared with E− plants except at 5 mg kg^{-1} Cd at the growing stage. However,

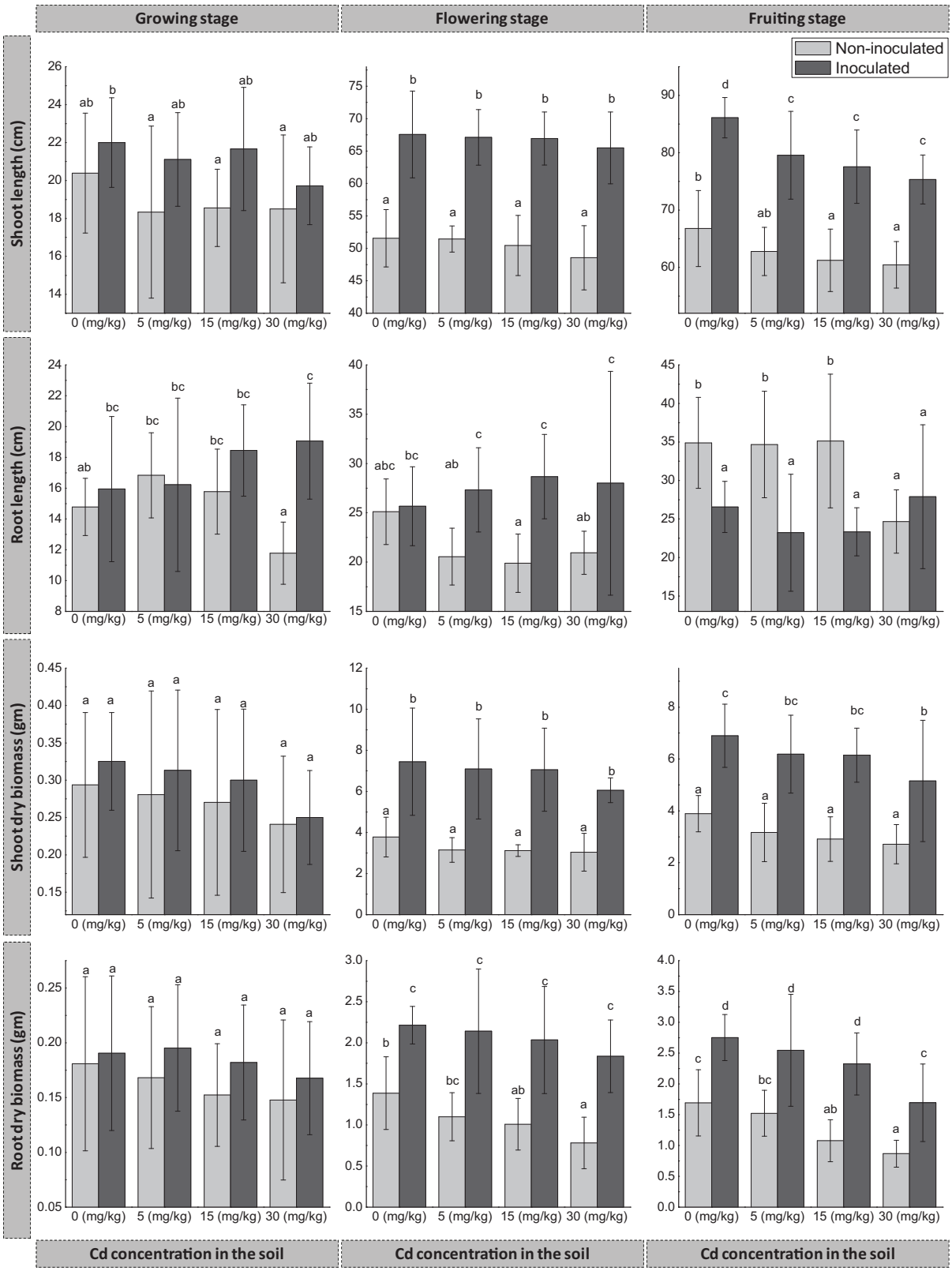

FIGURE 1 | Effect of seed endophyte FZT214 on *D. ambrosioides* growth under different Cd concentrations at different developmental stages (growing, flowering, and fruiting). The different lowercase letters indicate significant variation between treatments ($p < 0.05$) according to Duncan's test of one-way ANOVA. The shoot and root length, dry weight (mean ± STD, $n = 9$).

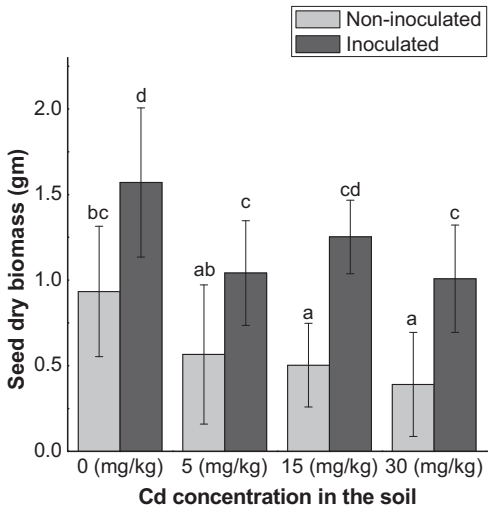

FIGURE 2 | Seed production affected by endophyte FZT214 under variable Cd stress. The different lowercase letters indicate significant variation between treatments ($p < 0.05$) according to Duncan's test of one-way ANOVA (mean ± STD, $n = 6$).

the difference was non-significant ($p > 0.05$) in most of the tested conditions except for Cd accumulation in aboveground parts at 15 mg kg^{-1} Cd during the flowering and fruiting stage, and at 30 mg kg^{-1} Cd during the growing stage.

The total and the bioavailable Cd content of the rhizospheric soil are presented in **Table 2**. In general, the total and bioavailable Cd in the rhizospheric soil of E+ plants was less than that of E− plants, except for 5 and 15 mg kg^{-1} Cd in the growing and flowering stages. The BAF and TF are presented in **Table 3**. The results indicated that the inoculation of FZT214 to *D. ambrosioides* did not significantly ($p > 0.05$) affect the BAF and TF for Cd, with only a few exceptions.

Effects of FZT214 on the Biochemical Factors of *D. ambrosioides*

Chlorophyll Content

The inoculation of FZT214 to *D. ambrosioides* had a positive effect on the total chlorophyll content of host plants at all three developmental stages (**Figure 3**). During the growing stage, the total chlorophyll content of E+ plants was more than that of E− plants, and the difference was significant ($p < 0.05$) at 0 and 30 mg kg^{-1}. During the flowering and fruiting stages, the total chlorophyll content of E+ plants was significantly ($p < 0.05$) more than that of E− plants at all concentrations of Cd. At the same time, with the increase in soil Cd concentrations, the total chlorophyll content was decreased for both E+ and E− plants in most of the cases, except at 30 mg kg^{-1} for E+ plants during the growing stage.

Glutathione and Malondialdehyde

The inoculation of FZT214 to *D. ambrosioides* affected host plants' GSH and MDA content (**Figure 3**). The GSH content of E+ plants was more than that of E− plants. The difference was significant ($p < 0.05$) at most conditions during different developmental stages, except at 30 mg kg^{-1} during the flowering stage ($p > 0.05$) and 0 mg kg^{-1} during the fruiting stage ($p > 0.05$). The increase in soil Cd concentrations supported the increase of GSH content in both E+ and E− plants at most of the cases except at 30 mg kg^{-1} for E+ plants during the flowering stage. The MDA content of E+ plants was less than that of E− plants. The difference was significant ($p < 0.05$) at most conditions during different developmental stages, except at 0 and 5 mg kg^{-1} during the growing and fruiting stages ($p > 0.05$).

Effects of FZT214 on the Phytohormone Content of *D. ambrosioides*

The inoculation of FZT214 to *D. ambrosioides* affected host plants' phytohormone content. However, the change was variable with Cd concentration in the soil and developmental stages (**Figure 4**). The inoculation increased host plants' IAA content except at 30 mg kg^{-1} Cd at the fruiting stage. However, the difference was only significant ($p < 0.05$) at 5 and 15 mg kg^{-1} Cd during the growing stage (**Figure 4**). GA content of E+ plants was more than that of E- plants, except at 5 mg kg^{-1} Cd during all developmental stages and 0 mg kg^{-1} Cd during the growing stage. The difference was significant ($p < 0.05$) during the flowering stage at all Cd concentrations, while the difference was non-significant ($p > 0.05$) during the growing stage at all Cd concentrations. During the fruiting stage, the difference was significant ($p < 0.05$) at all Cd concentrations

TABLE 1 | Effects of endophyte FZT214 on Cd accumulation in aboveground and belowground tissues of *D. ambrosioides* grown under different Cd concentrations at different developmental stages (mean ± STD, $n = 3$).

		0 mg Cd kg^{-1} soil		5 mg Cd kg^{-1} soil		15 mg Cd kg^{-1} soil		30 mg Cd kg^{-1} soil	
		Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated	Non-inoculated	Inoculated
Tissue cadmium content (mg kg^{-1} dry weight)									
Aboveground parts	Growing stage	0.30 ± 0.05a	0.30 ± 0.03a	12.30 ± 1.71b	12.07 ± 1.63b	21.31 ± 3.52cd	17.62 ± 4.04c	23.63 ± 3.31d	28.80 ± 0.48e
	Flowering stage	0.38 ± 0.12a	0.27 ± 0.02a	6.41 ± 0.77b	4.95 ± 0.89b	11.13 ± 1.94d	8.33 ± 0.51c	11.13 ± 1.45d	9.21 ± 1.54cd
	Fruiting stage	0.20 ± 0.02a	0.13 ± 0.00a	5.47 ± 1.50b	5.83 ± 1.16b	9.82 ± 2.27c	6.56 ± 1.10b	10.69 ± 1.58c	10.27 ± 0.60c
Belowground parts	Growing stage	0.59 ± 0.13a	0.26 ± 0.03a	15.48 ± 2.18ab	19.40 ± 5.68b	22.70 ± 6.60b	18.68 ± 4.25b	47.74 ± 23.29c	39.05 ± 7.86c
	Flowering stage	0.78 ± 0.32a	0.31 ± 0.04a	17.74 ± 2.31bc	13.63 ± 3.62b	17.45 ± 1.59bc	17.15 ± 2.06bc	20.85 ± 1.96c	20.30 ± 7.46c
	Fruiting stage	0.56 ± 0.10a	0.45 ± 0.18a	19.44 ± 2.27bc	11.75 ± 1.08b	22.79 ± 5.54bc	13.53 ± 2.19bc	20.45 ± 0.71c	17.25 ± 5.95c

The different lowercase letters indicate significant variation between treatments ($p<0.05$) according to Duncan's test of one-way ANOVA.

TABLE 2 | Effects of endophyte FZT214 on total and bioavailable Cd in the soils at different developmental stages.

	0 mg Cd kg^{-1} soil		5 mg Cd kg^{-1} soil		15 mg Cd kg^{-1} soil		30 mg Cd kg^{-1} soil	
	Non-inoculated	**Inoculated**	**Non-inoculated**	**Inoculated**	**Non-inoculated**	**Inoculated**	**Non-inoculated**	**Inoculated**
Total Cd content of the rhizospheric soil after harvest								
Growing stage	0.36 ± 0.16a	0.20 ± 0.065a	7.64 ± 0.85b	10.31 ± 2.10b	36.78 ± 2.30d	29.42 ± 0.79c	96.27 ± 2.09f	70.23 ± 7.12e
Flowering stage	0.47 ± 0.12a	0.36 ± 0.04a	35.63 ± 2.66c	22.52 ± 3.71b	44.33 ± 7.93d	60.53 ± 6.84e	66.66 ± 3.93e	63.78 ± 7.08e
Fruiting stage	0.63 ± 0.03a	0.53 ± 0.05a	37.13 ± 7.88b	27.30 ± 2.20b	67.09 ± 3.69c	63.53 ± 5.45c	85.65 ± 8.29d	62.68 ± 12.20c
Bioavailable Cd content of the rhizospheric soil after harvest								
Growing stage	0.29 ± 0.14a	0.18 ± 0.06a	5.36 ± 0.55b	6.98 ± 1.31b	27.11 ± 1.17d	20.36 ± 0.53c	71.90 ± 1.05f	54.32 ± 5.14e
Flowering stage	0.37 ± 0.14a	0.26 ± 0.04a	24.38 ± 1.90c	15.14 ± 2.60b	31.16 ± 5.58c	45.57 ± 5.21d	51.08 ± 1.72d	48.17 ± 8.79d
Fruiting stage	0.54 ± 0.03a	0.43 ± 0.05a	27.91 ± 6.75c	17.76 ± 2.00b	53.70 ± 5.47d	45.78 ± 4.61d	73.92 ± 1.34e	47.07 ± 9.82d

The different lowercase letters indicate significant variation between treatments ($p < 0.05$) according to Duncan's test of one-way ANOVA.

TABLE 3 | Effects of the endophyte (FZT214) inoculation on bioaccumulation factor (BAF) and translocation factor (TF) (mean ± STD, n = 3) of *D. ambrosioides* grown under different Cd concentrations at different developmental stages (growing, flowering, and fruiting).

		5 mg Cd kg^{-1} soil		15 mg Cd kg^{-1} soil		30 mg Cd kg^{-1} soil	
		Non-inoculated	**Inoculated**	**Non-inoculated**	**Inoculated**	**Non-inoculated**	**Inoculated**
BAF	Growing stage	1.61 ± 0.08d	1.20 ± 0.29c	0.58 ± 0.13b	0.60 ± 0.15b	0.25 ± 0.03a	0.41 ± 0.04ab
	Flowering stage	0.18 ± 0.02ab	0.23 ± 0.07ab	0.26 ± 0.09b	0.14 ± 0.02a	0.17 ± 0.03ab	0.15 ± 0.04a
	Fruiting stage	0.15 ± 0.01ab	0.21 ± 0.05c	0.15 ± 0.03ab	0.10 ± 0.01a	0.12 ± 0.01ab	0.17 ± 0.03bc
TF	Growing stage	0.82 ± 0.22abc	0.64 ± 0.10ab	0.97 ± 0.22c	0.94 ± 0.06bc	0.55 ± 0.16a	0.76 ± 0.13abc
	Flowering stage	0.36 ± 0.02a	0.37 ± 0.04a	0.63 ± 0.06b	0.49 ± 0.08ab	0.54 ± 0.10ab	0.51 ± 0.23ab
	Fruiting stage	0.29 ± 0.11a	0.50 ± 0.11ab	0.44 ± 0.08ab	0.49 ± 0.11ab	0.52 ± 0.09ab	0.65 ± 0.24b

The different lowercase letters indicate significant variation between treatments ($p < 0.05$) according to Duncan's test of one-way ANOVA.

except at 15 mg kg^{-1} Cd. On the contrary, JA content of E+ plants was comparatively less than that of E− plants during all developmental stages (**Figure 4**). During the growing stage, the difference was significant ($p < 0.05$) at 0 and 15 mg kg^{-1}. During the flowering stage, the difference was only significant ($p < 0.05$) at 0 mg kg^{-1}. During the fruiting stage, the difference was significant ($p < 0.05$) under various Cd exposures except ($p > 0.05$) at 0 mg kg^{-1} Cd.

DISCUSSION

Growth Improvement

Previous studies have demonstrated that endophytes can enhance host plant growth under Cd stress (Soleimani et al., 2010; Khan et al., 2015; Zhu et al., 2018; He et al., 2019; Shahid et al., 2019; Zhang et al., 2019). In the present study, it was found that fungal endophyte FZT214 improved *D. ambrosioides* growth under Cd stress. The dry biomass of both aboveground and belowground parts of E+ plants was more than that of E− plants. However, the difference depends on the developmental stage of plants and the concentration of Cd in the soil. At the flowering and fruiting stages, the shoot length and dry biomass of aboveground parts were significantly ($p < 0.05$) improved, while at the growing stage, there was an increase but not significant ($p > 0.05$). At the growing and flowering stage, the root length of E+ plants was longer than that of E− plants at all Cd concentrations except at 5 mg kg^{-1} in the growing stage. This trend was opposite during the fruiting stage, except at 30 mg kg^{-1} in the soil (**Figure 1**). Understanding this phenomenon (less biomass and more length) needs more work. Overall, E+ plants had shown positive developmental effects than E− plants under various Cd stress levels. However, signs of Cd toxicity were observed after 90 days on the leaves of plants grown in 15 and 30 mg kg^{-1} Cd in the soil, regardless of inoculation; these plants exhibited some yellow spots especially on the old leaves' surface. The reason for this phenomenon could be various physiological changes like a surge of abscisic acid or ethylene as a result of the Cd stress (Zhou and Qiu, 2005).

Seed endophytes have been associated with enhanced plant growth–related properties such as nutrient uptake, reduced susceptibility to heat and drought stress, and improved seed production (Hubbard et al., 2012, 2014; Li et al., 2019). This study indicated a comparative reduction in the seed dry biomass with the increase in the soil Cd both in E+ and E− plants except at 15 mg kg^{-1} Cd for E+ plants (**Figure 2**). This can be due to the effect of Cd stress. An important fact to be noted is that the dry seed biomass of E+ plants was significantly ($p < 0.05$) higher than that of E− plants. Indeed, we also observed early flowering in E+ plants as compared with E+ plants. Similarly, Gao and Shi (2018) reported successful transmission of seed endophyte *Herbaspirillum frisingense* RE3-3 to the seedlings, which improved seedling development and growth under Cd stress. These results suggest the possible survival strategy of plants

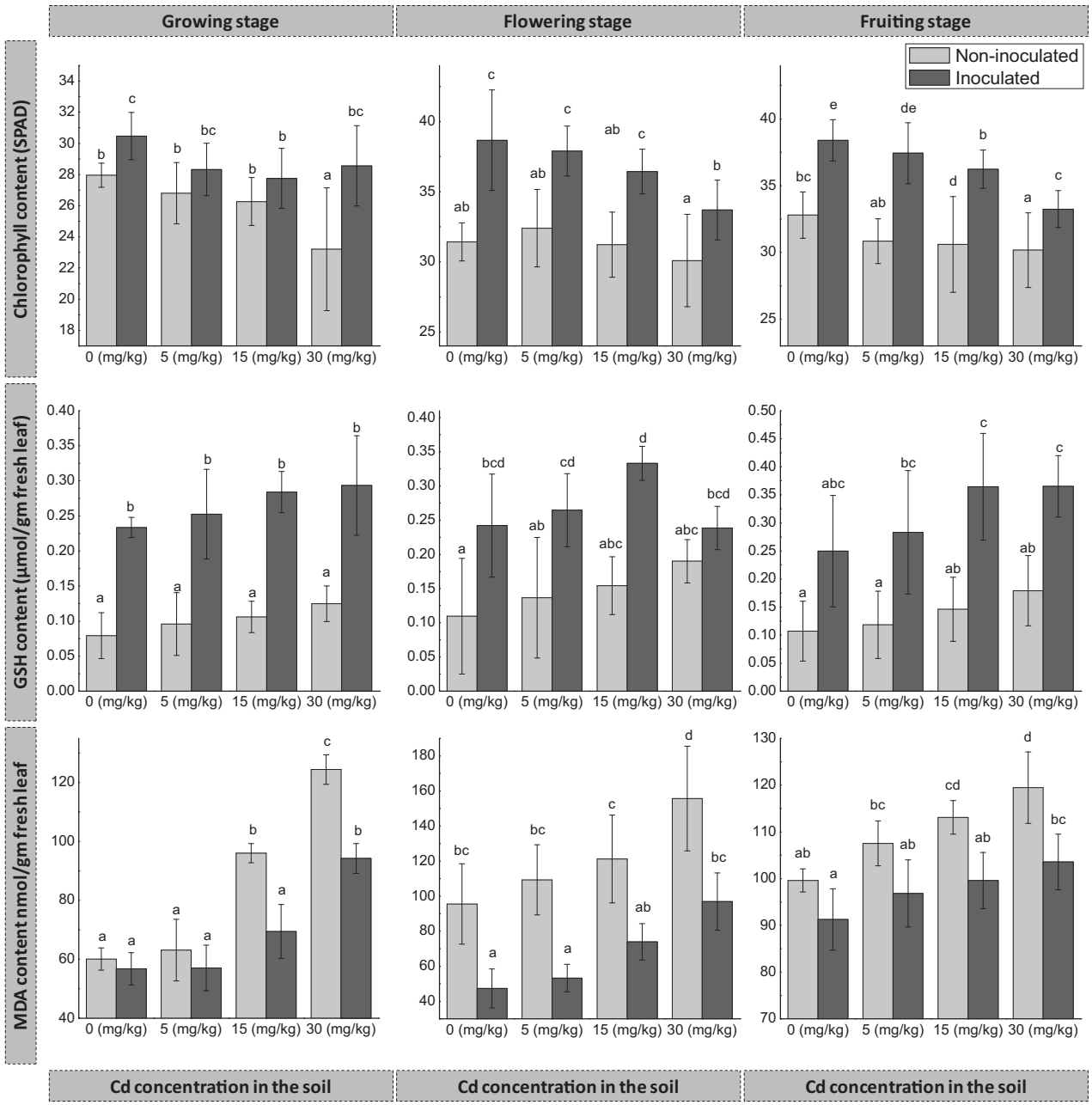

FIGURE 3 | Effects of endophyte FZT214 on the chlorophyll (mean ± STD, $n = 9$), GSH, and MDA (mean ± STD, $n = 3$) content of *D. ambrosioides* grown under different Cd concentrations at different developmental stages. The different lowercase letters indicate significant variation between treatments ($p < 0.05$) according to Duncan's test of one-way ANOVA.

under Cd stress conditions where the symbiotic association with the seed endophyte provides growth and tolerance benefits as well as helps the host plant to produce enough seeds for the next generation.

Cd Accumulation and Translocation

As described previously, *D. ambrosioides* is a Pb hyperaccumulator that can be used for phytoextraction and/or phytostabilization. For the effective and successful phytoremediation process, it is of utmost importance that the plant used could be able to withstand high metal stress. In addition, constant metal absorption and translocation to the aboveground parts is also very crucial. In general, the Cd concentration was relatively less in the aboveground and belowground tissues of E+ plants in most of the treatments (**Table 1**). This finding was similar to the results of the previous studies that observed lower Cd in inoculated plants than non-inoculated plants (Wang et al., 2016; He et al., 2017;

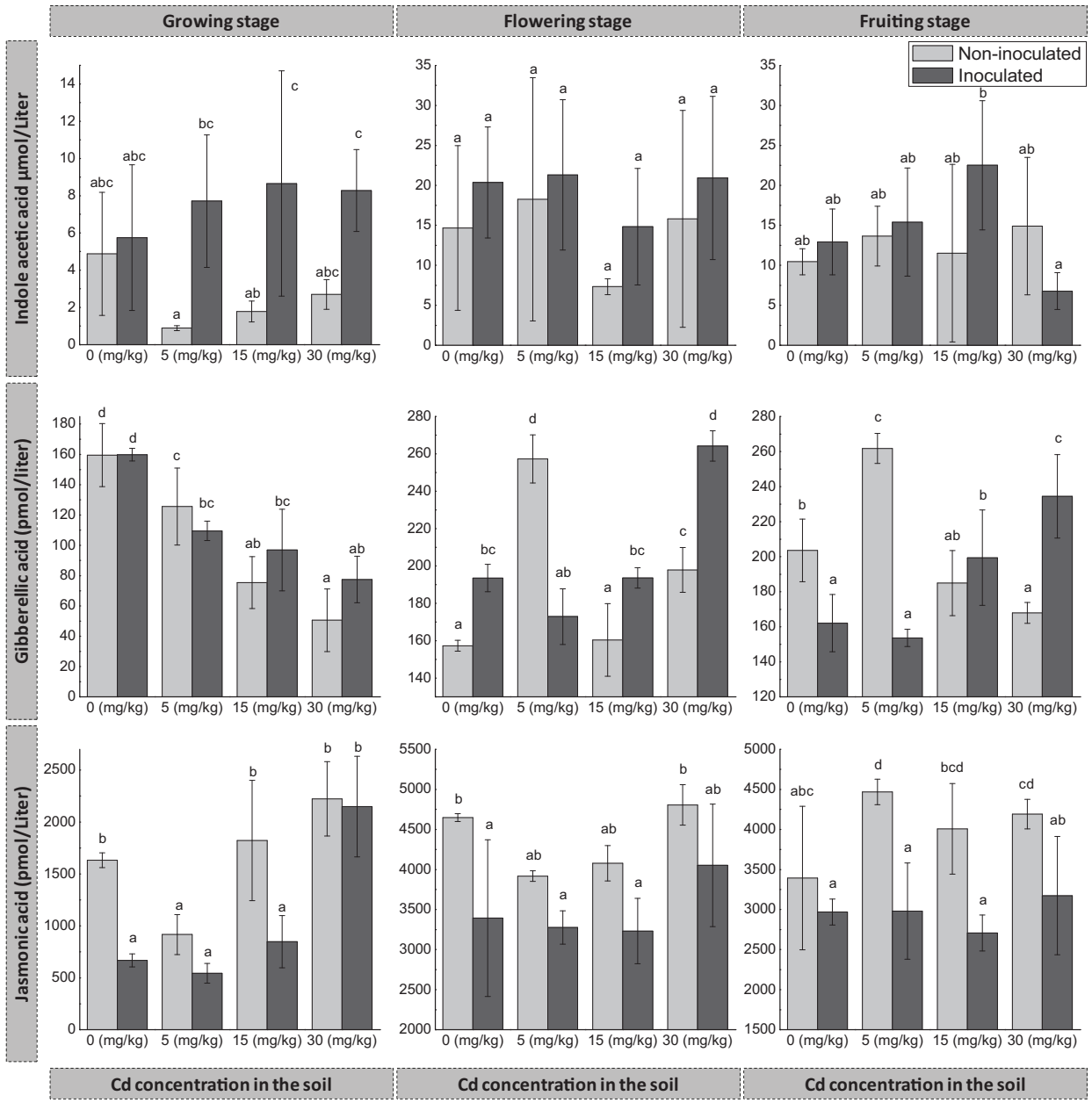

FIGURE 4 | Effects of endophyte FZT214 on the endogenous phytohormone content of *D. ambrosioides* grown under different Cd concentrations at different developmental stages. The different lowercase letters indicate significant variation between treatments ($p < 0.05$) according to Duncan's test of one-way ANOVA (mean ± STD, n = 3).

Zhan et al., 2017; Zhu et al., 2018; Shahid et al., 2019). However, in this study, the difference was non-significant ($p > 0.05$) in most of the tested conditions (**Table 1**).

Khan et al. (2015) recorded lower Cd in the leaf and roots of *Serratia* sp.–inoculated *Solanum nigrum* grown under Cd stress, and higher Cd in the stems of the inoculated plants. Wang et al. (2016) also observed that the dark septate endophyte colonization to *Zea mays* reduced Cd in the shoots and roots under Cd stress. He et al. (2017) reported that overall Cd content was less in the shoots and roots of inoculated plants in most of the treatments. However, the total Cd accumulated per plant was higher in the inoculated plants due to their higher biomass. This "dilution mechanism" can be related to the lower Cd toxicity in plant tissue and subsequently increased plant biomass in the inoculated plants (Guo et al., 2013). The induced growth and tolerance by the inoculated endophyte can be due to the relative decrease in metal uptake into the above and belowground tissues and the selective translocation of Cd from roots to aboveground tissues (Zhu et al., 2018; Shahid et al., 2019). Generally, metal contents in plant samples are subject to the availability of metals in the

soil (Vivas et al., 2006). Other possible reasons for lower Cd in the inoculated plants could be Cd sequestration in the root zone, siderophore secretion and production of exopolysaccharides, excretion of Cd from the cell by efflux pumps, and reduced phytotoxicity through secretion of antioxidants (Shahid et al., 2019; Wang et al., 2019).

The metal accumulation by a plant depends on the availability of the metal in the root zone of the plant (Hinsinger et al., 2009); therefore, the rhizospheric soil of E+ and E− plants was tested for the Cd concentration after the harvest. The metal content and its bioavailability in the rhizospheric zone of the plant are often considered as the better indicator of the metal translocation to the plant than the total soil (Cheng et al., 2018; Campillo-Cora et al., 2019). In general, the total and bioavailable Cd in the rhizospheric soil of E+ plants was less than that of E− plants, but the difference was non-significant ($p > 0.05$), except for some conditions (**Table 2**). Besides, the difference between E+ and E− plants was inconsistent with the Cd concentration in the soil and developmental stage of host plants. In addition, the belowground tissues accumulated more Cd than the aboveground tissues; it indicates that only little Cd was translocated to the aboveground parts. Khan et al. (2017) reported greater Cd contents in the *Solanum nigrum* roots, which suggest the role of these plants as a phytostabilizer. More Cd accumulation in the belowground tissue also indicates that the roots are the major site for storage of metals (Shahid et al., 2019).

Interestingly, the Cd concentration in the aboveground parts was more during the growing stage than the latter stages, irrespective of the soil Cd concentration and the endophyte inoculation. It indicates that the Cd accumulation and translocation were relatively higher during the growing stage than the latter stages. The BAF and TF were comparatively higher at the growing stage (**Table 3**). Therefore, the impact of Cd was greater while the plants were young; this could be the possible reason that the difference between shoot and root dry biomass of inoculated and non-inoculated plants was lower ($p > 0.05$) at the growing stage while higher ($p < 0.05$) at the latter stages (**Figure 1**).

Physiological and Biochemical Changes

The chlorophyll content is a significant indicator of plant growth status (Chen et al., 2010). Moreover, Cd-induced toxicity in plants can negatively influence the biosynthesis of chlorophyll by preventing protochlorophyllide reductase activity required for chlorophyll synthesis and altering the photosynthetic electron transport at PS-II (Somashekaraiah et al., 1992; Neelu, Kumar et al., 2000). In the present study, the observed decrease in the chlorophyll content with the increase of Cd concentration in the soil was probably due to Cd stress (**Figure 3**); a similar trend was also reported by several previous researchers (Zhang et al., 2010; Kamran et al., 2015). Our finding of relatively higher chlorophyll content in E+ plants agrees with the finding of Hunt et al. (2005) that endophytic-inoculated perennial ryegrass plants had higher chlorophyll content.

High exposure of Cd to the plants induces Cd stress that, in turn, increases ROS in the plant tissues. ROS in the form of superoxide anion and hydrogen peroxide mimic and disrupt the regular cellular functions by shifting the oxidation/reduction cycle (Khan et al., 2015). Glutathione is one of the important ROS scavenging molecules in plants. The free glutathione exists chiefly in its reduced form (GSH), its relatively higher production in the stress-adapted plants is due to strong activation of the defense cycle (Tausz et al., 2004). In this study, GSH content of E+ plants was more than that of E− plants (**Figure 3**), which indicates the counteractive mechanism developed by inoculated endophyte to check oxidative stress due to Cd. The results were consistent with previous studies, which observed endophytic inoculation may improve the host plant growth and tolerance to Cd stress through GSH regulation in different host plants (Khan et al., 2015; Wang et al., 2016; He et al., 2017; Zhan et al., 2017; Zhang et al., 2019).

Lipid peroxidation is an indicator of oxidative damage of the plant under metal stress; primarily it disturbs functions and integrity of cell membrane, and the damage is often irreversible (Khan and Lee, 2013; Khan et al., 2015; Bilal et al., 2018). Malondialdehyde (MDA) is a secondary breakdown product of lipid peroxides; its low levels imply a lesser degree of lipid peroxidation. The relatively lower MDA in E+ plants is due to lower lipid peroxidation, which suggests that the endophyte FZT214 interacts with the host plant and has some synergistic role in the protection of the host from Cd stress. The results were consistent with previous studies that lower MDA contents were found in endophyte-infected plants (Khan and Lee, 2013; Khan et al., 2015; Wang et al., 2016; Bilal et al., 2018; Zhu et al., 2018). The increase in soil Cd concentrations showed some signs of Cd toxicity in terms of an increase in the MDA content for both E+ and E− plants, but still E+ plants were more tolerant with lesser MDA content.

Previous studies have shown that some endophytic fungi can exogenously produce phytohormones that can help the host plant to mitigate the effects of abiotic stress (Khan et al., 2012). In liquid culture, the secondary metabolites of FZT214 showed the presence of IAA, GA, and JA (**Supplementary Table 1**). Interestingly, FZT214 inoculation increased IAA content of E+ plants as compared with E− plants (**Figure 4**). IAA has been reported to play a key role in apical dominance, cell elongation, and development of vascular tissue (Wang et al., 2001). It can also modulate plant growth under stress conditions (Eyidogan et al., 2012). The relatively higher endogenous IAA production in E+ plants than E− plants in the current study may be related to the increased shoot length and biomass in the inoculated plants (**Figures 1**, **4**).

GA is an important phytohormone that plays a critical role in plant development such as stem elongation, flower and trichome initiation, seed germination, fruit development, and leaf expansion (Yamaguchi, 2008; Liu et al., 2009). JA had also been demonstrated to be able to promote plant performance under unfavorable conditions such as metal stress (Bilal et al., 2018; Per et al., 2018). The relatively higher endogenous GA production in E+ plants than E− plants especially at 15 and 30 mg kg^{-1} Cd in the current study indicates better plant growth and can also be related to the increased seed production (**Figure 2**). JA was reported to alter antioxidant potential and reducing H_2O_2, MDA concentrations, and additionally improve photosynthetic

pigment concentrations under Pb and Cd stress in different plants (Piotrowska et al., 2009; Ahmad et al., 2017). JA is related to plant response to Cu and Cd toxicity with differential effects amidst the plant and growth stage (Maksymiec et al., 2005). It induces the production of defensive proteins called jasmonate inducible proteins and other metabolic changes (Farmer et al., 2003; Maksymiec et al., 2005). These changes have been related to several developmental processes of plants; for example, it may reduce photosynthetic activity and growth processes, and commence harmful effects (Maslenkova et al., 1990; Maciejewska and Kopcewicz, 2002; Maksymiec et al., 2005).

The relatively lower JA observed in E+ than E− plants indicates better plant tolerance to Cd stress. Kim et al. (2014) also observed reduced contents of endogenous JA in rice under Cd stress. Bilal et al. (2018) observed higher GA, and lower ABA and JA in the endophyte inoculated plants under Al/Zn stress. To further test the role of FZT214 in host-plant physiology under Cd-exposed conditions, this isolate can also be assessed in the future on phytohormone-deficient mutant plant cultivars, e.g., GA-deficient mutant rice cultivar (Waito-C) and GA cultivar (Dongjin-byeo) (Khan et al., 2012). The symbiotic interaction of the plant–endophyte alleviates the abiotic stress by inducing various physiological changes like reduced glutathione, catalase, peroxidase, and polyphenol oxidase, besides endophytic interaction modulates the plant hormone levels such as downregulation of abscisic acid, change in jasmonic acid, and increase in salicylic acid contents (Waqas et al., 2012; Bilal et al., 2018).

CONCLUSION AND REMARKS

If not all, most seed endophytes are directly transferred from their parents to progeny. Furthermore, their existence or colonization could play an essential role in the successive germination and plant growth. This study explores the role of fungal seed endophyte FZT214 to *D. ambrosioides* during its different developmental stages under various Cd stress conditions. The results showed that FZT214 colonization supported *D. ambrosioides* growth under variable Cd stress, through all three developmental stages as evident by the increased plant growth parameters, higher chlorophyll content, lower oxidative damage revealed by the lower lipid peroxidation, and higher GSH content. The beneficial effects of the FZT214 were more pronounced under Cd-spiked soil, explaining its effectiveness in decreasing the Cd toxicity in E+ plants.

The FZT214 colonization also significantly improved the seed production of *D. ambrosioides* under Cd stress conditions and induced early flowering. The results suggested that endophyte-colonized *D. ambrosioides* may have an ecological and evolutionary advantage in the metal-contaminated environment as compared with the non–endophyte-treated plants. Seed endophytic isolate FZT214 was recorded to exogenously secrete phytohormones such as IAA, GA, and JA. The phytohormone-producing characteristics of this fungal isolate might be the key mechanism of the host plant growth promotion.

Moreover, a deep understanding of the molecular mechanism of the interaction between host plant–endophytes–metal is a crucial requirement in the future, and more studies are required concerning FZT214 potential in other plants for phytoremediation and growth promotion in the Cd-contaminated environment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SP, VKS, and HL hypothesized the research work. SP and VKS performed the research, analyzed the data, wrote the manuscript, and review, and edited the manuscript. TL performed the methodology. WT investigated the project and resources, project administration. HL acquired the funding and supervised the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.783475/full#supplementary-material

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Epichloë Fungal Endophytes Influence Seed-Associated Bacterial Communities

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Seeds commonly harbour diverse bacterial communities that can enhance the fitness of future plants. The bacterial microbiota associated with mother plant's foliar tissues is one of the main sources of bacteria for seeds. Therefore, any ecological factor influencing the mother plant's microbiota may also affect the diversity of the seed's bacterial community. Grasses form associations with beneficial vertically transmitted fungal endophytes of genus *Epichloë*. The interaction of plants with *Epichloë* endophytes and insect herbivores can influence the plant foliar microbiota. However, it is unknown whether these interactions (alone or in concert) can affect the assembly of bacterial communities in the produced seed. We subjected *Lolium multiflorum* plants with and without its common endophyte *Epichloë occultans* (E+, E-, respectively) to an herbivory treatment with *Rhopalosiphum padi* aphids and assessed the diversity and composition of the bacterial communities in the produced seed. The presence of *Epichloë* endophytes influenced the seed bacterial microbiota by increasing the diversity and affecting the composition of the communities. The relative abundances of the bacterial taxa were more similarly distributed in communities associated with E+ than E- seeds with the latter being dominated by just a few bacterial groups. Contrary to our expectations, seed bacterial communities were not affected by the aphid herbivory experienced by mother plants. We speculate that the enhanced seed/seedling performance documented for *Epichloë*-host associations may be explained, at least in part, by the *Epichloë*-mediated increment in the seed-bacterial diversity, and that this phenomenon may be applicable to other plant-endophyte associations.

Keywords: seed microbiota, plant-associated bacterial communities, *Epichloë* endophytes, plant-microbe interactions, herbivory

INTRODUCTION

Plant seeds commonly harbour a diverse community of microbes including bacteria and fungi (Nelson, 2018). The seed-associated microbes are generally critical for plant fitness since they can confer protection against herbivores and pathogens, increase germination, enhance seedlings growth, and increase plant tolerance to stresses (Truyens et al., 2015; Bastias et al., 2017; Rochefort et al., 2021). Members of the seed microbiota can be acquired from different sources namely certain mother plant tissues, plant reproductive structures and the environment (Shade et al., 2017; Fort et al., 2021). Those that are inherited from mother plants are termed as 'vertically transmitted microbes', whereas those recruited from the external environment are termed as 'horizontally transmitted microbes' (Shade et al., 2017). Since the mother plant's microbiota is one of the main sources of microbes for seeds, any ecological factor influencing this source could eventually affect the diversity and composition of the seed's microbial community (Chesneau et al., 2020; Fort et al., 2021). In fact, it is well documented that plant leaf microbial communities can be influenced by the interaction of plants with beneficial persistent microorganisms and herbivores (Nissinen et al., 2019; Seabloom et al., 2019; Humphrey and Whiteman, 2020). However, it is not fully understood whether the effects of these ecological factors on the mother plant microbiota can persist and affect the assemblies of microbes associated with the produced seeds (Wei and Jousset, 2017). A remarkable symbiotic system to evaluate the effects of ecological interactions experienced by mother plants on seed-associated microbial communities is the association between cool-season grasses and asexual fungal endophytes of the genus *Epichloë*. These endophytes are persistent symbionts of grasses (they grow in foliar host tissues during the entire plant life) and are vertically transmitted through the seeds (Schardl et al., 2004). Because *Epichloë* endophytes confer antiherbivore protection and can modulate the leaf microbiota of mother plants (e.g., Bastias et al., 2017; Nissinen et al., 2019), we hypothesise that these symbionts may also influence the diversity and composition of the seed microbiota.

The potential effects of *Epichloë* endophytes on the diversity and composition of seed microbial communities have been barely explored (e.g., Tannenbaum et al., 2021). *Epichloë* may affect the seed microbiota though its effects on the microbial assemblies of mother plants. For example, the presence of *Epichloë coenophiala* endophytes modified the leaf-associated fungal communities in *Festuca arundinacea* plants (Syn. *Schedonorus phoenix*; Nissinen et al., 2019). In addition, *Epichloë festucae* var. *lolii* endophytes re-organised the bacterial communities associated with seedlings of *Lolium perenne* (Tannenbaum et al., 2020). *Epichloë* symbionts may also affect the seed microbiota *via* the transport of certain endophytic microbes into the developing seed. At the host reproductive stage, *Epichloë* extend hyphae into fertilised ovaries and later, into embryos and other seed structures as part of the vertical transmission process (Gundel et al., 2011b; Liu et al., 2017). Therefore, microbes located on fungal hyphae of *Epichloë* spp. could eventually be transmitted to seeds. In fact, bacteria inhabiting the external surface of *Epichloë* mycelia have been recently isolated (Bastías et al., 2020). The potential regulation of the seed microbiota by *Epichloë* endophytes could also arise due to *Epichloë*-mediated changes on seed chemical composition. *Epichloë* endophytes can modify the concentrations of sugars (i.e., mannitol, ribitol and thehalose) and antioxidants (i.e., tocochromanols and glutathione) in seeds (Gundel et al., 2018; Zhang et al., 2019; Hettiarachchige et al., 2021). In addition, the accumulation of *Epichloë*-derived alkaloids has been shown to modulate bacterial communities associated with host plant leaves (Roberts and Lindow, 2014) and as such may also influence the seed microbiota (Hewitt et al., 2020). *Epichloë* could also modify the seed microbial composition *via* competition for plant resources. The successful competition for plant resources by *Epichloë occultans* endophytes seemed to exclude the pathogen *Claviceps purpurea* from *Lolium multiflorum* plant flowers (Pérez et al., 2013).

The interaction of mother plants with herbivores can also affect their foliar-associated microbial communities and, eventually, the microbiota of their produced seed. For example, the bacterial diversity in the leaves of *Cardamine cordifolia* plants was modified by the attack of the foliar chewing insect *Scaptomyza nigrita* (Humphrey and Whiteman, 2020). The herbivore-mediated changes in leaf plant microbiota could be explained by the activation of plants defences (acting negatively on microbes), the release of plant nutrients from damaged tissues, and the inoculation of microbes from the herbivore into plant tissues (e.g., microbes inhabiting its oral secretions; Smets and Koskella, 2020). In the case of the interaction between *C. cordifolia* plants and *S. nigrita* insects, the plant activation of defences responses due to herbivore attack (i.e., activation of jasmonic acid-dependent responses), explained the changes in bacterial diversity (Humphrey and Whiteman, 2020). Interestingly, since *Epichloë* spp. can reduce herbivore pressure on plants, due to the production of anti-herbivore alkaloids (Bastias et al., 2017), the effects of herbivores on the mother plant's microbiota (and eventually on their produced seeds) might be reduced in *Epichloë* infected associations. In regard to plant defences, it is important to underscore that *Epichloë* can also modulate host plant immune responses (i.e., salicylic acid and jasmonic acid signalling pathways; Bastias et al., 2017). This endophyte-modulation of host plant immunity could affect directly the mother plant's microbiota *via* excluding microbial groups (Shi et al., 2020; Kou et al., 2021), and/or indirectly though changing the levels of plant resistance to herbivores (e.g., Bastías et al., 2018).

Here, we studied the effects of the interaction between *Epichloë* endophytes and insect herbivory experienced by mother plants on the diversity and composition of seed-associated bacterial communities. We subjected mother plants of *L. multiflorum* (common name: Italian ryegrass) with and without its common endophyte *E. occultans* to a challenge with *Rhopalosiphum padi* aphids to further characterise the bacterial communities associated with seed produced by these

plants. This aphid species commonly interacts with the grass *L. multiflorum* in natural grasslands and cultivated pastures (Omacini et al., 2001; Weibull, 1993). *Epichloë occultans* is a vertically transmitted fungus that produces loline alkaloids, compounds with known bioactivity against *R. padi* aphids (Bastías et al., 2017). Due to the documented effects of *Epichloë* endophytes on the mother plant-associated microbiota and seed biochemical composition, we hypothesise that seed-associated bacterial microbiota will be influenced by the *Epichloë* presence. We also hypothesise that assemblies of seed-associated bacterial communities will vary due to the herbivory history of mother plants. However, since *Epichloë* endophytes also modulate plant-herbivore interactions, the potential effect of the herbivory experienced by mother plants on seed-associated bacterial communities may be attenuated.

MATERIALS AND METHODS

Biological Material

We used the symbiotic interaction between *L. multiflorum* and the fungal endophyte *E. occultans* as our study system. *Lolium multiflorum* is an annual grass native to Mediterranean region (currently worldwide distributed in ecosystems with temperate humid climates), and naturally associated with the fungal endophyte *E. occultans* (Moon et al., 2000). The vertical transmission efficiency of this endophyte species is usually high (close to 100%; Gundel et al., 2011a).

In our group, we maintain two plant biotypes for experimentation, endophyte-symbiotic (E+) and endophyte-free (E-) plants. Endophyte free plants were initially obtained by removing the endophyte from naturally E+ plants with a systemic fungicide (Gundel et al., 2012). Since then, we have been cultivating every year E+ and E- plants as to produce fresh seeds for experiments. We grow and maintain 25 E+ and 25 E- plants during the whole growing cycle to produce seeds. The plants are randomly sowed on a square plot (distance between plants: 0.50 m). The plot is maintained clean from weeds, and plants are watered on demand. Biotypes are maintained as true to type since *E. occultans* is only vertically transmitted through the seed (this endophyte species cannot be horizontally transmitted between plant biotypes; Gundel et al., 2011b). Furthermore, *L. multiflorum* is a self-incompatible and wind-pollinated species, thus the exchange of pollen between plants at flowering avoids the genetic differentiation between the E+ and E- biotypes (Gundel et al., 2012). Ripened seeds from each individual plant are harvested every year and the endophytic status assessed (using the 'seed squash' technique) prior to generate E+ and E- pools of seed for experimentation (Card et al., 2011).

We used the generalist phytophagous herbivore aphid *R. padi* L. (Hemiptera; Aphididae) for experimentation. This aphid species is common pest in cereals and grasses (Dixon, 1971; van Emden and Harrington, 2017). *Rhopalosiphum padi* aphids were collected from volunteer plants, transferred to Petri plates containing leaves of *Avena sativa* L., and maintained for 5 days to eliminate parasitised insects. Apterous adults free of parasites were placed on young *A. sativa* plants to initiate the insect population for experimentation. Plants were periodically watered and constantly replaced by healthy ones. The aphid populations were grown under optimal controlled conditions [temperature: 23°C (±1), photoperiod: L16-D8 h, radiation: 150 $\mu mol\, m^{-2}\ s^{-1}$].

Experimental Design

We sowed six E+ and six E- plants. Each *L. multiflorum* plant was individually grown in 3 L pots filled with a commercial potting mix substrate. The experiment was carried out outdoors (at spring season), placing plants on 1-m-high benches, and maintaining 0.2 m of distance between pots. One month after sowing, three plants of each symbiotic biotype were randomly assigned to any of the following treatments: herbivore-free (H-) or herbivory-challenged (H+) with *R. padi* aphids. Plants were at tillering stage with 6–10 tillers each. In the herbivory treatment, 10 apterous adult aphids were placed on each plant, and resulting populations were allowed to grow for 21 days. Each plant was individually enclosed with a tubular plastic net (0.05 mm mesh) to maintain the aphids under confinement, and to avoid the entrance of any external insect. At day 21, aphids and tubular nets were removed from the experimental plants, and these plants continued growing in the same conditions (on benches, outdoors) until the end of the growing cycle (they were periodically inspected to avoid water deficit and pest attacks). At flowering stage, plants were allowed to exchange pollen freely (with experimental and surrounding plants). At the end of the growing cycle, plants were characterised measuring dry weight (DW) shoot biomass, seed production, and the *Epichloë* presence within the produced seed. Shoot plant biomass was determined after drying the foliar tissues in an oven (60–70°C) for 48 h. Mature seeds were harvested from each mother plant and placed in paper bags. All the seed bags ($n = 12$) were stored at 4°C in plastic boxes containing silica gels until DNA extraction (see below). Endophyte presence was determined by inspecting 10 seeds per mother plant using the 'seed squash' technique (Card et al., 2011).

Characterisation of Seed-Associated Bacterial Communities

DNA Extraction, Library Preparation and Sequencing

Bacterial communities were characterised by sequencing the bacterial 16S ribosomal RNA (rRNA) gene though Illumina MiSeq sequencer (paired end, 2x300bp; Illumina, United States). For the DNA extraction, 25 seeds were randomly selected from each seed lot. Seeds were surface sterilised with 70% ethanol solution (Ma et al., 2015), and homogenised with sterile ceramic beads using a TissueLyser II (Qiagen, United States). DNA was extracted on 50 mg of homogenised seed material using the GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, United States) following manufacturer's instructions. Samples were processed under laminar flow chambers to avoid environmental contamination. DNA integrity was assessed by

electrophoresis on 1% agarose gels while DNA purity and concentration were checked with a Nanodrop spectrophotometer (Nanodrop Technologies, United States). Libraries for sequencing were prepared by Macrogen Inc. (Seoul, South Korea) using universal primers targeting the V3/V4 region within the 16S rRNA gene sequence (primers 341F: CCTACGGGN GGCWGCAG; 805R: GACTACHVGGGTATCTAATCC). These primers have been frequently used in bacterial metabarcoding studies (Herlemann et al., 2011).

Processing of Sequencing Data

The sequencing instrument produced ~1.22 million read pairs with an average of 102 K read pairs per sample. The amplicon reads were processed using a modified pipeline from Camarinha-Silva et al. (2012). The sequence reads were paired using the program FLASH2 (Magoč and Salzberg, 2011), and the paired reads were quality trimmed using Trimmomatic (Bolger et al., 2014). The trimmed reads were reformatted into FASTA, and read headers were modified to include sample names. All reads were compiled in a single file. The Mothur program suit was used to remove reads with homopolymers longer than 10 nucleotides, and to collapse the reads into unique representatives (Schloss et al., 2009). The Swarm program was used to cluster the collapsed reads (Mahé et al., 2014). The clustered reads were filtered based on their abundances, maintaining the reads that were (a) present in one sample with a relative abundance >0.1%, (b) present in >2% of the samples with a relative abundance >0.01% or (c) present in 5% of the samples at any abundance level. The selected reads were annotated using the QIIME program (version 1.9.1) with the Silva database (version 138; Caporaso et al., 2010; Quast et al., 2013). A Ruby program that implements the above-described abundance filter is available in the AgResearch Gitea website: https://gitea.agresearch.co.nz/JAUREGUIR/Microbiomics. The taxonomic affiliation of each operational taxonomic units (OTU) was verified using the Ribosomal Database Project (RDP) classifier and the 16S rRNA training set 18 as reference database (Wang et al., 2007). OTUs from chloroplast or mitochondrial plant sequences were removed from the database prior to analyses.

The final table included 99 bacterial OTUs representing 282,784 high-quality sequence reads (see **Supplementary Table S1**). All rarefaction curves associated with seed samples reached asymptotes (number of OTUs vs. number of sequencing reads) (**Supplementary Figure S1**), indicating that the sequencing depth described most of the bacterial assemblages (Wooley et al., 2010).

A phylogenetic tree was constructed for dominant seed-associated bacterial taxonomic groups using MEGA X software (Kumar et al., 2018). The sequence alignments were performed with MAFFT in Geneious software using default parameters (Kearse et al., 2012). Phylogenetic reconstruction was carried out using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm with a bootstrap test (9,999 replicates) and default parameters (Hall, 2013).

Statistical Analyses

The effect of the plant symbiosis status and the herbivory history on the response variables 'plant shoot biomass', 'seed production', 'bacterial abundance (number of reads)', 'species richness (number of OTUs)', 'species evenness' and 'Shannon diversity' of the OTUs were analysed using linear effect models with the *gls* function from the *nlme* R package (Pinheiro et al., 2009). We assumed independent, identically distributed normal random errors. The models included the plant's symbiotic status (E+, E-) and herbivory history (H+, H-) as categorical factors. VarIdent variance structures were used on the interaction between symbiotic status and herbivory history to accommodate deviations in variance homogeneity in the bacterial abundance response variable. Assumptions of each analysis of variance were met before to perform the analyses.

The effect of the plant symbiosis status and the herbivory history on the composition of bacterial assemblages of plant seed were analysed using permutational multivariate analysis of variance (PERMANOVA; 9,999 permutations), based on Bray-Curtis distances, with the function *adonis* from the *vegan* R package (Anderson, 2001; Oksanen et al., 2020). PERMANOVA allows testing for differences between groups when multivariate responses are measured. Similarly, the model included the plant's symbiotic status (E+, E-) and herbivory history (H+, H-) as categorical factors. To characterise the composition differences between bacterial assemblages associated with seed, non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities were performed using the *vegan* R package (Anderson, 2001; Oksanen et al., 2020). A Shepard diagram correlating NMDS ordination distances and Bray-Curtis community dissimilarities was used to validate NMDS ordination analysis. A high correlation indicates that the calculated NMDS ordination is a good representation of community structures (Leeuw and Mair, 2015). All the presented values in the result section are means ± standard errors (S.E.M). All the analyses were performed on R software (version 3.1.1; R Core Team, 2014).

RESULTS

The plant shoot biomass was not affected by either the plant symbiosis status ($F_{(1,8)} = 2.78$, $p = 0.134$) or the aphid herbivory ($F_{(1,8)} = 0.00$, $p = 0.978$). E+ plants marginally produced more seed than E- plants ($F_{(1,8)} = 4.96$, $p = 0.05$). The aphid herbivory did not affect the plant seed production ($F_{(1,8)} = 0.04$, $p = 0.835$). All seed produced by E+ plants contained *Epichloë* endophytes, whereas seed from E- plants were free of these endophytes. The aphid herbivory treatment did not affect the *Epichloë* seed transmission (**Table 1**).

We identified 99 bacterial OTUs in seed produced by *L. multiflorum* plants, with an average of 56.58 ± 2.11 OTUs per seed sample (i.e., from a mother plant). These bacterial OTUs were identified in phyla Proteobacteria (74% of OTUs), Actinobacteria (13% of OTUs), and Firmicutes (4% of OTUs; the remaining 9% of OTUs could not be classified at the phylum level; **Supplementary Table S1**). Considering the

experimental conditions (i.e., endophyte presence within seed and plant herbivory history), bacterial assemblages were dominated by 6–13 OTUs (relative abundances > 1%; number of OTUs: E-/H- = 6, E-/H+ = 6, E+/H- = 11, E+/H+ = 13; **Figure 1**). Mostly these OTUs were identified in phylum Proteobacteria (i.e., *Pantoea*, *Pseudomonas*, *Erwinia* and *Kosakonia*) with only one belonging to Actinobacteria (i.e., *Curtobacterium*; **Figure 1**).

TABLE 1 | Shoot biomass, seed production, seed number and endophyte seed transmission in *Lolium multiflorum* plants symbiotic (E+) and non-symbiotic (E-) with the fungal endophyte *Epichloë occultans*, that were challenged (H+) or not (H-) with *Rhopalosiphum padi* aphids.

Symbiosis/ herbivory	Shoot plant biomass (g DW • plant^{-1})	Seed production (g • plant^{-1})	Seed number (# • plant^{-1})	*Epichloë* seed transmission (%)
E-/H-	12.52 ± 0.66a	2.53 ± 0.36a	~1372.07	0
E+/H-	12.06 ± 0.91a	2.89 ± 0.33b	~1386.76	100
E-/H+	12.37 ± 1.58a	1.64 ± 0.25a	~890.09	0
E+/H+	13.04 ± 1.03a	3.48 ± 0.26b	~1668.26	100

Different letters in values indicate significant differences ($p < 0.050$). Endophyte transmission was determined by inspecting 10 seeds per mother plant. Values are means ± SEM ($n = 3$ plants).

The composition of the seed bacterial assemblages differed between endophyte-symbiotic and endophyte-free *L. multiflorum* plants, but it was independent on the plant herbivory history (**Table 2**). This was visualised with a NMDS ordination that showed that bacterial communities were mainly grouped by the symbiotic status of seeds rather than by the maternal herbivory history (**Figure 2**). The Shannon diversity index increased *ca.* 42% by the endophyte presence (E+ = 2.03 ± 0.06, E- = 1.17 ± 0.07; **Table 2**; **Figure 3A**). Whereas the bacterial richness associated with plant seed was not affected by the treatments, the evenness of bacterial assemblages increased *ca.* 42% by the endophyte presence (E+ = 0.50 ± 0.01, E- = 0.29 ± 0.01; **Table 2**; **Figures 3B,C**). In agreement with the endophyte-mediated effect on the evenness of bacterial communities, the relative abundances of the dominant taxonomic groups (relative abundances >1%) were more similarly

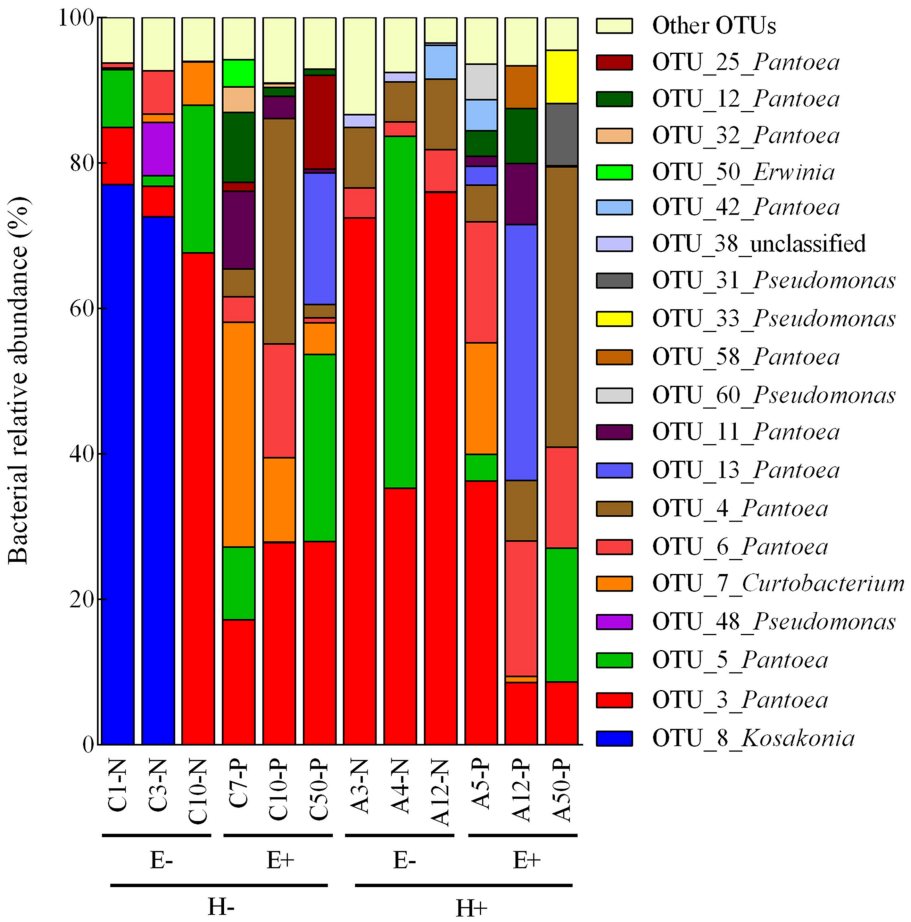

FIGURE 1 | Relative abundance of dominant bacterial OTUs (operational taxonomic units) associated with individual seed samples of *Lolium multiflorum* plants symbiotic (E+) or not (E-) with the fungal endophyte *Epichloë occultans*, and challenged (H+) or not (H-) with the aphid herbivore *Rhopalosiphum padi*. Dominant bacterial OTUs were those with relative abundances above 1%, on average, in at least one group (i.e., E-/H-, E+/H-, E+/H- and E+/H+). Dominant bacterial OTUs represented 86-95% of the abundances of sequence reads associated with each seed sample. OTUs with relative abundances below 1% were compiled in the 'other OTUs' group.

TABLE 2 | Effect of the endophyte symbiotic status and maternal herbivory history on the bacterial composition, bacterial diversity (Shannon index, H'), bacterial richness and bacterial abundance (evenness index, J') of seed of *Lolium multiflorum* plants symbiotic or not with the fungal endophyte *Epichloë occultans*, and challenged or not with the aphid herbivore *Rhopalosiphum padi*.

		Bacterial composition (based on OTUs)			Shannon diversity index (H' of OTUs)		Bacterial richness (# of OTUs)		Bacterial evenness index (J' of OTUs)	
Treatment	***df***	***F*-values**	**R^2**	***p***	**F-values**	***p***	**F-values**	***p***	***F* values**	***p***
Symbiosis	1,8	2.89	0.21	**0.004**	72.35	**<0.001**	0.20	0.661	106.87	**<0.001**
Herbivory	1,8	1.42	0.10	0.204	0.19	0.669	0.60	0.458	1.16	0.312
Symbiosis x Herbivory	1,8	1.56	0.11	0.157	0.58	0.466	1.36	0.276	0.08	0.782

Bacterial composition data were analysed with permutational multivariate analysis of variance (PERMANOVA). Bacterial diversity, richness, and abundance data were analysed with analyses of variance. OTUs means operational taxonomic units. Statistically significant effects ($p < 0.050$) are highlighted in bold ($n = 3$ plants).

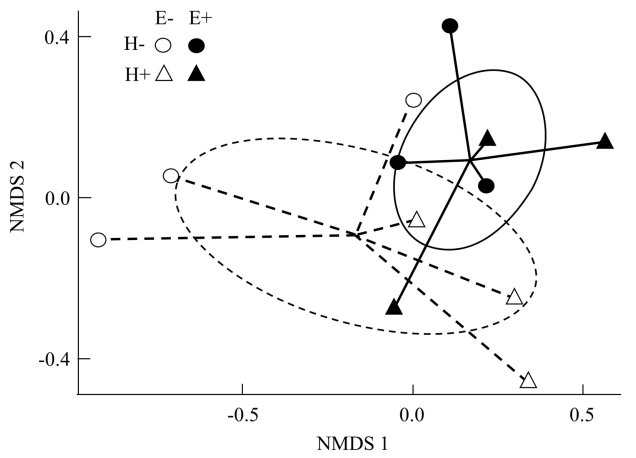

FIGURE 2 | Non-metric multidimensional scaling ordinations (NMDS; stress = 0.09) for bacterial assemblages associated with seed of *Lolium multiflorum* plants symbiotic (E+, filled symbols) or not (E-, open symbols) with the fungal endophyte *Epichloë occultans*, and challenged (H+, triangles) or not (H-, circles) with the aphid herbivore *Rhopalosiphum padi*. Lines indicate compositional distances between centroids and E+ and E- seed-associated bacterial communities (continuous and discontinuous lines, respectively). Ellipses represent 95% confidence intervals around centroids and show clustering of bacterial compositions in seed based on the presence/absence of *Epichloë* endophytes (continuous and discontinuous lines, respectively). The Shepard diagram (**Supplementary Figure S2**) showed a good linear fit between ordination distances and Bray-Curtis dissimilarities ($n = 3$ plants).

distributed in endophyte-symbiotic seed compared with their non-symbiotic counterparts (**Figure 4**).

DISCUSSION

We hypothesised that vertically transmitted *Epichloë* endophytes influence the composition and diversity of the seed-associated bacterial microbiota of their host plants. Furthermore, we posited that the herbivory experienced by mother plants also affect the composition and diversity of seed bacterial communities, but this effect of herbivores would interact with the presence of *Epichloë* endophytes in mother plants. Our results showed that *Epichloë* indeed influenced the composition of the bacterial communities in host seeds. The seed-associated bacterial communities were more diverse in endophyte-symbiotic than endophyte-free seeds. Instead of an increase in species richness, this higher diversity was attributed to the relative abundances among bacterial taxa being more similar in communities associated with E+ than E- seed (bacterial communities in E- seed were dominated by few taxa). Contrary to our expectation, seed bacterial communities were not affected by the herbivory experienced by mother plants.

The seed-associated bacterial communities are generally simpler in terms of composition and diversity than communities from soils, seedlings and adult plant tissues (Chesneau et al., 2020; Rochefort et al., 2021; Walsh et al., 2021). Bacteria within phylum Proteobacteria, including members from *Pantoea* spp. and *Pseudomonas* spp., normally dominate seed bacterial communities (Truyens et al., 2015; Kim and Lee, 2021). In agreement with this, bacterial communities in *L. multiflorum* seeds were dominated by members from the Proteobacteria, including the above-mentioned genera and a few other groups (e.g., *Erwinia* spp.). This pattern of bacterial diversity in *L. multiflorum* seeds is similar to those documented in seeds from other grass species such as *Lolium perenne* (Tannenbaum et al., 2020, 2021), *Elymus nutans* (Guo et al., 2021), *Oryza sativa* (Eyre et al., 2019; Wang et al., 2020a) and *Triticum aestivum* (Hone et al., 2021; Walsh et al., 2021). Several beneficial bacteria within the *Pantoea* and *Pseudomonas* genera have been isolated from plant seeds (Feng et al., 2006; Díaz Herrera et al., 2016; Verma et al., 2018). Experimental evidence has shown that beneficial seed-inhabiting *Pantoea* and *Pseudomonas* can efficiently colonise seedlings and that the colonisation of these bacteria can provide advantages to seedlings during establishment (Ferreira et al., 2008; Pavlova et al., 2017; Verma et al., 2017; Morella et al., 2019; Soluch et al., 2021).

Epichloë fungal endophytes can enhance the performance of host seed and seedlings (Clay, 1987; Novas et al., 2003; Stefanoni-Rubio et al., 2021). This *Epichloë*-mediated enhancement in host performance can be even more significant when symbiotic seed and seedlings experience stressful conditions (e.g., pathogens, salinity, drought, soil contamination and nutrient limitation; Gwinn and Gavin, 1992; Gundel et al., 2006; Zhang et al., 2010; Vázquez de Aldana et al., 2014; Ding et al., 2015; Pérez et al., 2016; Wang

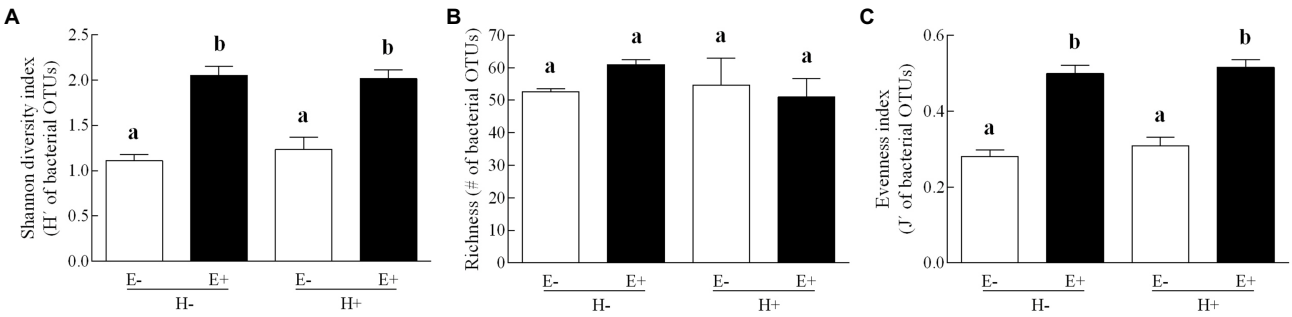

FIGURE 3 | Bacterial diversity indexes (panel **A**: Shannon index; panel **B**: richness; panel **C**: evenness index) associated with seed of *Lolium multiflorum* plants symbiotic (E+, filled bars) or not (E-, open bars) with the fungal endophyte *Epichloë occultans*, and challenged (H+) or not (H-) with the aphid herbivore *Rhopalosiphum padi*. OTUs means operational taxonomic units. Different letters indicate significant differences at $p < 0.050$. Bars represent means values ± SEM ($n = 3$ plants).

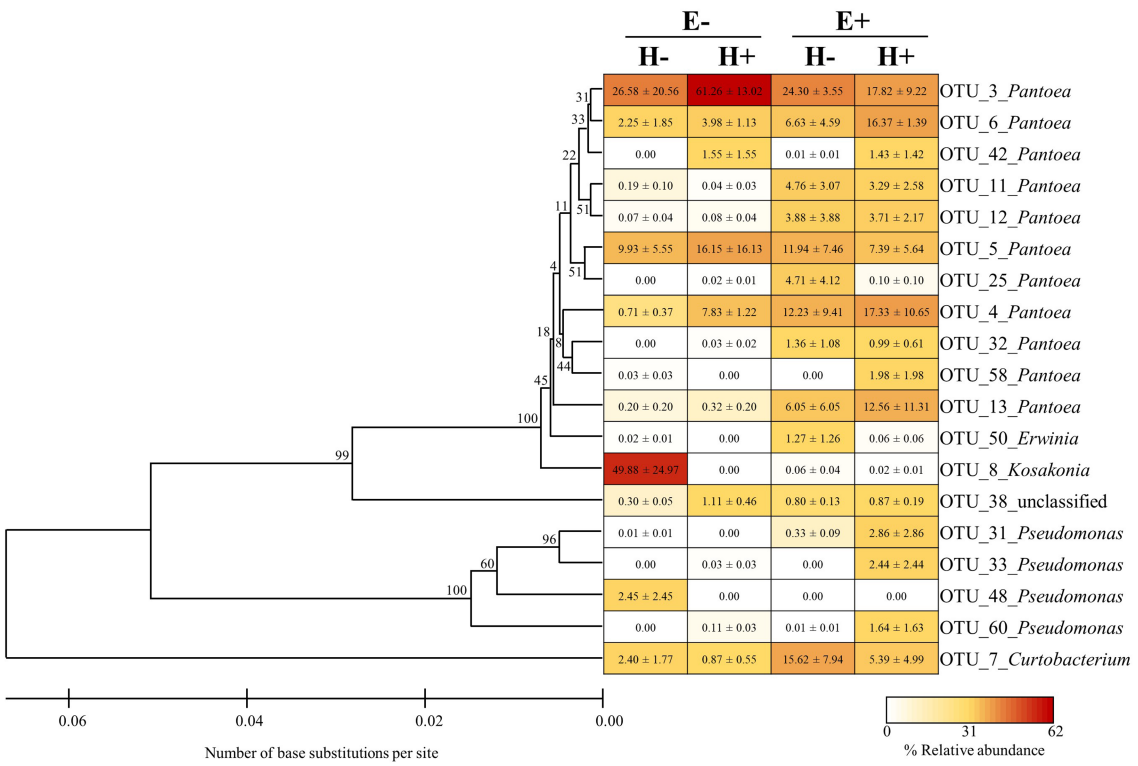

FIGURE 4 | Phylogenetic tree and relative abundance of bacterial OTUs (operational taxonomic units) associated with seed of *Lolium multiflorum* plants symbiotic (E+) or not (E-) the fungal endophyte *Epichloë occultans*, and challenged (H+) or not (H-) with the aphid herbivore *Rhopalosiphum padi*. The tree was inferred using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm with a bootstrap test (9,999 replicates). Bootstrap values for ingroup clades are shown next to branches. Evolutionary distances were calculated using the Maximum Composite Likelihood method. The heat map represents the relative abundances of dominant bacterial OTU associated with each seed group. Dominant bacterial OTUs (>1% relative abundance) represented 91-94% of the abundances of sequence reads associated with each seed group. Values in cells indicate means ± SEM ($n = 3$ plants).

et al., 2020b). The increased performance of symbiotic seed/seedlings has been normally attributed to the biochemical changes exerted by *Epichloë* on host plants (e.g., alkaloids, antioxidants and sugars; Gundel et al., 2018; Zhang et al., 2019; Hewitt et al., 2020; Ueno et al., 2020). However, enhanced performance could also be explained, at least in part, by the beneficial activities derived from other microbial symbionts co-inhabiting *Epichloë*-symbiotic seed and seedlings. In the present study, *Epichloë* endophyte presence was associated with increased diversity and changes on composition of seed-associated bacterial communities, particularly in members from *Pantoea* and *Pseudomonas*. Interestingly, both genera contain species/strains described to

provide a suite of benefits to plants such as growth promotion, nitrogen fixation, phosphate solubilisation and protection against pathogens/plant competitors (Elmore et al., 2019; Guo et al., 2021). For instance, several plant-growth promoting *Pseudomonas* isolates increased the seed germination and seedling growth of *Solanum lycopersicum* plants (through production of auxins, phosphate-solubilising compounds and another growth-promoting compounds; Qessaoui et al., 2019). The stress-protective *Pantoea alhagi* strain NX-11 bacterium increased the salt stress resistance of *Oryza sativa* seedlings (*via* exopolysaccharide production, compounds that inhibit the plant absorption of salt; Sun et al., 2020). The antimicrobial-producing *Pseudomonas* sp. strain SY1 bacterium protected seedlings of *Oryza sativa*, *Cynodon dactylon* and *Poa annua* from the fungal pathogen *Fusarium oxysporum* (Verma et al., 2018). Therefore, it is likely that the performance of symbiotic seed/seedlings can be also increased by the action of those beneficial bacteria (e.g., *Pantoea* spp. and *Pseudomonas* spp.) promoted by the association of host plants with *Epichloë* endophytes.

We did not detect an effect of the mother plant herbivory (or *via* interaction with the endophyte presence) on the seed-associated bacterial communities. In the present experiment, plants were interacting with aphids for 21 days (plants were at tillering stage), and the rest of the growing cycle were maintained free of herbivores (i.e., *ca.* 45 days). It is possible that any impact from the aphid treatment was transitory such that changes in the plant associated microbiota did not persist until the plant flowering stage when microbes are transmitted into seeds. This seems likely since herbivory triggers changes in plant defence and plant nutrient release, both of which likely impact the plant microbiota indirectly, in addition to microbes being added directly through the herbivore itself (Smets and Koskella, 2020). It is also possible that we observed minimal herbivory-associated changes on the plant's bacterial communities because these are driven more by the colonisation of microbes from external sources such as the soil, air and biotic vectors (Tombolini et al., 1999). In fact, experimental evidence has shown that plant microbial communities can recover their pre-perturbation structures by colonisation of microbes from environmental sources (Haas et al., 2018; Stone and Jackson, 2021).

In conclusion, this study highlights the influence of *Epichloë* endophytes in the diversity of bacterial communities associated with seed. Our results indicate that these endophytes affect the seed bacterial microbiota by modifying the composition and increasing the diversity of these bacterial communities. *Epichloë* fungal endophytes may increase diversity by reducing the overall bacterial load and by preventing establishment of dominance of certain groups. It is likely that this *Epichloë*-mediated increment in bacterial diversity contributes to the documented enhanced performance of endophyte-associated seed/seedlings (e.g., Clay, 1987; Gwinn and Gavin, 1992; Pérez et al., 2016). Furthermore, due to the lack of effect of our herbivory treatment, we speculate that stresses occurring close to the plant flowering stage, when microbes are transmitted, may have larger impacts on seed-associated microbial communities than stresses acting at earlier stages (Shade et al., 2017). In fact, it has been documented that certain seed attributes in *L. multiflorum* plants can be affected by stresses occurring at, or close to, the reproductive stage (e.g., seed production and longevity; Ueno et al., 2020, 2021). Considering the relative abundances of bacterial taxa across treatments, *Kosakonia* was remarkable because this taxon exhibited high abundance and was virtually only present within E-/H- seed. Perhaps *Kosakonia* spp. were particularly susceptible to potential chemical changes exhibited by seed due to the experimental treatments. In agreement with this hypothesis, Hou et al. (2020) found that the abundance of *Kosakonia* spp. effectively depended on the plant chemical composition (in this study, *Kosakonia* spp. were also associated with only E- plants). Further experiments that isolate bacteria from *Epichloë*-associated seed will be essential to characterise the microbiota and also to identify those beneficial bacteria for plants. Sequencing of whole bacterial genomes, transcriptomes and/or inoculation of bacterial isolates into plants can be useful tools for characterising the isolated bacteria (Bastías et al., 2020; Ju et al., 2021; Li et al., 2021).

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI Short Read Archive (SRA), accession number PRJNA782330.

AUTHOR CONTRIBUTIONS

DB, LB, and PG conceived the experiment. LB and AB performed the experiments. DB, RJ, IA-R, and PG analysed data. IA-R, RJ, MM-M, and PG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.795354/full#supplementary-material

Supplementary Figure 1 | Rarefaction curves of bacterial OTUs (operational taxonomic units) associated with each seed sample used in the present study.

Supplementary Figure 2 | Shepard diagram of the non-metric multidimensional scaling (NMDS) ordination related to **Figure 2**.

Supplementary Table 1 | Matrix of bacterial OTUs (operational taxonomic units) vs. read counts associated with seed samples.

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Epichloë Endophyte-Promoted Seed Pathogen Increases Host Grass Resistance Against Insect Herbivory

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Plants host taxonomically and functionally complex communities of microbes. However, ecological studies on plant–microbe interactions rarely address the role of multiple co-occurring plant-associated microbes. Here, we contend that plant-associated microbes interact with each other and can have joint consequences for higher trophic levels. In this study we recorded the occurrence of the plant seed pathogenic fungus *Claviceps purpurea* and aphids (*Sitobion* sp.) on an established field experiment with red fescue (*Festuca rubra*) plants symbiotic to a seed transmitted endophytic fungus *Epichloë festucae* (E+) or non-symbiotic (E–). Both fungi are known to produce animal-toxic alkaloids. The study was conducted in a semi-natural setting, where E+ and E– plants from different origins (Spain and Northern Finland) were planted in a randomized design in a fenced common garden at Kevo Subarctic Research Station in Northern Finland. The results reveal that 45% of E+ plants were infected with *Claviceps* compared to 31% of E– plants. Uninfected plants had 4.5 times more aphids than *Claviceps* infected plants. By contrast, aphid infestation was unaffected by *Epichloë* symbiosis. *Claviceps* alkaloid concentrations correlated with a decrease in aphid numbers, which indicates their insect deterring features. These results show that plant mutualistic fungi can increase the infection probability of a pathogenic fungus, which then becomes beneficial to the plant by controlling herbivorous insects. Our study highlights the complexity and context dependency of species–species and multi-trophic interactions, thus challenging the labeling of species as plant mutualists or pathogens.

Keywords: fungal endophyte, *Claviceps*, aphid, symbiosis, mutualism, herbivory, plant-microbe interactions, alkaloids

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INTRODUCTION

Plants, similar to all higher organisms, interact with an abundant and diverse microbiota, which is discovered to affect plant fitness (Zilber-Rosenberg and Rosenberg, 2008; Vandenkoornhuyse et al., 2015; Roughgarden et al., 2018; Enebe and Babalola, 2019; Saikkonen et al., 2020). Microbes, such as mycorrhizae, nitrogen-fixing bacteria, and asymptomatic endophytes, live symbiotically with their host plant. These symbionts can, for instance, affect the nutrient acquisition, phytohormone regulation, abiotic stress tolerance, and herbivory and pathogen resistance of their host

(Ortíz-Castro et al., 2009; Berendsen et al., 2012; Dupont et al., 2015; Hassani et al., 2018; Nelson, 2018; Compant et al., 2019). Accordingly, these symbiotic microbes are commonly regarded as defensive plant mutualists, contrary to antagonistic parasitic and pathogenic microbes that have negative effects on host fitness (Clay, 2009).

However, the ecological roles of microbes in plant–microbe interactions are often complex, diverse, and prone to change from mutualistic to antagonistic and vice versa, depending on the abiotic and biotic conditions (Hayward, 1974; Carroll, 1988; Ahlholm et al., 2002; Partida-Martinez and Heil, 2011; Marsberg et al., 2017; Marchetto and Power, 2018; Shaffer et al., 2018; Meier and Hunter, 2019; Afkhami et al., 2020; Franklin et al., 2020; Laihonen et al., 2020; Petipas et al., 2020; Saikkonen et al., 2020). This continuum of antagonistic to mutualistic interactions in space and time (Saikkonen et al., 1998; Hirsch, 2004; Drew et al., 2021) must be considered in studies on plant microbe interactions. Furthermore, plants interact with myriads of organisms that probably interact not only with plants but also with each other (Lamichhane and Venturi, 2015; Bass et al., 2019). Despite the increasing number of studies characterizing plant microbiomes, ecological studies on the functional role of plant-associated microbes have only rarely examined the joint effects of the co-occurring microbes on the host plant. All this complexity of plant microbiomes shows the importance of comprehensive studies on reputed plant mutualistic or antagonistic microbes.

In this study, we examine the four-way interaction between red fescue (*Festuca rubra* L.), two fungal species, a vertically transmitted endophyte (*Epichloë festucae* Leuchtm., Schardl & M.R. Siegel) and a grass seed pathogen (*Claviceps purpurea* [Fr.] Tul.), and a phloem-feeding aphid (*Sitobion* sp.). *F. rubra* is a fine-leaved perennial grass growing in a wide range of habitats in the northern temperate zone. Some *Epichloë* species are common mutualistic symbionts of cool-season grasses. These systemic fungi grow asymptomatically in the aboveground parts of their hosts and reproduce asexually via host plant seeds (Schardl, 1996; Saikkonen et al., 2016). They often enhance their host's ability to endure abiotic stress, such as salinity or drought, or increase resistance against herbivores and pathogens (Schardl, 1996; Saikkonen et al., 1998, 2010). By contrast, *C. purpurea*, the ergot fungus, is widely recognized as a plant pathogen that infects the inflorescences in the Poaceae family. The fungus sterilizes the ovaries in flowers and utilizes the resources of its host to develop a sclerotium, preventing the development of a host plant seed. However, the pathogen usually castrates only a few flowers in a host plant inflorescence (Luttrell, 1980). Even though ecologically different, both *Epichloë* and *Claviceps* belong to the same fungal family, Clavicipitaceae.

Plant interactions with *Epichloë* and *Claviceps* have been thoroughly studied, but separately. Existing studies show that the presence of *Epichloë occultans* in *Lolium multiflorum* and *Epichloë gansuensis* in *Achnatherum inebrians* is correlated with a decreased frequency of *C. purpurea* infection, indicating that *Epichloë* mediates protection against *C. purpurea* (Pérez et al., 2013, 2017; Zhang et al., 2021). The increased pathogen resistance may result from the *Epichloë*-increased immunocompetence of the host, or from a direct competition between the invading microbe and the endophytic *Epichloë* (Saikkonen et al., 2016; Malinowski and Belesky, 2019). However, four-way interactions, including herbivores, have not been studied in this context. Both *Epichloë* and *Claviceps* produce animal-toxic alkaloids (Saikkonen et al., 2013; Miedaner and Geiger, 2015; Florea et al., 2017) that may synergistically increase herbivore resistance. The *Epichloë*-increased anti-herbivory defenses are primarily accounted for fungal-origin alkaloids including pyrrolizidines (lolines), ergot alkaloids, indolediterpenoids (lolitrems), and pyrrolopyrazines (peramine) (Saikkonen et al., 2013, 2016; Schardl et al., 2013). The sclerotia of *C. purpurea* contain high concentrations of ergot alkaloids that deter herbivores and fungivores (Miedaner and Geiger, 2015). Vertebrate herbivores avoid seed sets with only a few *C. purpurea* sclerotia which can increase host plant fitness in highly grazed environments. This attribute questions the strict pathogenic nature of *C. purpurea* (Wäli et al., 2013).

Here, in a four-way interaction, we examine how symbiotic *E. festucae* affects the natural colonization of *C. purpurea* and how these two fungi jointly affect herbivores in their common host plant in a semi-natural subarctic setting. We investigated in particular, whether:

1. The infection frequency of *Claviceps* differs between *Epichloë* symbiotic (E+) and *Epichloë* free (E–) plants
2. Aphid infestation on the plants is affected by either of the two plant-associated fungi
3. The chemical profile of ergot alkaloids can explain the infestation rate of aphids on the plants

All three species compete for the host plant's resources; however, we assume their ecological interactions with the host are as follows: *Epichloë* commonly forms mutualistic interaction with the host plant; *C. purpurea* is mainly antagonistic but potentially becomes beneficial under high herbivory pressure; and aphids as herbivores are pure antagonists to their host. Based on existing literature, we hypothesize that symbiosis with *Epichloë* decreases *Claviceps* infection frequency, and both fungal species contribute to plant defense, negatively affecting aphid performance. We discuss our findings regarding multiple contexts and defensive mutualism hypotheses.

MATERIALS AND METHODS

Study System

The plants used in this study were part of a transplantation experiment designed to examine the importance of *E. festucae* in wild populations of red fescue (*F. rubra*) (Leinonen et al., 2019). We established identical experiments in three locations: Northern Finland; Southern Finland; and Salamanca, Spain. For this study, we utilized the Northern Finland experiment at Kevo Subarctic Research Institute (N 69.757 E 27.011, WGS84) to monitor naturally occurring herbivores and plant pathogens. The area was fenced to exclude large mammalian herbivores.

We planted *F. rubra* plants in a balanced and randomized common garden design in local sandy soil in 2018. Half of the plants originated from Northern Finland (in this study: "local

plants") and the other half came from inland Spain. The plants were collected from three Spanish and three Northern Finland populations (see details of locations in Leinonen et al., 2019). Half of the experimental plants harbored symbiotic *E. festucae* (E+), and the other half were endophyte-free (E–). A total of 120 plant individuals (genets) were divided into small ramets (clones) that were then planted. In the experiment, the plant clones were randomized to a block (12 × 10 plants), which was replicated five times (see Leinonen et al., 2019 for a detailed description of the entire setup). Thus, our experiment consisted of a total of 600 plants.

Data Collection

Field Data

We recorded the occurrence of *C. purpurea* and the number of aphids on the experimental plants in their third growing season on August 12, 2020. We selected our timing according to two natural phenomena. First, *Claviceps*-infected plants were abundant that year in the region. Second, aphids were concentrated in inflorescences to feed on the nutrient-rich phloem, as flowering of the plants had just ended and seeds started to develop. This was the only time during our monitoring, since the establishment of the experimental field in 2018, when *Claviceps* infection and aphid infestation co-occurred on the experimental plants. As *Claviceps* infection visibly affects only the developing seeds that turn into sclerotia, we collected the data from all flowering individuals. We did not detect aphids feeding outside of the inflorescences, and we excluded non-flowering plant individuals from the data. We then identified the aphid species from photographs.

We seldom observed more than one *Claviceps* sclerotium in a single inflorescence, and thus we did not count the number of sclerotia per inflorescence. Our observation is in line with the literature stating that most host plant seeds develop normally (Luttrell, 1980).

Alkaloid Analyses

We analyzed ergot alkaloids from the inflorescences of 40 plants. All plants were of local origin. The selected plants evenly represented both endophyte-symbiotic (E+) and endophyte-free plants (E–), as well as *Claviceps*-infected (C+) and non-infected (C–) plants. The aphid distribution in the chosen plants appropriately represented the same pattern as in the original data. We clipped the plant inflorescences and stored them in deep freeze conditions until the analyses.

Plant samples were freeze dried and disrupted in a Mini-Beadbeater (Biospec Scientifica) and analyzed for ergot alkaloids following the procedure of Fuchs et al. (2013) with some modifications. In brief, samples (20 mg) were extracted with 150 μL of methanol and 150 μL of methylene chloride. After centrifugation two times for 10 min, the organic phases were combined. A 200-μL aliquot was evaporated, and the residue was dissolved in 25 μL of 80% methanol and analyzed using Liquid Chromatography Mass Spectrometry (LC/MS).

The analyses were performed using a UHPLC system (Agilent 1290 infinity II) coupled to a quadrupole time-of-flight mass spectrometer QTOF (Agilent G6546A). The separation of chemical compounds was conducted using a Zorbax Eclipse Plus C18 HD column (50 mm × 2.1 mm, 1.8 μm particle size; Agilent) at 30°C using the following solvents: solvent A consisted of aqueous formic acid (0.1%), and solvent B consisted of acetonitrile (100%). Gradient elution was performed using 100% B at a flow rate of 0.3 mL min^{-1} for 7 min and 100% B to 5% B in 3 min.

For the analysis of alkaloids, electrospray was operated in the positive ionization mode with the following settings: 225°C gas temperature, 13 L min^{-1} gas flow rate, 30 psgi nebulizer pressure, 350°C sheath gas temperature, and 7.5 L min^{-1} sheath gas flow. Ergot alkaloids (ergonovine, ergosine, ergotamine, ergocornine, α-ergocryptine, and ergocristine) were identified using the METLIN Metabolomics Database, and ergovaline was verified with a standard. All the alkaloids were quantified using ergotamine tartrate (Merck) as the standard.

Data Accessibility

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Statistical Analyses

We ran all the analyses using the Statistical Analysis Software SAS 9.4. The FREQ procedure was used to describe the data. Given that several aphids on the plant were always present or not present at all, we conducted analyses for both the aphid number and the aphid presence/absence (binary variable).

We used the LOGISTIC procedure to conduct logistic regressions for *Claviceps* occurrence (C+ or C–) and aphid presence, with endophyte presence (E+ or E–), plant origin (Spain or local), genet, and block as explanatory variables. *Claviceps* occurrence was also an explanatory variable for aphid presence. We conducted a generalized linear model using the GLM procedure, in which the number of aphids was explained with *Claviceps* occurrence, plant's endophyte status and plant origin as fixed factors and block and genet as random factors. We used Tukey adjustment when comparing the aphid numbers between the groups.

Three E–C– alkaloid samples were removed from the analyses due to contamination. We used Student's *t*-tests (TTEST procedure) to compare the amounts of each distinguished ergot alkaloid in *Epichloë*-symbiotic and -free (E+, E–) plants and in *Claviceps*-infected and non-infected (C+, C–) plants. The GLM procedure with Tukey-adjusted *post-hoc* tests was used for pairwise comparisons among all four *Epichloë–Claviceps* combinatory plant groups (E–C–, E–C+, E+C–, and E+C+). Finally, we calculated Pearson correlation coefficients between the amounts of those alkaloids and aphid numbers in the sampled plants.

RESULTS

Field Data

Our final field data resulted from a set of 176 flowering plants, since the rest of the plants did not produce inflorescences. A proportion of 46% of these plants were endophyte-free (E–). The

TABLE 1 | Summary of the fixed effects used in the models.

	All plants			Local plants only		
Effect	X^2	*df*	*p*	X^2	*df*	*p*
***Claviceps* occurrence**						
Endophyte	4.050	1	**0.044**	4.812	1	**0.028**
Plant origin	0.672	1	0.413			
Endophyte × plant origin	1.185	1	0.276			
Aphid numbers						
Claviceps	34.55	1,169	**<0.001**	35.47	1,159	**<0.001**
Endophyte	0.88	1,169	0.349	1.42	1,159	0.235
Claviceps × endophyte	0.16	1,168	0.685	0.38	1,158	0.537
Plant origin	2.25	1,168	0.135			
Claviceps × plant origin	1.00	1,167	0.319			
Endophyte × plant origin	0.08	1,167	0.775			
Aphid presence						
Claviceps	14.089	1	**<0.001**	13.649	1	**<0.001**
Endophyte	0.052	1	0.820	0.004	1	0.952
Claviceps × endophyte	1.734	1	0.188	2.056	1	0.152
Plant origin	0.708	1	0.400			
Claviceps × plant origin	0.045	1	0.833			
Endophyte × plant origin	0.002	1	0.968			

All analyses were conducted for all plants and for local plants. Endophyte affected the occurrence of Claviceps, and Claviceps affected both aphid numbers and presence. Statistically significant (<0.05) p-values are bolded. $N_{all\ plants} = 176$; $N_{local\ plants} = 166$.

majority of the plants were of local origin, as only 10 flowering plants (2 E– and 8 E+) were from Spain.

Claviceps infection was more common if the plant harbored the endophyte, occurring on 45% of the E+ plants compared with 31% of the E– plants (**Table 1**). *Claviceps* occurrence differed between the blocks [$X^2_{(4)} = 15.714$, $p = 0.003$]; however, the interactions between the block and other variables were statistically non-significant.

Aphid numbers were 4.5 times higher in non-infected plants (C–) than in plants infected with *Claviceps* (C+). However, aphid numbers did not differ between endophyte-symbiotic (E+) and endophyte-free (E–) plants (**Table 1** and **Figure 1**). The plants selected for chemical analysis represented the same pattern (**Figure 2A**). Regarding presence data, aphids were not found in 59% of the *Claviceps*-infected (C+) plants compared with 29% of the non-infected (C–) plants [**Table 1**; block: $X^2_{(4)} = 28.326$, $p = <0.001$, interactions with block non-significant].

The genet and all interactions between the explanatory variables were not statistically significant and were removed from the final models.

As almost 95% of the plants within the final dataset were of local origin, we also ran the analyses exclusively for the local-origin plants. The results were similar to those presented above (**Table 1**).

Initially, we ascribed the aphids to a single species in the genus *Sitobion*. Aphid specialist Anders Albrecht (Finnish Museum of Natural History) confirmed that they were most likely *Sitobion avenae* (Fabricius, 1775), although *Sitobion fragariae* (Walker, 1848) could not be completely ruled out based on identification from photographs (**Figure 1**).

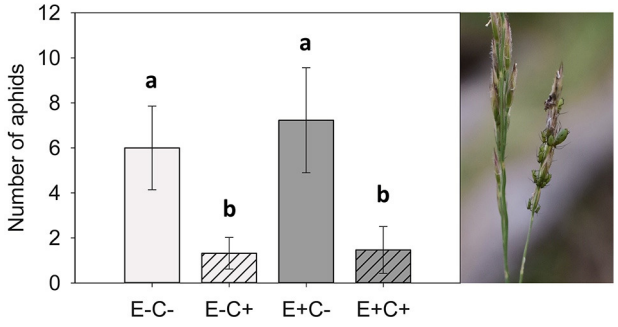

FIGURE 1 | Number of aphids on the study plants. There were over 4.5 times more aphids on the plants without the *Claviceps* (C) infection. *Epichloë* (E) did not affect the amount of aphids on the plant. Generalized linear model, Tukey-adjusted pairwise comparisons: E–C– and E–C+: $t_{167} = 3.48$, $p = 0.004$; E–C– and E+C–: $t_{167} = 1.13$, $p = 0.670$; E–C– and E+C+: $t_{167} = 3.93$, $p = 0.001$; E–C+ and E+C–: $t_{167} = 4.36$, $p = <0.001$; E–C+ and E+C+: $t_{167} = 0.24$, $p = 0.995$; E+C– and E+C+: $t_{167} = 4.92$, $p = <0.001$. Error bars represent 95% confidence intervals. $N = 176$.

Alkaloid Analyses

We identified seven different ergot alkaloids in plant extracts: ergovaline, ergonovine, ergosine, ergotamine, ergocornine, α-ergocryptine, and ergocristine. Highest ergovaline concentrations were detected in inflorescences of *Epichloë*-symbiotic (E+) plants, and its concentrations correlated negatively with aphid numbers (**Table 2**). We found ergosine, ergocornine, α-ergocryptine, and ergocristine mainly in

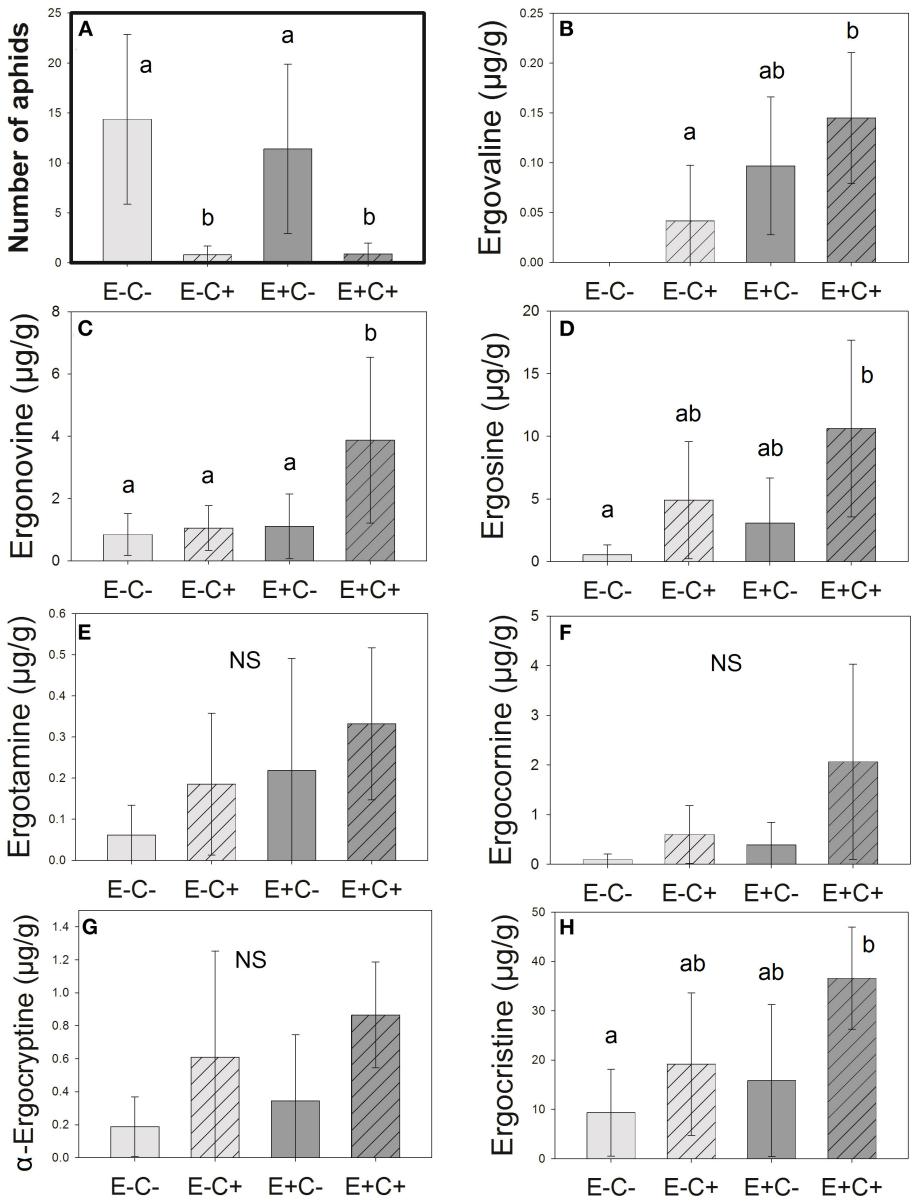

FIGURE 2 | Number of aphids **(A)** next to the ergot alkaloid concentrations **(B–H)** in the samples analyzed. Alkaloid concentrations tend to be the highest in plants that are both *Epichloë*-symbiotic and *Claviceps*-infected (E+C+). Letters represent statistically significant differences ($p < 0.05$, Tukey adjusted). Error bars represent 95% confidence intervals. $N = 37$.

inflorescences having sclerotia of *Claviceps* (C+). Ergonovine concentrations were associated with endophytic *Epichloë*, but association with *Claviceps* was also marginally significant. We could not associate ergotamine with either fungi. The concentrations of ergovaline, ergotamine, and ergocristine correlated negatively with aphid numbers. However, correlations with other alkaloids were also close to statistical significance (**Table 2**).

Some seemingly uninfected plants contained low alkaloid amounts (**Figures 2B–H**). This finding is probably due to the emerging, still invisible *Claviceps* infections or sporal contaminations. *Epichloë*-symbiotic plants that were also *Claviceps* infected (E+C+) had significantly higher concentrations of ergonovine than other plants (**Figure 2C**). Concentrations of ergovaline were higher in the E+C+ plants than in the E–C– or E–C+ plants (**Figure 2B**), and the concentrations of ergosine and ergocristine were significantly higher in E+C+ than those in the E–C– (**Figures 2D,H**). We did not find statistically significant differences in the pairwise comparisons between any *Epichloë–Claviceps* combinatory plant groups in ergotamine, ergocornine, and α-ergocryptine concentrations (**Figures 2E–G**).

TABLE 2 | Analysis of ergot alkaloids showing which alkaloids were associated with *Epichloë* symbiosis and visible *Claviceps* infection and how their concentration correlated with the number of aphids in the same plants.

		Ergovaline	Ergonovine	Ergosine	Ergotamine	Ergocornine	α-Ergocryptine	Ergocristine
Epichloë	t	3.58	2.10	1.54	1.53	1.66	0.75	1.67
	df	35	22.6	35	35	23.3	35	35
	p	**0.001**	**0.047**	0.132	0.135	0.111	0.457	0.105
Claviceps	t	−1.18	−1.99	−2.63	−1.11	−2.17	−2.25	−2.32
	df	35	25.5	27.8	35	21.5	35	35
	p	0.247	0.058	**0.014**	0.276	**0.042**	**0.031**	**0.026**
Aphids	*r*	−0.325	−0.302	−0.309	−0.338	−0.243	−0.319	−0.405
	p	**0.049**	0.069	0.062	**0.041**	0.147	0.054	**0.013**

Statistically significant (<0.05) p-values are bolded. N = 37.

DISCUSSION

Our results do not support the hypothesis that defensive mutualism against invertebrate herbivores and pathogens drives endophyte–plant symbiosis. Contrary to our predictions that *E. festucae* should increase the resistance of its host grass, *F. rubra*, the aphid numbers were similar on *Epichloë*-symbiotic (E+) and *Epichloë*-free (E−) plants, and the infection frequencies of pathogenic *Claviceps* were higher in E+ plants than in E− ones. However, as expected, *Claviceps* provided notable protection against aphid herbivory, suggesting that the net effects on the host can remain positive as the infection causes only minor seed loss (Luttrell, 1980; Wäli et al., 2013). These results emphasize the importance of understanding the structure and functional complexity of plant microbiomes by demonstrating that plant–pathogen interactions are context dependent, ranging from antagonistic to mutualistic, rather than always disadvantageous to the host plant (Saikkonen et al., 1998, 2020; Vázquez de Aldana et al., 2010; Wäli et al., 2013; Rybakova et al., 2016; Selosse et al., 2018).

As the defensive mutualism between grasses and *Epichloë* species is primarily attributable to fungal origin alkaloids (Saikkonen et al., 2010; Schardl et al., 2013), our results suggest that the fungal genotypes associated with the plants could not provide protection against aphids by producing a sufficient amount of alkaloids. Our previous transplant study with *F. rubra* plants collected from the same wild populations revealed that *E. festucae* can produce peramine and ergovaline; however, alkaloid profiles in plants varied among the geographic origin and growth conditions of the symbiotum (Vázquez de Aldana et al., 2020). Peramine was only produced by the Spanish genotypes. Ergovaline was detected in E+ plants across Europe, but the concentrations varied among fungal strains, and the profiles changed when the symbiotum was transplanted to new environments. For example, none of the Spanish E+ plants contained detectable amounts of ergovaline in Northern Finland. In line with our previous study, ergot alkaloid concentrations varied considerably among E+ plants in the present study. Similarly, many other studies with other grass species have revealed that the quantities of *Epichloë* alkaloids vary among geographic origin and genetic plant–fungus combinations and can be plastic during the growing season depending on environmental conditions (Siegel et al., 1990; Thom et al., 2014; Helander et al., 2016; König et al., 2018; Fuchs et al., 2020).

In this study, we focused on the putative anti-herbivore properties of ergot alkaloids, which are a diverse family of mycotoxin compounds with a common origin (Panaccione, 2005). Unlike the majority of past studies treating ergot alkaloids as a pooled ensemble, we identified seven different ergot alkaloids from plant extracts (ergovaline, ergonovine, ergosine, ergotamine, ergocornine, α-ergocryptine, and ergocristine) and examined their potential in modulating host plant quality to aphids. Ergovaline is the main ergot alkaloid produced by *Epichloë* in most host grasses and a minor component of *C. purpurea* (Garner et al., 1993); the other six compounds are the main alkaloids of *C. purpurea* (Miedaner and Geiger, 2015). Our alkaloid results are in line with this literature, but ergonovine was also present in E+ plants, and we were not able to clearly associate presence of ergotamine with *Claviceps* infection only. The few earlier studies on individual alkaloids have shown variable effects on herbivores. For example, ergonovine is responsible for aphid mortality on sleepygrass (*Achnatherum robustum*) (Shymanovich et al., 2015), and similarly ergocryptine markedly explains the variation in fall armyworm dry weight on perennial ryegrass (*Lolium perenne*) (Salminen et al., 2005). By contrast, ergovaline appears to be insignificant for the performance of root or shoot aphids (Siegel et al., 1990; Popay et al., 2021). The issue becomes even more complicated when other trophic layers are involved. For example, Kunkel et al. (2004) showed that ergot alkaloids can mediate cascading effects in food webs, thereby indirectly benefiting herbivores due to their toxic effects on their natural enemies. In concordance with these studies, our results demonstrate dissimilar effects of individual ergot alkaloids on herbivores. Overall, the concentrations of all examined alkaloids were highest in *Claviceps*-infected E+ plants, suggesting the synergistic effect of both fungi on the alkaloid profile of the host grass. Concentrations of three out of the seven examined alkaloids (ergovaline, ergotamine, and ergocristine) were negatively correlated with the number of aphids on the plants. *Epichloë* symbiosis explained only the presence of ergovaline and ergonovine, whereas *Claviceps* explained the concentrations of ergosine, ergocornine, α-ergocryptine, and

ergocristine. Furthermore, ergocristine was associated with both *Claviceps* infection and the low number of aphids on the plants. We acknowledge that the chemical ecology underlying these results should be interpreted cautiously, since we studied only ergot alkaloids, and the analyses do not allow us to distinguish other *Epichloë*- and *Claviceps*-origin alkaloids in the samples. Still, since we found no compelling evidence that *Epichloë* symbiosis confers protection against aphids, we suggest that *Claviceps* is primarily responsible for the production of anti-herbivore compounds and for the reduction of aphid infestation. However, we acknowledge that additional chemical changes may occur (volatiles, etc.), which when combined with the examined alkaloids might reduce the aphid infestation in C+ plants.

The lack of *Epichloë*-enhanced host grass resistance to aphids can be partly explained by the high variation among the examined fungal lineages in their ability to produce alkaloids due to their genetic and chemotypic differentiation in Spanish and Finnish plants under different selection pressures. Empirical evidence supports the idea that post-glacial colonization history and contrasting climatic environments have resulted in local adaptations and genetic differentiation in symbiotum across its range in Europe (Dirihan et al., 2016; Leinonen et al., 2019; von Cräutlein et al., 2019, 2021). Strong seasonal changes in temperature, including short growing seasons and long winters, and variation in day length and light quality characterize environments at high latitudes, whereas plants in Spanish semiarid grasslands must adapt to seasonal drought (Zabalgogeazcoa et al., 2006; Leinonen et al., 2019). Genetic potential for diverse alkaloid production is remarkable in southern populations due to the prevalence of sexual reproduction in Spanish *Epichloë* populations (von Cräutlein et al., 2019, 2021). Although the northern populations have not adapted to strong invertebrate herbivory, the typically high *Epichloë* frequencies in wild *F. rubra* populations across its range in Europe are likely to be attributable to other benefits associated with *Epichloë*. Our previous transplant experiment with plants collected from the same geographic regions suggests that abiotic factors have not played a significant role in maintaining *Epichloë* symbiosis (Dirihan et al., 2016; Leinonen et al., 2019). Many examined European *F. rubra* populations with high frequencies of E+ commonly show strong vertebrate grazing—for example, by sheep in the Faroe Islands and Iceland and large ungulates in Spain, Switzerland, and northern Finland (Dirihan et al., 2016). This finding suggests that vertebrate grazing has been among the main selective forces driving the coevolution of *E. festucae* and *F. rubra* in Europe.

Increased susceptibility of E+ plants to the pathogenic *Claviceps* fungus compared with their E-conspecifics suggests that reciprocal changes during the long coevolutionary history of *Epichloë*-species and their host grasses involve the loss of host traits that prevent microbial invasions. These changes may result from modulated recognition, signaling, and defense responses (Saikkonen et al., 2013; van Overbeek and Saikkonen, 2016; Schmid et al., 2017; Bastías et al., 2018; Compant et al., 2019; Nissinen et al., 2019). Our results demonstrate that the consequences of such a predisposition to pathogens can be advantageous to the host plant in environments where a pathogen with only marginal damage to the host provides reinforced protection against pests. This finding questions whether these changes in host traits resulted from tripartite coevolution with reciprocal changes in the partners.

CONCLUSIONS

We propose that the independent coevolution of these two closely related fungi with their shared host plant might have resulted in the detection of complementary protection against herbivores and other benefits to each other as by-products without reciprocity and cooperation (Leimar and Connor, 2003). Vertical transmission and alkaloid production ability of *Epichloë*-species have selected benign symbiosis with the host grass, particularly in environments driven by strong herbivory pressure. The fitness of *Claviceps* is similarly highly dependent on the protection of host inflorescences to ensure successful sclerotium development to complete its life cycle. However, the occurrence and distribution of *Claviceps* is primarily dependent on the presence of the host and favorable weather and climatic conditions, whereas the heritable infections of *Epichloë* species depend on the host fitness. Although host protection can be regarded as an indirect by-product of mutualism, our results are consistent with a few other recent findings, suggesting that *Epichloë* species must be considered keystone species in shaping the microbial communities of their shared hosts (Nissinen et al., 2019).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

ML and BF originally formulated the idea, performed the experiments, and analyzed the data. ML, BF, KS, and MH conceived and designed the experiments. BV and IZ performed the chemical analyses. ML, KS, MH, BV, IZ, and BF wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Cytobacts: Abundant and Diverse Vertically Seed-Transmitted Cultivation-Recalcitrant Intracellular Bacteria Ubiquitous to Vascular Plants

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We have recently described 'Cytobacts' as abundant intracellular endophytic bacteria inhabiting live plant cells based on the observations with callus and cell suspension cultures of grapevine and other plant species with the origin ascribable to field explants. In this study, we investigated the prevalence of such cytoplasmic bacterial associations in field plants across different taxa, their cultivability, and the extent of taxonomic diversity and explored the possibility of their embryo-mediated vertical transmission. Over 100 genera of field plants were surveyed for 'Cytobacts' through bright-field live-cell imaging as per our previous experience using fresh tissue sections from surface-sterilized shoot-tissues with parallel cultivation-based assessments. This revealed widespread cellular bacterial associations visualized as copious motile micro-particles in the cytoplasm with no or sparse colony forming units (CFU) from the tissue-homogenates indicating their general non-cultivability. Based on the ease of detection and the abundance of 'Cytobacts' in fresh tissue sections, the surveyed plants were empirically classified into three groups: (i) motile bacteria detected instantly in most cells; (ii) motility not so widely observed, but seen in some cells; and (iii) only occasional motile units observed, but abundant non-motile bacterial cells present. Microscopy versus 16S-rRNA V3–V4 amplicon profiling on shoot-tip tissues of four representative plants—tomato, watermelon, periwinkle, and maize—showed high bacterial abundance and taxonomic diversity (11–15 phyla) with the dominance of Proteobacteria followed by Firmicutes/Actinobacteria, and several other phyla in minor shares. The low CFU/absence of bacterial CFU from the tissue homogenates on standard bacteriological media endorsed their cultivation-recalcitrance. Intracellular bacterial colonization implied that the associated organisms are able to transmit vertically to the next generation through the seed-embryos. Microscopy and 16S-rRNA V3–V4 amplicon/metagenome profiling of mature embryos excised from fresh watermelon seeds revealed heavy embryo colonization by diverse bacteria with sparse or no CFU. Observations with grapevine fresh fruit-derived seeds and seed-embryos

endorsed the vertical transmission by diverse cultivation-recalcitrant endophytic bacteria (CREB). By and large, Proteobacteria formed the major phylum in fresh seed-embryos with varying shares of diverse phyla. Thus, we document 'Cytobacts' comprising diverse and vertically transmissible CREBs as a ubiquitous phenomenon in vascular plants.

Keywords: Brownian motion, endophytic bacterial diversity, metagenomics 16S, next-generation deep sequencing, plant cell biology, plant kingdom, plant tissue culture, uncultivable bacteria

INTRODUCTION

All plants and plant organs are known to harbor endophytic bacteria, the prokaryotes that inhabit plants internally without apparent adverse effects on the host (Hallmann et al., 1997; Kandel et al., 2017). Bacterial endophytes are known to be acquired by plants primarily from the rhizosphere, occasionally from the atmosphere, and also as seed-borne microbial communities (Hardoim et al., 2015; Walsh et al., 2021). Field plants show the highest population and diversity of endophytic bacteria in soil-embedded roots (Conn and Franco, 2004; Liu et al., 2017). Lower bacterial diversity commonly observed in root tissues relative to the rhizosphere has contributed to the assumption that plants selectively recruit a subset of their choice organisms from soil as endophytes (Compant et al., 2010, 2021; Hardoim et al., 2015). One major and initial route of plant entry of endophytic bacteria is through root hairs (Prieto et al., 2011; Compant et al., 2021). This is particularly applicable for seed-associated bacteria making their entry at seed germination from the spermosphere (Nelson, 2018). Once inside root the epidermis, the microorganisms find their way to the root vascular tissues traversing the parenchymatous cortex at which phase is mostly intracellular (Hallmann et al., 1997; Compant et al., 2005). As per the general understanding, the organisms move further upward through the vascular system and reach various plant parts displaying intercellular colonization (Compant et al., 2005, 2008, 2011; Kandel et al., 2017; Liu et al., 2017). Thus, the endophytic bacterial association is generally known to be intercellular or apoplastic (Sattelmacher, 2001; Hardoim et al., 2015; Liu et al., 2017). Plant acquisition of endophytes from the aerial parts through natural openings such as stomata, lenticels, and injuries incited by insects and other pests is also proposed, but in such cases too, the colonization is generally intercellular (Frank et al., 2017; Kandel et al., 2017).

We have recently documented abundant and diverse cytoplasmic/intracellular bacteria, termed 'Cytobacts,' in healthy plant cells as per microscopic observations including fluorescent *in situ* hybridization (FISH) targeting different classes of bacteria and 16S rRNA gene amplicon profiling on long-term actively maintained cell-suspension and callus cultures of grapevine and other plant species (Thomas and Franco, 2021). The ability to maintain *in vitro* cultures protected from external microorganisms offered the strong point in drawing the aforementioned conclusion contrary to the general practice of using *in situ* or *ex situ* plants for studying the endophytic microorganisms. Further, the organisms proved to be not amenable to cultivation which to some extent explained why this association went unnoticed so long. 'Cytobacts' were first described with banana shoot-tip tissues and *in vitro* cultures as conventionally uncultivable abundant bacteria colonizing the cytoplasm and also as adherents to the plasma membrane or organelles (Thomas and Reddy, 2013; Thomas and Sekhar, 2014). Subsequently they were documented with the field plants and *in vitro* cultures of papaya (Thomas et al., 2019). This conclusion on cytoplasmic bacteria was further strengthened with the isolated reports on intracellular bacteria documented in the meristem tissue of pine (Pirttilä et al., 2000), axenically grown pineapple and orchids (Esposito-Polesi et al., 2017), and 'Bacteriosomes' observed in axenic peach palm plants (de Almeida et al., 2009). The elucidation of 'Cytobacts' was facilitated through bright-field live-cell imaging displaying abundant motile micro-particles in the cytoplasm resembling the cells of common endophytic bacteria, supported by live bacterial staining using the DNA stain SYTO-9 (Thomas and Reddy, 2013; Thomas and Sekhar, 2014). Extension of this study to long-term actively maintained cell and callus cultures of grapevine and other plant species supported by fluorescence and confocal microscopy employing bacterial stains, FISH, and next-generation deep sequencing revealed cytoplasmic bacterial association as a wide-spread phenomenon for *in vitro* cultures across plant species (Thomas and Franco, 2021). An exploration on to the possible sources of these cytoplasmic bacteria in cultured plant cells indicated the prevalence of such intracellular bacteria in field-pants. In these microscopic studies, no bacteria could be detected in the intercellular regions to support the broad inference of apoplastic colonization by endophytic bacteria (Hallmann et al., 1997; Sattelmacher, 2001; Hardoim et al., 2015). Although there is substantial literature on endophytic bacterial associations covering their biology, diversity, functions in plants, and their potential exploitation for optimizing crop production, very few studies have provided microscopic data on tissue colonization, which too in most instances have targeted just the root system (White et al., 2014, 2018; Shehata et al., 2017). Most of these reports and reviews go with the general statements on endophytic bacterial colonization as intercellular and/or intracellular (Hardoim et al., 2015; Kandel et al., 2017; Liu et al., 2017).

The wide-spread cytoplasmic bacterial associations observed in cultured cells of different plant species *in vitro* and the occasional reports on intracellular bacteria cited above made it imperative to investigate the prevalence of such associations in field plants across diverse taxa. Cultivation

Abbreviations: CREB, cultivation-recalcitrant endophytic bacteria; FDW, filter-sterilized distilled water post-autoclaving; NA, nutrient agar, SDW, sterile distilled water, SATS, spotting-and-tilt-spreading; SP-SDS, single plate – serial dilution spotting; TH, tissue homogenate, TSA, trypticase soy agar.

versus microscopy-based studies on field plant tissues of banana earlier indicated that the associated organisms were normally uncultivable (Thomas and Sekhar, 2014). Scientific studies employing molecular tools such as 16S rRNA ribotyping; 16S rRNA gene amplicon profiling; or whole genome metagenomics on banana, grapevine, papaya, etc., have brought to light a huge diversity of uncultivable/cultivation-recalcitrant endophytic bacteria (CREB) (Thomas and Sekhar, 2017; Thomas et al., 2017, 2019). Generally, Proteobacteria formed the major phylum followed by Actinobacteria, Firmicutes, and Bacteroidetes, and several other phyla including some candidate phyla with no or sparse cultivable counterparts.

One major question with respect to the intracellular bacterial colonization pertained to how the organisms gain cytoplasmic entry throughout the plant system without damaging the cells, concurring with the widely accepted concept of plant recruitment of endophytic bacteria from soil or atmosphere. This becomes feasible in the case of embryo-mediated vertical transmission (Thomas and Sahu, 2021). Intracellular colonization implies that the organisms are able to move to the daughter cells/gametes through mitosis/meiosis and then to the embryo, which allows their distribution to all parts of the new plant similar to cell organelles. This aspect is best studied by analyzing the seed-embryos for any associated bacteria, taking care to exclude all no-embryonic seed-tissues. Seed transmission of bacterial endophytes is a topic of much recent interest suggesting the possibility of vertical microbial movement from one generation to the next (Nelson, 2018; Shahzad et al., 2018; White et al., 2019; Matsumoto et al., 2021). Seed-associated microbes could inhabit the seed internal tissues and/or the external tissues, and hence, the seed association does not essentially mean vertical transmission unless the organisms colonize the embryo *per se* (Thomas and Shaik, 2020). Most studies on seed transmission of endophytic bacteria have explored this aspect primarily employing surface-sterilized seeds (Khalaf and Raizada, 2016; Glassner et al., 2018; Nelson, 2018). In such studies, there is a possibility of carry-over of externally associated bacteria despite extensive surface-sterilization treatments. It is essential that the embryo be studied distinctly excluding the seed coat tissues to confirm the vertical transmission (Thomas and Sahu, 2021). The report on 'Cytobacts' as a widespread association in different plant tissues and as a general phenomenon in cell and callus cultures across plant species substantiated the possibility of their movement to the gametes, possible integration in the new embryos during fertilization, and the continued perpetuation in the new plant. This is substantiated by the report on pollen or ovule mediated-transmission of endophytic bacteria (Frank et al., 2017; Maniranjan et al., 2017).

Thus, the present study was taken up as a continuation of the observations on the prevalence of abundant and diverse endophytic bacteria in cell cultures of different plant species with their origin traced to the field explants (Thomas and Franco, 2021) and their possible vertical transmission (Thomas and Sahu, 2021). Further, we found bright-field live-tissue imaging as an easy and simple way to observe the intracellular bacteria (Thomas and Sekhar, 2014; Thomas and Franco, 2021), whereas the deep-sequencing or omics-based tools help in studying the diversity of conventionally uncultivable plant microbiome (Kaul et al., 2016; Matsumoto et al., 2021). The present study had the objectives of assessing the prevalence of intracellular bacteria in field plants across different functional and taxonomic categories, estimating the gross taxonomic diversity including CREB, and investigating the embryo-mediated vertical transmission.

MATERIALS AND METHODS

General Experimental Design

Live cell bright-field microscopy imaging under high magnification (1000×) without stains or using low levels of Safranin/Grams crystal violet and micro-videography on microbial cell-motility was employed as a general tool for surveying different plant species for the cellular bacterial associations (Thomas and Sekhar, 2014; Thomas and Franco, 2021). A parallel cultivation-based assessment of bacterial CFU from surface-sterilized tissues was undertaken on representative plants. Further, a cultivation-independent determination of endophytic bacterial microbiome diversity adopting 16S rRNA gene V3–V4 taxonomic profiling of four selected/representative field plants was undertaken. In order to verify the possible embryo-mediated vertical transmission of endophytic bacteria, bacterial taxonomic profiling on clearly excisable embryos from mature watermelon seeds through 16S rRNA gene V3–V4 profiling and whole genome metagenome profiling was undertaken. Considering that grape cell cultures formed the main experimental material for the elucidation of intracellular bacteria in the earlier study (Thomas and Franco, 2021), bacterial taxonomic profiling on whole grape seeds gathered aseptically from ripe berries and the seed-embryos excised from hard grape seeds after alkali treatment were employed in 16S rRNA gene taxonomic profiling with parallel cultivation-based bacterial monitoring. As a prelude to this study, pure cultures of some bacterial strains isolated as endophytes, namely, *Klebsiella pneumonia* (medium motile rods), *Microbacterium esteraromaticum* (small motile rods), and *Micrococcus terrus* (motile cocci) were captured under bright-field microscopy for a comparison of their size, shape, and motility patterns to the motile micro-particles in tissue sections or the issue homogenates along with a common laboratory contaminant *Bacillus pumilus* (longer motile rods).

Field Plants for Microscopic Observations

This involved about 100 genera of field-plants covering cereal, pulse, oilseed, fruit, vegetable, ornamental, medicinal, tuber and forage crops, grasses, weeds, and forest species (**Table 1**). The list included herbs, shrubs, climbers, and trees across annual and perennial species covering dicots and monocots mostly from India (Bengaluru, Karnataka State, or parts of neighboring Kerala and Tamil Nadu States) and some Australian plants (Adelaide, SA, Australia). Tissue segments from tender shoot or petiole tissues of field-grown plants were generally used. Wherever field plants were not accessible, seedlings/plants raised in glasshouse on soil-based medium were employed. Tissues were

TABLE 1 | List of plant species taken up for the bright-field microscopy-based survey for endophytic bacteria and an empirical classification of plants based on the ease of observing motile intracellular bacteria in fresh tissue sections.

	(i) Motile bacteria detected instantly in most cells				(ii) Motility not so widely observed in all cells, but seen in some cells with non-motile bacteria obvious in most cells				(iii) No obvious motile bacteria but abundant non-motile micro-particles corresponding to bacteria present		
	Genus/species	**Category[#]**	**Tissue**		**Genus/species**	**Category[#]**	**Tissue**		**Genus/species**	**Category[#]**	**Tissue**
1	*Acalypha hispida*	A	Petiole	1	*Allamanda cathartica*	P	Petiole	1	*Abelmoschus esculentus* (**Supplementary Movie 12**)	A	Petiole
2	*Amaranthus* sp.	A	Petiole	2	*Allium cepa*	A	Leaf base	2	*Anacardium occidentale*	P	Petiole
3	*Ananas comosus*	S	Crown	3	*Arabidopsis thaliana*	A	Hypocotyl	3	*Annona reticulata*	P	Petiole
4	*Aster patens*	A	Stem	4	*Averrhoa carambola*	P	Petiole	4	*Anthurium andraeanum*	S	Petiole
5	*Beta vulgaris*	A	Stem	5	*Begonia semperflorens-cultorum*	S	Petiole	5	*Araucaria excelsa* (**Supplementary Movie 13**)	P	Rachis
6	*Bougainvillea glabra*	P	Stem	6	*Brassica nigra*	A	Stem	6	*Azadirachta indica*	P	Petiole
7	*Brassica oleracea*	A	Midrib	7	*Carica papaya*	P	Petiole	7	*Casuarina equisetifolia*	P	Petiole
8	*Canna generalis*	S	Leaf base	8	*Cicer arietinum*	A	Hypocotyl	8	*Cosmos bipinnatus*	A	Petiole
9	*Capsicum annuum* (**Supplementary Movie 06**)	A	Petiole, Stem	9	*Citrus aurantifolia*	P	Petiole	9	*Cycas revoluta*	P	Rachis
10	*Catharanthus roseus*	S	Petiole	10	*Citrus pummelo*	P	Petiole	10	*Dracaena kaweesakii*	S	Leaf base
11	*Celosia argentea*	A	Stem	11	*Codiaeum variegatum*	P	Petiole	11	*Dypsis lutescens*	P	Rachis
12	*Centella asiatica*	A	Petiole	12	*Cynodon dactylon*	S	Stem	12	*Eupatorium odoratum*	S	Petiole
13	*Chlorophytum comosum*	S	Leaf base	13	*Cyprus rotundus*	S	Leaf base	13	*Garcinia indica*	P	Petiole
14	*Chrysanthemum indicum* (**Supplementary Movie 09**)	S	Stem, petiole	14	*Dendrobium* sp.	P	Leaf base	14	*Gladiolus palustris*	S	Leaf base
15	*Citrullus lanatus*	A	Stem	15	Duranta goldiana	P	Petiole	15	*Grevillea robusta*	P	Stem
16	*Colocasia esculenta*	S	Petiole	16	*Eleusine coracana*	A	Coleoptile	16	*Hibiscus rosa-sinensis*	P	Stem, petiole
17	*Coriandrum sativum*	A	Stem	17	*Geranium* sp.	S	Petiole	17	*Hylocereus undatus*	P	Stem
18	*Cucurbita moschata*	A	Stem	18	*Impatiens balsamina*	A	Stem	18	*Lagerstroemia speciosa*	P	Stem
19	*Dracaena braunii*	S	Leaf base	19	*Ipomoea indica*	S	Stem	19	*Lantana camera*	P	Stem
20	*Epipremnum aureum*	S	Petiole	20	*Ixora coccinea*	P	Petiole	20	*Lilium longiflorum*	S	Leaf
21	*Euphorbia pulcherrima*	S	Petiole	21	*Jasminum sambac*	S	Stem	21	*Mimosa pudica*	S	Stem
22	*Fragaria* × *ananassa*	S	Petiole	22	Leucaena leucocephala	S	Stem	22	*Mangifera indica*	P	Petiole
23	*Gerbera* sp.	A	Petiole	23	*Lonicera sempervirens*	S	Stem	23	*Maranta arundinacea*	S	Petiole

(Continued)

TABLE 1 | (Continued)

	(i) Motile bacteria detected instantly in most cells				(ii) Motility not so widely observed in all cells, but seen in some cells with non-motile bacteria obvious in most cells				(iii) No obvious motile bacteria but abundant non-motile micro-particles corresponding to bacteria present		
	Genus/species	**Category[#]**	**Tissue**		**Genus/species**	**Category[#]**	**Tissue**		**Genus/species**	**Category[#]**	**Tissue**
24	*Mentha* × *piperita*	A	Stem	24	Michelia champaca	P	Petiole	24	*Nephrolepis exaltata*	S	Rachis
25	*Mirabilis jalapa*	S	Stem	25	*Manihot esculenta*	S	Petiole	25	*Peltophorum pterocarpum*	P	Petiole
26	*Momordica charantia*	A	Stem	26	*Mussaenda erythrophylla*	S	Petiole	26	*Philodendron bipinnatifidum*	S	Leaf
27	*Mucuna bracteata*	S	Stem	27	*Oryza sativa* (**Supplementary Movie 10**)	A	Coleoptile	27	*Phyllostachys aurea*	S	Leaf base
28	*Musa* sp.	P	Shoot tip	28	*Oxalis* sp.	A	Petiole	28	*Polyalthia longifolia*	P	Stem
29	*Nicotiana tabacum*	A	Stem, Petiole	29	*Passiflora edulis*	P	Petiole	29	*Psidium guajava*	P	Petiole
30	*Ocimum sanctum*	S	Stem	30	*Polianthes tuberosa*	S	Leaf base	30	Ricinus communis	S	Petiole
31	*Pachystachys lutea*	S	Stem	31	*Punica granatum*	P	Petiole	31	*Spathiphyllum wallisii*	S	Leaf base
32	Pentas lanceolata	P	Stem	32	*Rosa indica*	P	Petiole	32	*Syzygium cumini*	P	Petiole
33	*Phaseolus vulgaris* (**Supplementary Movie 08**)	A	Petiole	33	*Sesbania drummondii*	S	Stem	33	*Syzygium samarangense*	P	Petiole
34	*Pisum sativum* (**Supplementary Movie 07**)	A	Petiole	34	*Strelitzia reginae*	S	Leaf base	34	*Tecoma grandiflora*	P	Petiole
35	*Raphanus sativus*	A	Petiole	35	*Triticum aestivum* (**Supplementary Movie 11**)	A	Coleoptile	35	*Terminalia catappa*	P	Petiole
36	*Salvia splendens*	A	Stem	36	*Vitis vinifera*	P	Petiole	36	*Thuja occidentalis*	P	Stem
37	*Solanum lycopersicum* (**Supplementary Movie 05**)	A	Stem, Petiole	37	*Zea mays*	A	Leaf base	37			
38	*Solanum melongena*	A	Petiole								
39	*Spinacia oleracea*	A	Petiole								
40	*Syngonium podophyllum*	A	Petiole								
41	*Tagetes patula*	A	Petiole								
42	*Zingiber officinale*	A	Petiole								
43	*Portulaca grandiflora*	S	Petiole								

[#]*Category: A, annual; P, perennial; S, semi-perennial/semi-annual.*

used after surface sterilization, which involved two initial rinses in autoclaved distilled water (ADW) containing 0.1% Tween-20 and 5–6 min sodium hypochlorite (2% available chlorine; Fisher Scientific, Mumbai, India) treatment with a change of disinfectant after 3 min, and final six rinses in sterile water. When tender tissues such as coleoptile or hypocotyl of seedlings was used, the duration of chemical treatment (3 min) or the strength of disinfectant was reduced (1% chlorine).

Tissue Microscopy

Preparation of thin tissue sections using a Cryotome FSE (Thermo Fisher Scientific, Cheshire, United Kingdom) was initially considered for bight-field live-cell imaging on surface-sterilized tender shoots of grapevine 'Flame Seedless.' Cryo-sections, however, showed tight adhesion to the glass slide due to the cryo-gel. Further, the cryo-sections mounted in water did not show any micro-particle motility. The tissue sections also failed to take the live bacterial stain SYTO-9 suggesting cryo-gel imparted inhibition of DNA staining.

Thin free-hand tissue sections prepared from tender shoot tissues—terminal stem tissue, petiole, leaf base, or rachis—were employed for bright-field cell imaging. Surface sterilized tissues were sectioned over a sterile glass slide (washed in soap solution, wiped with 70% ethanol and flamed twice with absolute alcohol dip) using a stainless razor blade (Vidyut Super-Max, Thane, India), and 4–5 thin sections (∼30–50 μm) were transferred to 0.2 μm filtered double-autoclaved distilled water (FDW) on a fresh glass slide. After two rinses in FDW, they were observed directly in FDW or in 0.001% safranin at 1000× under oil immersion using Leica DM2000 microscope (Leica Biosystems, Wetzlar, Germany) as described elsewhere (Thomas and Sekhar, 2014).

Assessing Cultivable Endophytic Bacteria in Shoot-Tip Tissues

Shoot-tip tissues from field plants were surface-sterilized as above and then treated with 2.0% $Na_2S_2O_3$ for 10 min to remove any residual toxic chloramines. After three rinses in sterile distilled water (SDW), the tissues were aseptically homogenized with a micro-pestle (1.5 ml tube) and the tissue homogenate (TH; 100 mg ml^{-1} FDW) was spread on nutrient agar (NA) through the SP-SDS method using 20-μl lots (Thomas et al., 2015) employing four replications. Prior to the TH preparation, the last two wash solutions post-surface sterilization (200 μl) were plated on NA as per spotting-and-tilt-spreading (Thomas et al., 2012), and the CFU estimates for TH were accepted after ensuring the effectiveness of surface sterilization for 2–4 days.

16S rRNA Gene V3–V4 Amplicon Profiling on Field Samples

Four plant species, namely tomato (*Solanum lycopersicum*), watermelon (*Citrullus lanatus*), periwinkle (*Catharanthus roseus*), and maize (*Zea mays*) were selected as representatives for cultivation-independent bacterial diversity assessment adopting 16S rRNA gene-V3–V4 amplicon profiling. Tomato, watermelon, and maize are common annual crops accessible the world over, the former two representing dicots and the latter, a monocot. The first two also formed the candidates for seed and embryo bacterial transmission studies (Thomas and Shaik, 2020; Thomas and Sahu, 2021). Periwinkle, a semi-perennial shrub, formed the source of cell cultures employed for the elucidation of intracellular bacteria (Thomas and Franco, 2021) along with grapevine which was covered in detail earlier (Thomas et al., 2017).

A pooled tissue sample was drawn from the field shoot-tip tissues (25–50 g) of 1- to 2-month-old seed-derived field plants except in perennial periwinkle, and about 5 g tender tissues were surface-sterilized as above. To ensure effective tissue surface disinfection, the wash solutions were plated on NA and monitored for CFU for a week during which the tissue samples were stored at −20°C. After a week, the samples were thawed and homogenized in a mortar, and the TH (1 g FDW in 10 ml) was incubated at 4°C for 2 h to allow the larger particles to settle down. The tissue homogenates were tested for the share of cultivable bacteria through SP-SDS plating of decimal dilutions on NA and trypticase soy agar (TSA). DNA was extracted from the upper 3–4 ml clearer sample after high speed pelleting (18,000 × *g*) employing the Qiagen DNeasy PowerFood Microbial Kit (Qiagen GmbH, Hilden Germany; Cat. No. 21000-100) as per the extended lysis protocol. After preliminary quality and quantity assessments, one pooled DNA sample from three replications each were submitted to M/s Eurofins Genomics, Bengaluru[1] for 16S rRNA V3–V4 taxonomic profiling.

16S rRNA gene amplicon libraries were prepared at M/s Eurofins Genomics targeting the V3–V4 hypervariable region as per the standard Illumina 16S Metagenomic Sequencing Library preparation protocol. Library sequencing on Illumina MiSeq platform (2 × 300 bp), quality filtrations, stitching, and QIIME (Quantitative Insights Into Microbial Ecology) bioinformatics analysis were done as described elsewhere (Thomas and Sekhar, 2017). Two rounds of QIIME analyses were undertaken as per Thomas and Sekhar (2017), the first round on the cleaned up data and the second round on the filtered data excluding sequences that corresponded to plastid and mitochondria 16S rRNA identified in the first round. Taxonomic assignments were made with Greengenes as the reference database.

Cultivation Versus Microscopy-Based Assessment of Endophytic Bacteria in Watermelon Seed-Embryos

Seed-embryos of watermelon (cv. Arka Manik) were employed to assess the prospect of vertical transmission of bacterial endophytes considering the feasibility of clear excision of embryos excluding the seed external tissues. Fruits weighing about 3–4 kg after repeated surface wipes with 10% Domex were used as the source of fresh seeds. Seeds (50 nos) were extracted from the middle part of the fruits in a laminar air-flow (LAF), shaken vigorously in 0.01% Tween-20, rinsed thrice in SDW, treated with 70% ethanol (1 min), and air-dried over sterile tissue-paper. Twenty seeds each were collected per fruit and decoated

[1]http://www.eurofinsgenomics.co.in

aseptically using a sterile nail cutter. The excised embryos were treated with 90% ethanol (60 s) and then NaOCl (4% chlorine) for 5 min followed by six rinses in SDW, soaked in 2.0% $Na_2S_2O_3$ for 10 min and then rinsed thrice in SDW with the plating of 400 μl of the last wash solution. Surface-sterilized seed-embryos were homogenized in a mortar employing 1 ml FDW per embryo which yielded a milky suspension. Original embryo-homogenate and five decimal dilutions were assessed for cultivable bacteria through SP-SDS using 20 μl lots (Thomas et al., 2015) on four replicate TSA plates while the 10^0–10^{-2} lots were assessed on NA through spotting-and-tilt-spreading (SATS) (Thomas et al., 2012) and observed for CFU for 2 weeks at 30°C. Altogether, 10 fruits were studied as the source of seed-embryos in different batches.

16S rRNA Gene V3–V4 Amplicon Profiling of Watermelon Seed-Embryos

The embryo homogenates from the above 10 fresh fruit-derived seeds were stored at −20°C while waiting for the results from cultivation-based assessments. The homogenate from five seed lots which did show any cultivable bacteria for 1–2 weeks were used for the cultivation-independent analysis to avoid the over-representation of OTUs from easily cultivable organisms. The thawed embryo homogenates were pooled and incubated at 4°C for 2 h for the larger particles to settle down. The upper clearer 4 ml suspension with abundant microscopic motile particles was pooled and used for microbial DNA isolation after pelleting at 18,000 × *g* for 2 min. The supernatant part which showed some motile micro-particles was re-spun twice in a fresh tube. The pooled pellet from the three spins was used for DNA extraction employing (i) PowerFood Microbial DNA isolation (PF) kit (Laboratories Inc., Carlsbad, CA, United States) with extended 10 min at 70°C before the bead-beating step, or (ii) AxyPrep bacterial DNA isolation (AP) kit (Cat #: AP-MN-BT-GDNA-50; Axygen Biosciences, Union City, CA, United States). After preliminary quantity and quality assessments, the embryo-derived DNA pooled from different fruit sources were pooled and submitted to M/s Xcelris Labs Ltd. (Ahmedabad, India)[2] for 16S rRNA gene V3–V4 taxonomic profiling (sample IDs MG09 and MG10, respectively, for PF and AP kits).

16S rRNA gene V3–V4 region amplicon library preparation, library sequencing, quality filtrations, stitching, and two rounds of QIIME bioinformatics analysis were done as described above. Further, the 16S V3–V4 reads were used for functional analysis through PICRUSt (Langille et al., 2013) as described elsewhere (Thomas et al., 2019).

Whole Genome Metagenome Taxonomic and Functional Profiling of Watermelon Seed-Embryos

Whole genome metagenome (WMG) analysis involved taxonomic and functional profiling using the −20°C stored embryo-homogenate from five fruits (original 50 seed-embryos). The pooled embryo-homogenate was used directly for DNA extraction employing a PF kit without separating the upper clearer portion. After preliminary quality and quantity assessments, the DNA sample was submitted to M/s SciGenom Labs Private Limited, Cochin, India[3], for NGS-mediated WMG profiling. This involved the standard Illumina protocol including genomic DNA fragmentation, adenylation, adapter ligation, PCR amplification, library preparation, size distribution analysis, and library sequencing on the Illumina HiSeq with 2 × 250 bp high-quality paired-end (PE) reads as described earlier for grapevine (Thomas et al., 2017). After read quality checks and data processing, metagenome assembly was carried out *de novo* using the Ray-Meta program and taxonomy tree construction was done using the MEGAN software. Functional annotation of all the contigs were carried out by SEED classification employing MEGAN software from DIAMOND BLASTX results. KEGG pathway analysis was performed for each contig sequences by assigning KEGG Orthology (KO) numbers obtained from known reference hits. Further, enzyme and pathway information were assigned to contigs based on KO.

Assessing Seed and Seed-Embryo Bacterial Microbiome in Grapevine

Since grape (*Vitis vinifera* L.) cell cultures formed the primary tool for the elucidation of cytoplasmic bacterial associations in the previous study (Thomas and Franco, 2021), grape seeds/embryos formed the candidates to assess the extent of embryo-mediated vertical bacterial transmission and the comparative assessment of bacterial diversity in seeds *versus* seed-embryos. Seeds were collected aseptically from ripe berries and the seed-embryos were excised as described below.

Aseptic Collection of Mature Grape Seeds

Ripe fruits of 'Red Globe' (RG) imported from California, United States, to Bengaluru, India, and the locally grown 'Bangalore Blue' (BB) were collected from local home groceries. Bold berries (25 nos) from a single bunch with no external injuries or blemishes were gathered retaining 2–4-mm stalk-bits. To assess initial external microbial load, berries were washed five times in SDW with wash solution monitoring by spotting (10 × 1-μl lots) on NA. Berries were surface-sterilized using 90% ethanol (1 min) and NaOCl (4% available chlorine) for 10 min followed by six SDW washes and wash solution monitoring. After removing the stalk-bits, berries were opened in a LAF cabinet, and the seeds were collected. After blotting dry the seeds of any residual fruit pulp using sterile tissue paper, seeds were rinsed six times in FDW. One seed each from 25 berries was pooled, weighed aseptically (1.35 g for 'RG' and 1.45 g for 'BB' for 25 seeds) and then crushed in a mortar followed by extended manual grinding (15 min) using 1 ml FDW per 100 mg seed yielding a thick milky solution with black particles. The upper part of the suspension after 1 h at 4°C was used for seed DNA isolation.

Aseptic Collection of Seed-Embryos From Mature Grape Seeds

Fifty seeds each of 'RG' and 'BB' collected from bold and ripe berries in the above experiment were used for this. Mature grape

[2]www.xcelrislabs.com

[3]www.scigenom.com

seeds proved very hard to open manually. Based on pre-trials, seeds were soaked in the identified seed softening solution (20% NaOH in 0.1% Tween-20) for 18–24 h at room temperature (24°C–26°C), washed 10 times in SDW, scrub-dried on tissue paper, and then de-coated aseptically with the help of a pair of forceps and surgical blade. The whole and broken embryos were gathered, weighed aseptically, and washed 10 times in FDW during which most of the perisperm membrane got dislodged. The wash solutions were monitored on NA and TSA and the seed-embryos were stored at −20°C. Two weeks later, with no CFU from wash solutions and embryo imprints on NA and TSA, the seed-embryos (280 mg for 'RG' and 300 mg for 'BB' mg) were thawed in FDW, cleared of all floating particles and the perisperm tissue, and washed recurrently in FDW (with wash solution monitoring) followed by grinding to a fine slurry in a mortar (50 mg ml^{-1} FDW). The embryo homogenate was assessed for any cultivable bacteria through SP-SDS on NA and SATS on TSA for 1 week before proceeding to 16S rRNA taxonomic profiling.

Microbial DNA Extraction and 16S rRNA Gene V3–V4 Amplicon Profiling

The supernatants from the seed tissue homogenates of 'RG' and 'BB' after standing 1 h at 4°C (to allow the settling of black seed-coat particles) were used for the whole seed DNA extraction while the embryo-homogenates was used wholly for DNA isolation soon after preparation. After 4 min of spinning (12,000 × *g* in 1.8 ml × 2 columns) the seed and embryo homogenates gave thick pellets with cloudy supernatants. The supernatant part was collected, re-spun twice, and then pooled. The combined pellet was used for microbial DNA isolation using Qiagen DNeasy PowerFood Microbial Kit as per the outlined extended protocol with the final DNA elution in a 60-μl buffer. After preliminary quality and quantity assessments, the DNA samples were taken up for 16S rRNA gene V3–V4 profiling as per the standard Illumina protocol at M/s. Eurofins Genomics India, Bengaluru[1] (see text footnote 1) with two rounds of QIIME analysis to remove the interfering plant chloroplast and mitochondrial sequences.

Documentation, Data Analysis, and Accession Numbers

The field plants were observed mainly though bright-field live cell imaging on fresh tissue sections from surface-sterilized tissues, and the documentation involved mainly micro-videography under 100× oil immersion objective (effective 1000× magnification). The videos were captured in 'avi' format and converted to 'mp4' with the help of WinX HD Video Converter Deluxe 2018 Trial Version. Parallel cultivation-based CFU assessments were made on the shoot-tips of common plant species through SP-SDS. Analysis of 16S V3–V4 sequence data involved two rounds of QIIME to exclude the plant chloroplast and mitochondrial and originally unassigned sequences while the WMG data analysis was done through MEGAN. Only one bio-replicate sample was employed in different experiments keeping the primary objective of assessing whether the bacterial diversity was small or large in order to substantiate the microscopic observations and to serve as guide for future experiments.

The 16S V3–V4 NGS data generated on the shoot-tip tissues of four plant species have been deposited with NCBI under the project "Shoot- tip endophytic bacterial microbiome" with the Bioproject ID PRJNA705572, Bio-sample acc. nos. SAMN18092256 (tomato), SAMN18092258 (watermelon), SAMN18092402 (periwinkle) and SAMN18092404 (maize), NGS data for watermelon seed and embryo samples under the project title "Cucurbit Seed, Embryo & Seedling Microbiome" with the Bioproject ID PRJNA564696, Bio-sample acc. nos. SAMN12726312 for MG-09 and SAMN12726313 for MG.10. For grape seed and seed-embryos, the bacterial microbiome NGS data has been deposited under the Bioproject ID PRJNA701136, Bio-sample acc. nos. SAMN17848617 for EG-Gr.MG01 (RG-Seed), SAMN17848618 for EG-Gr.MG02 (BB-Seed), SAMN17848891 for EG-Gr.MG03 (RG-Embryo), and SAMN17848989 for EG-Gr.MG04 (BB-Embryo).

RESULTS

Preliminary Microscopic Observations

Pure cultures of different bacteria isolated as endophytes were observed under bright-field microscopy, and the captured videos for some representative organisms—*Klebsiella pneumoniae*, *Microbacterium esteraromaticum, Micrococcus terrus*—have been provided to serve as a reference to assess the similarity of the motile micro-particles in the tissue sections to the bacterial cells in terms of size, shape, and motility patterns (**Supplementary Movies 01–03**) along with *Bacillus pumilus* (**Supplementary Movie 04**) isolated as external contaminant. Initial attempts employing cryo-microtome sections from grape shoot and petiole tissues did not work satisfactorily due to the tight adhesion of tissue sections to the glass slide and no micro-particle motility observed under bright-field. Live microscopic imaging on thin fresh tissue sections from tender shoots or petioles (∼30–50 μm) offered the best means to detect cellular bacteria directly or with safranin (0.005%).

Bright-Field Live Microscopy on Diverse Field-Plants

Microscopic observations on fresh tissue sections derived from field gown plants, covering >100 genera/species of vascular plants, revealed cytoplasmic bacteria 'Cytobacts' readily in most cases. The ease with which motile bacterial cells were detected in tissue sections showed some variation (**Table 1**). Thus, three distinct plant categories were observed: (i) motile bacteria detected instantly in most cells, as documented with tomato, sweet pepper, hot pepper, egg-plant, garden peas, cowpea, French bean, coriander, amaranth, chrysanthemum, marigold, gerbera, crossandra, centella, money plant, and pineapple (**Supplementary Movies 05–09**); (ii) motility not so widely observed, but seen in some cells with non-motile bacteria obvious in most cells (e.g., rice, wheat, grape, citrus, etc.; **Supplementary Movies 10**, **11**); and (iii) no or occasional motile bacteria observed but abundant non-motile micro-particles corresponding to bacteria present (e.g., hibiscus, okra, mango, pine, etc.; **Supplementary Movies 12**, **13**). This may

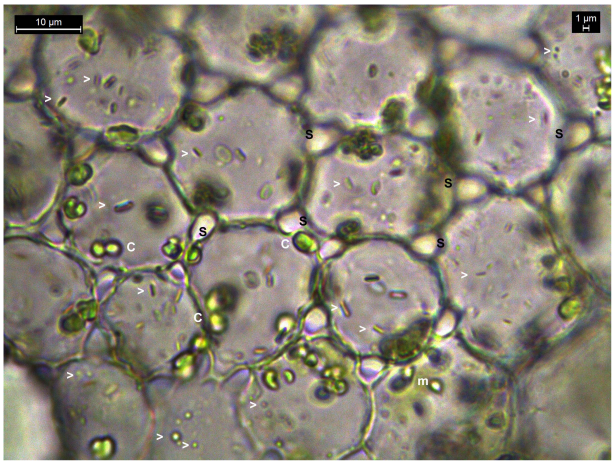


FIGURE 1 | Bright-field microscopy on thin tissue section of water lettuce petiole (1000×). Tissue section showing abundant bacteria in the intracellular region. Arrowheads indicate bacterial cells; c, chloroplasts; m, mitochondria; s, inter-space between cells where no bacteria are observed.

be an empirical list, but the observations explicitly indicated the ubiquitous presence of cellular bacteria across plant species/genera. Intracellular bacterial motility did not appear dynamic always with the active particle motility obvious in all plant hosts and across all host cells in a microscopy field. Cells exhibiting intact structural organization displayed bacteria aligning along the plasma membrane or adhering to organelles. The detection of 'Cytobacts' was enabled with the motility behavior for which cell disruption appeared a necessity. Bacterial motility was perhaps induced with the disruption of the cell cytoskeleton at tissue preparation, the vulnerability to which apparently varied with the plant species, tissue type, tissue age, and other factors.

Another noteworthy observation during microscopic explorations on tissue sections was the absence of obvious intercellular spaces or the bacterial cells in between the cells as generally reported for endophytic bacteria (**Supplementary Figure 1** and **Supplementary Movies 05–13**). On the other hand, a direct snap shot of a bright field image with bacterial staining employing dilute crystal violet or safranin, or even without any stains, showed abundant intracellular bacteria in some instances (**Figure 1**). Even in cases where some intercellular spaces were noticed, no bacteria were observed in those regions, and the amount of microparticles seen inside the cells far outnumbered any possible inhabitants in the intercellular region.

Cultivation- Versus Microscopy-Based Assessment of Endophytic Bacteria in Field Plant Shoot-Tip Tissues

Tissue homogenate from surface-sterilized field tissues of glasshouse or field grown plants yielded no colony forming units (CFU) on NA for 55% of 40 plant genera explored through the cultivation-based approach while the rest showed a few CFU (10^3 g^{-1} tissue in 15%, 10^4 in 20%, and 10^5 in 10%) with one to five colony morphotypes for a plant species. This CFU appeared far too low compared with the abundant bacterial cells as documented earlier with the shoot-tip tissue sections, or observed with the tissue homogenates (**Supplementary Movie 14**), indicating the general non-cultivability of associated bacteria.

Cultivation Versus 16S rRNA Gene V3–V4 Amplicon Profiling of Shoot-Tip Tissues of Representative Plants

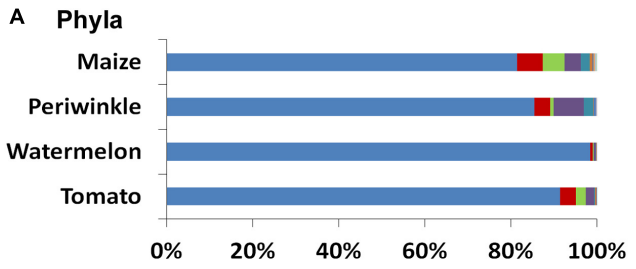
Tissue samples from the four plant samples—tomato, watermelon, periwinkle, and maize—showed low CFU (10^3–10^4 g^{-1} tissue) with one to four different colony types during the 1 week observation period. Considering that our interest was merely to get an estimate of cultivable organisms relative to the bacterial cells observed during microscopy or molecular based diversity analysis, the organisms were not taken up for identification or further characterization. The DNA yields appeared low (7.3 ng μl^{-1} in maize to 11 ng μl^{-1} in tomato), yet they yielded clear PCR amplicons and good quality amplicon libraries with 0.14–0.17 million reads (**Supplementary Table 1**). QIIME round-1 analysis showed a large share of reads corresponding to chloroplasts and mitochondria (46.3% in periwinkle to 62.6% in maize). QIIME round-2 taxonomic analysis excluding the plant reads showed OTUs in the range of 117 (periwinkle) to 179 (maize) with Shannon alpha diversity indices in the range of 2.04 (watermelon) to 3.80 (maize).

Taxonomic Diversity

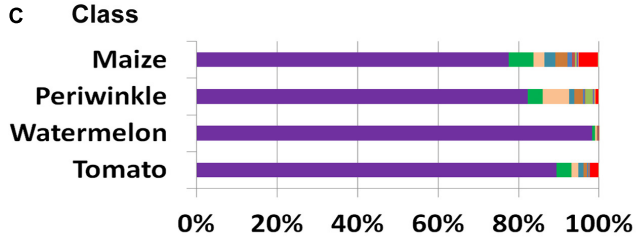
Majority of the OTUs belonged to Eubacteria (95% in maize to 99.8% in watermelon) and the rest to Archaea, the latter appearing noteworthy for maize (5%) and tomato (2.3%). Assessing the eubacterial diversity, 16 phyla were documented across the four plant species with seven common phyla (Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Planctomycetes, Verrucomicrobia, and Fusobacteria) (**Figure 2A**). Proteobacteria formed the major component in all samples (81.4%–98.4%). Actinobacteria (0.64%–5.98%), Firmicutes (0.5%–3.78%), and Bacteroidetes (0.1%–2.25%) constituted the other notable phyla while the rest constituted very low shares. Archaeal OTUs were confined to one phylum, Euryarchaeota.

Overall, 307 OTU categories were observed across the four plant species including 116 in tomato, 142 in watermelon, 117 in periwinkle, and 179 in maize. The four plant species showed 34 common eubacterial OTU categories constituting 77.7%–93.5% of the total OTUs and three Archaea forming 1.7%–17.3% OTUs, both together forming 79.5%–99.9% of the OTUs (**Figure 2B**). Thus, besides a high bacterial diversity, the four plant species also displayed considerable taxonomic similarity indicating a core microbiome across the species. The limited CFU observed during cultivation-based study versus the documented high diversity suggested the organisms were largely uncultivable.

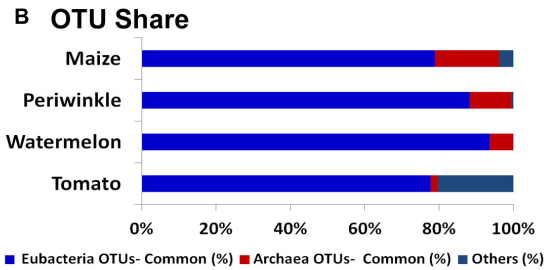
At the Class level, γ-Proteobacteria formed the major constituent in all the plant samples: 75.8% in maize to 98.3% in watermelon (**Figure 2C**). The next major classes included Actinobacteria followed by Bacilli for tomato, watermelon,



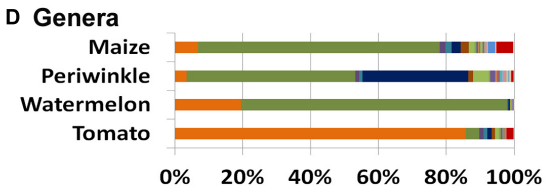
	Tomato	Watermelon	Periwinkle	Maize
Proteobacteria	91.45	98.44	85.45	81.44
Actinobacteria	3.61	0.64	3.72	5.98
Euryarchaeota	2.32	0.16	0.77	5.00
Firmicutes	2.01	0.51	6.96	3.83
Bacteroidetes	0.21	0.13	2.25	2.07
Planctomycetes	0.18	0.04	0.07	0.63
Verrucomicrobia	0.08	0.02	0.35	0.08
Tenericutes	0.04	0.02		0.08
MVP-21	0.04			
Fusobacteria	0.04	0.02	0.14	0.04
Elusimicrobia	0.02		0.07	0.08
TM7		0.01	0.07	0.27
[Thermi]			0.07	0.20
Lentisphaerae			0.07	0.04
Fibrobacteres				0.20
Cyanobacteria		0.01		
Acidobacteria				0.08



	Tomato	Watermelon	Periwinkle	Maize
EUBACTERIA				
Gammaproteobacteria	89.23	98.27	82.01	75.81
Actinobacteria	3.61	0.64	3.72	5.90
Bacilli	1.70	0.47	6.47	2.70
Betaproteobacteria	1.39	0.08	1.26	2.70
Alphaproteobacteria	0.80	0.08	2.18	2.93
Clostridia	0.31	0.04	0.49	1.09
Planctomycetia	0.18	0.04	0.07	0.63
Bacteroidia	0.10	0.10	1.90	0.51
Flavobacteriia	0.08	0.02	0.28	0.35
Fusobacteriia	0.04	0.02	0.14	0.04
Verrucomicrobiae	0.02	0.02	0.35	0.08
ARCHAEA				
Methanobacteria	2.09	0.16	0.70	4.61
Thermoplasmata	0.18	0.01	0.07	0.31



OTUs: Numbers	Tomato	Watermelon	Catharanthus	Maize
Bacterial OTUs common to four plants	3981	12611	1255	2011
Archaeal OTUs common to four plants	88	18	10	105
OTUs not common to all four plants	1054	862	158	443
Gross OTUs	5123	13491	1423	2559
a. Eubacteria OTUs common to 4 species (%)	77.71	93.47	88.19	78.78
b. Archaea OTUs common to 4 species (%)	1.72	6.38	11.1	17.3
Total (%)	79.43	99.85	99.29	96.08



	Tomato	Watermelon	Periwinkle	Maize
EUBACTERIA				
Pseudomonas (Gammaproteobacteria)	82.59	19.44	3.30	6.06
Unclassified Gammaproteobacteria	3.75	78.16	46.94	63.77
Klebsiella (Gammaproteobacteria)	1.25	0.08	1.05	1.52
Corynebacterium (Actinobacteria)	1.13	0.18	0.91	1.60
Acinetobacter (Gammaproteobacteria)	1.11	0.47	29.44	2.46
Propionibacterium (Actinobacteria)	1.05	0.14	1.34	2.15
Staphylococcus (Bacilli)	1.03	0.24	4.43	1.52
Micrococcus (Actinobacteria)	0.61	0.12	0.42	0.43
Brevibacillus (Bacilli)	0.29	0.07	1.26	0.35
Paracoccus (Alphaproteobacteria)	0.23	0.03	0.21	0.23
Unclassified Alphaproteobacteria	0.21	0.02	0.35	0.20
Enhydrobacter (Gammaproteobacteria)	0.14	0.02	0.42	0.16
Unclassified Actinobacteria	0.14	0.07	0.42	0.16
Unclassified Planctomycetia	0.12	0.02	0.07	0.63
Streptococcus (Bacilli)	0.12	0.10	0.07	0.31
Methylobacterium (Alphaproteobacteria)	0.08	0.01	0.28	0.20
Haemophilus (Gammaproteobacteria)	0.06	0.02	0.07	0.16
Ralstonia (Betaproteobacteria)	0.06	0.04	0.56	0.04
Prevotella (Bacteroidia)	0.06	0.03	0.70	0.39
Erwinia (Gammaproteobacteria)	0.04	0.03	0.56	0.23
Delftia (Betaproteobacteria)	0.04	0.01	0.21	1.88
Lactobacillus (Bacilli)	0.02	0.04	0.28	0.20
Rothia (Actinobacteria)	0.02	0.07	0.35	0.20
ARCHAEA				
Methanobrevibacter (Methanobacteria)	1.93	0.14	0.70	4.34
vadinCA11 (Thermoplasmata)	0.18	0.01	0.07	0.31

FIGURE 2 | Illumina MiSeq NGS-based bacterial diversity analysis on surface-sterilized shoot-tip tissues of four plant species. 16S rRNA gene V3–V4 region profiling of tomato, watermelon, periwinkle, and maize showing **(A)** distribution of OTUs under different phyla. **(B)** OTU shares common to different plant species. **(C)** Major classes and **(D)** major genera.

and maize while periwinkle had more of Bacilli followed by Actinobacteria. Tomato and maize displayed a notable share of Methanobacteria under phylum Euryarchaeota contrary to the minor shares of this in other plant species. Grossly, tomato harbored 20 classes with watermelon, periwinkle, and maize showing 17, 18, and 26 constituents, respectively, with 13 classes common for the four samples. Pseudomonadaceae formed the major family in tomato (83.3%) while others had Moraxellaceae in the lead (65.5%, 69.3%, and 56.2% for watermelon, periwinkle, and maize, respectively). The four plant species showed 56, 42, 48, and 69, constituents, respectively, although many of these were present in very small amounts.

At the Genus level, *Pseudomonas* formed the largest single constituent in tomato (82.6%) while the other three samples showed undefined Moraxellaceae as the largest constituent along with a notable share of unclassified *Pseudomonadaceae*

(**Figure 2D** and **Supplementary Dataset 1**). Periwinkle had *Acinetobacter* (Moraxellaceae) as the second major genus. Tomato showed OTU distribution under 76 eubacterial genera. The respective values for watermelon, periwinkle, and maize were 58, 63, and 87 with 27 genera common to the four species albeit the OTU shares were quite negligible in some instances. It was also striking to note six Archael taxa (*Methanobrevibacter, vadinCA11, Unclassified Methanobacteriaceae, Natronococcus, Methanosphaera*, and *Methanoplanus*) across the four plant species with the first two taxa observed in all of them. *Methanobrevibacter* constituted a major component in maize (4.4%) and tomato (1.9%). The observations here endorsed the prevalence of huge taxonomic diversity of Eubacteria and some amount of Archaea internally in field shoot-tip tissues in agreement with the abundant intracellular bacteria observed during microscopy.

PICRUSt Functional Analysis

KEGG pathway analysis showed the microorganisms largely involved in metabolic pathways followed by environmental information processing and genetic information processing (**Figure 3A**). COG description indicated diverse but similar functional profiles for the endophytic bacterial biome in different plant species with general functional prediction and amino acid metabolism on the top (**Figure 3B**). Other functional roles involved transcription, energy production and conversion, signal transduction mechanisms, inorganic ion transport and metabolism, cell wall/membrane/envelope biogenesis, translation, ribosomal structure and biogenesis, and others. Thus, the organisms appeared to be involved potentially in various plant cellular processes.

Cultivation- and Microscopy-Based Assessment of Endophytic Bacteria in Watermelon Seed-Embryos

The hard rind of watermelon fruits facilitated the gathering of seeds aseptically after surface sterilization, thus the isolation of seeds without external exposure (**Supplementary Figure 2**). Seed-embryos could be excised distinctly from air-dried seeds excluding the seed coat tissues with the help of a nail cutter. During NaOCl treatment, the membranous perisperm tissue covering the embryo got released leaving aside the clean seed-embryos. The embryo wash solutions post ethanol treatment of seeds and of embryos with NaOCl did not show any CFU on NA ensuring that no organisms external to the embryo were entering the processing line. The embryo homogenate—original stock and decimal dilutions—did not show any CFU on NA or TSA with both SATS and SP-SDS spreading procedures during the 1–2 weeks of observation. Monitoring the seeds from the subsequent fruits showed very few CFU from the original, 10^1 or 10^2 samples in some instances during the 1- to 2-week observation period, but none in others (**Supplementary Figure 3**).

The occasional CFU that developed from tissue homogenates after 1–2 weeks did not appear proportional to the dilution series, and it was translated to 10^1 to 10^3 CFU per embryo with just one to three colony morphotypes. These random CFUs proved too negligible considering the abundant motile bacterial cells observed during microscopic observations on the tissue homogenate (**Supplementary Movie 15**). This was particularly so considering that it warranted the use of 1:10 or 1:100 dilutions for microscopic observations since the original homogenate (1 embryo ml^{-1} FDW) was too dense for microscopy mounting. The observations indicated a huge bacterial presence in the embryo tissues considering that the 1:100 dilution barely had 10-μg tissues in a 20-μl sample loaded and that the microscopy field view covered only a negligible area of the homogenate spread. Here again, the organisms were not taken up for identification or further characterization since that was not a priority in this study.

16S rRNA Gene-Based Taxonomic Profiling on Watermelon Seed Embryos

Seed-embryo homogenate pooled from five seed lots that were not showing any bacterial CFU during cultivation-based monitoring were employed in this study. The sample showed relatively low DNA yields with the two kits (10.5 ng μl^{-1} for PF kit and 7.26 ng μl^{-1} for AP kit.), but both (MG09 and MG10, respectively) gave rise to good 16S V3–V4 amplicon libraries (**Supplementary Table 2**). A large share of NGS-derived OTUs as per QIIME round-1 analysis corresponded to Cyanobacteria/chloroplast or Proteobacteria/mitochondria leaving a smaller fraction attributable to bacteria. QIIME round-2 analysis after filtering out the plant sequences showed a high bacterial diversity influenced by the DNA isolation kit (22 and 16 phyla, respectively, for MG09 and MG10) with the dominance of Proteobacteria (41.0% and 95.7%, respectively) followed by Firmicutes (37.6% and 3.2%, respectively). The former also displayed small shares of Actinobacteria, Bacteroidetes, and Planctomycetes along with minor shares of 17 other phyla including several candidate phyla and some Euryarchaeota (0.2%), with the latter displaying much less diversity (**Figure 4A**).

At the Class level, MG09 and MG10 showed 49 and 31 constituents, respectively, with 27 taxa common to both (**Figure 4B**). Although Bacilli formed the major class in MG09 (30.6%), this branched to the families Leuconostocaceae, Aerococcaceae, Lactobacillaceae, and Streptococcaceae. Gamma-Proteobacteria formed the next major class (26.7%) followed by Alphaproteobacteria, Clostridia, Actinobacteria, Bacteroidia, and Betaproteobacteria. MG10, on the other hand, had nearly 88% OTUs under γ-Proteobacteria followed by β-Proteobacteria and Bacilli. At the Family level, MG09 showed 166 constituents with Moraxellaceae and Leuconostocaceae forming the largest families (**Figure 4C**). MG10 showed about 92 families with the dominance of Enterobacteriaceae. At the Genus level, MG09 comprised 244 constituents with 123 defined genera while MG10 bore 129 constituents comprising of 72 defined genera. The distribution of top 25 defined families and genera indicated that the high shares of Proteobacteria and Firmicutes in MG09 was primarily ascribable to *Acinetobacter* and *Leuconostoc* spp., whereas MG10 had mostly *Serratia* sp. (**Figure 4D**). The results in one way reflected a significant effect due to the DNA extraction kit on bacterial diversity but, on the other hand, emphasized the need

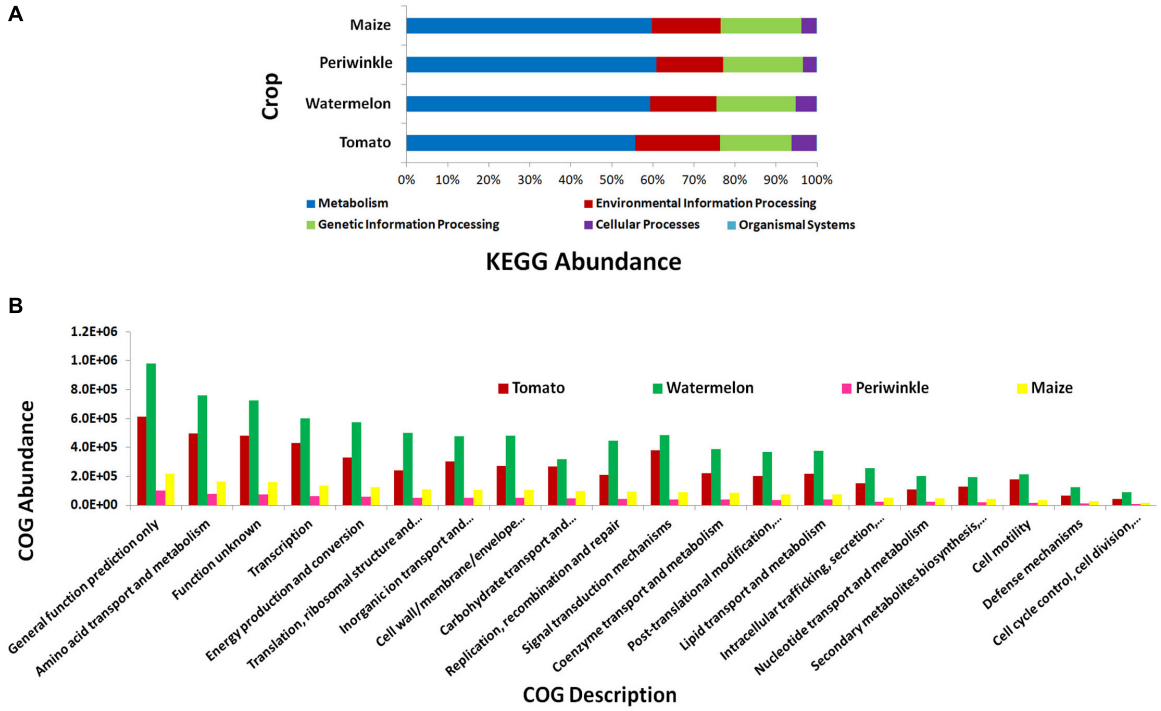


FIGURE 3 | PICRUSt functional analysis on 16S rRNA V3–V4 region profiles with Illumina MiSeq NGS platform on different plant species, tomato, watermelon, periwinkle/*Catharanthus*, and maize. **(A)** KEGG Level 1 functional analysis and **(B)** COG Level 1 functions.

for different DNA extraction approaches to bring out a larger diversity from phyla to genera levels (**Supplementary Dataset 2**). Spore-forming bacteria constituted a negligible fraction in both MG09 (*Lactobacillus* 1.56%, *Bacillus* 0.23%, *Marinilactibacillus* 0.20%; *Paenibacillus* 0.06%; *Lysinibacillus* 0.03%) and MG10 (*Lactobacillus* 0.19%; *Bacillus* 0.03%).

Whole Genome Metagenome Mediated Taxonomic and Functional Analyses on Watermelon Seed Embryos

The whole embryo homogenate used for DNA extraction gave better yields (24.4 ng μl^{-1}) than in the earlier instance of using the supernatant fraction for the MG09 sample using the same PF DNA isolation kit. Illumina HiSeq yielded 13 million raw reads with 0.375 million contigs available for downstream analysis and 0.3 million contigs aligning to bacteria (**Supplementary Table 3**).

MEGAN-mediated taxonomic profiling showed relatively less taxonomic diversity compared with the previous 16S rRNA gene V3–V4 profiling and a different taxonomic profile. Actinobacteria formed the most dominant phylum (47%) closely followed by Proteobacteria (42%) and then smaller shares of Planctomycetes (4%), Firmicutes (3%), and Bacteroidetes (2%) with the traces of Verrucomicrobia, Chloroflexi, and Thermobaculum (**Figure 5**). At the Class level, Actinobacteria formed the major constituent followed by γ-Proteobacteria, α-Proteobacteria, and β-Proteobacteria with Enterobacteriaceae constituting the dominant family. Spore-formers (*Bacillus* and *Paenibacillus* spp.) constituted a very minor share of 2% OTUs. Besides bringing out the prevalence of an array of diverse bacteria inside the embryos, the results endorsed the vertical transmission hypothesis in tune with the intracellular colonization.

SEED analysis of watermelon WMG data showed metabolism as the main functional role for the bacterial community covering amino acid, DNA, carbohydrate, nitrogen, protein, and RNA with other attributes including cell wall, stress response, motility, and others (**Figure 6A**). KEGG analysis also showed metabolism as the key function followed by genetic information processing, environmental information processing, and others with a major share (24%) remaining unclassified (**Figure 6B**).

Cultivation- and Microscopy-Based Testing of Grapevine Seeds and Seed-Embryos for Endophytic Bacteria

'Red Globe' had large bold berries compared with 'Bangalore Blue,' but the seed and embryo sizes were comparable for both grape varieties (**Figure 7**). Monitoring the berry wash solutions by spotting on NA showed a few colonies initially, but none after the ethanol step. Tracking the seed- and embryo-wash solutions did not show any bacteria in spotting tests or the plating of up to 400 μl of the last wash solution.

The whole seed tissue homogenates did not show any bacterial growth on NA or TSA for up to 2 weeks. Similarly, the embryo homogenates also did not display any microbial colonies for about 10 days, but thereafter, by 2–3 weeks, 'RG' homogenate showed multiple pigmented colonies (translated to about 1.0–2.5 × 10^3 CFU g^{-1} tissue), while

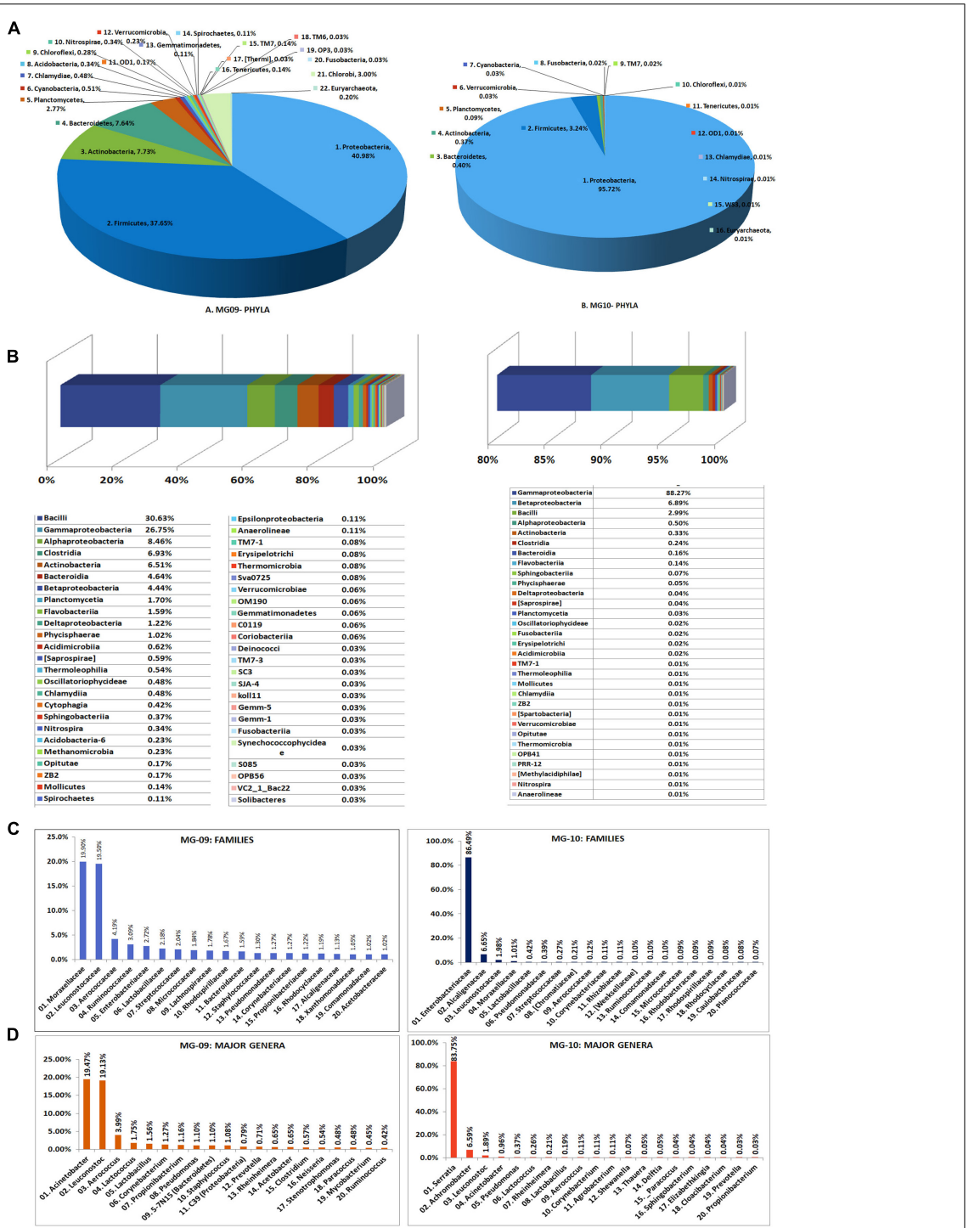


FIGURE 4 | Illumina MiSeq NGS-based bacterial diversity analysis on watermelon seed-embryos with DNA extracted using PowerFood microbial DNA isolation kit (MG09) or Axygen DNA isolation kit (MG10). Distribution of phylogenetic groups based on 16S rRNA gene V3–V4 region taxonomic profiling at **(A)** phyla and **(B)** class levels. **(C)** Major families. **(D)** Major genera.

'BB' occasionally showed a few white colonies from the thick homogenate applied spots of the original homogenate. Bright-field microscopy on seed and embryo homogenates, on the other hand, indicated abundant motile-bacterial cells which indicated that the CFU formed was far negligible relative to the cell numbers.

Three bacterial isolates were retrieved from 'RG' embryo-homogenate applied plates. Attempting 16S rRNA gene

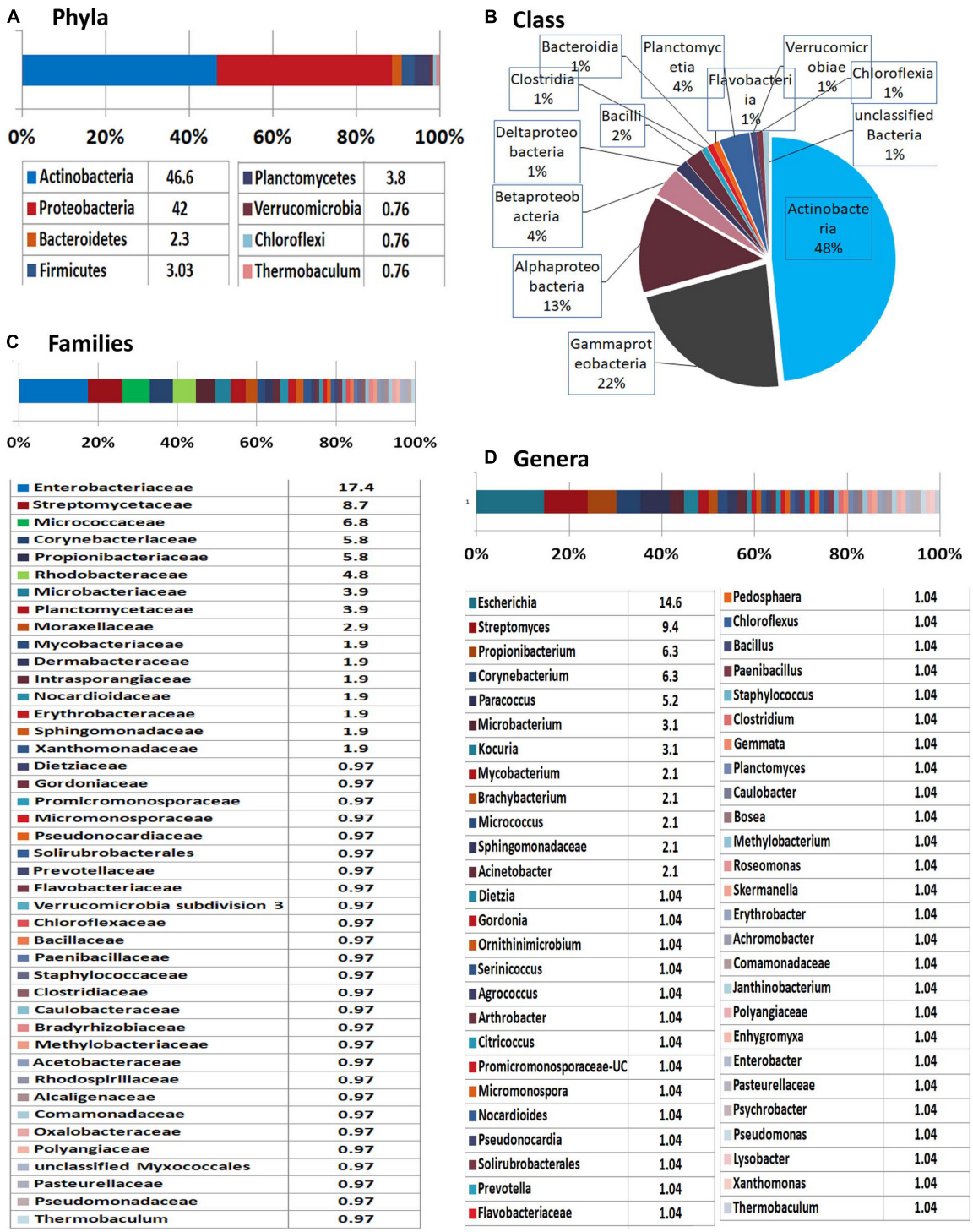


FIGURE 5 | Illumina HiSeq NGS based whole genome metagenomics on watermelon seed-embryos. MEGAN-based taxonomic profiling of bacterial microbiome at the **(A)** phyla, **(B)** class, **(C)** family, and **(D)** genera levels.

sequence-based bacterial identification, two isolates were established as *Roseomonas pecuniae* and *Kineococcus* sp. (Actinobacteria), while the third isolate turned out to be a mixture despite adopting three rounds of SP-SDS-mediated single-colony purification. All these isolates appeared short-lived failing to revive after 1-week storage under ambient-conditions or under refrigeration and, thus, could not be characterised. One isolate was obtained from

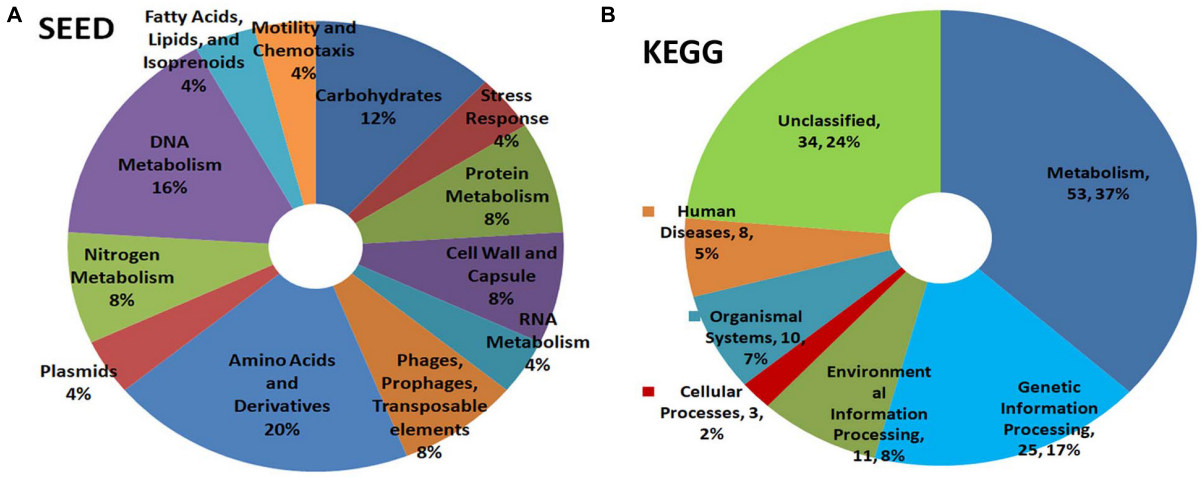


FIGURE 6 | Illumina HiSeq NGS based whole genome metagenomics on watermelon seed-embryo bacterial microbiome functional profiling as per **(A)** SEED and **(B)** KEGG analyses.

'BB' seed-embryo, which was identified as *Staphylococcus warneri* (Firmicutes).

16S V3–V4 Profiling of Seeds and Seed-Embryos in Grapevine

All the four samples, namely, 'RG' and 'BB' seeds and the excised seed-embryos, showed low DNA yields (3–5 ng μl^{-1}), but they gave rise to good amplicon libraries with 0.23–0.27 million reads for seed samples and 0.19–0.31 million reads for seed-embryos (**Supplementary Table 4**). A major share of these reads, however, corresponded to chloroplast and mitochondria for the seed-embryos (36%–50%), but this formed only a minor share for whole seeds (1.2%–1.5%). The seed samples exhibited about 1800 OTUs, while the seed embryos displayed 840 OTUs excluding the plant reads. More than 99% of the OTUs corresponded to Eubacteria with a minor share of Archaea.

The read/OTU distribution for seed tissues showed a high bacterial diversity with 28 phyla for 'RG' and 34 for 'BB' with a more or less identical pattern for both the cultivars with respect to major phyla. Proteobacteria constituted about 35.7% share followed by Firmicutes (23%), Bacteroidetes (18.7%), Actinobacteria (13.4%), and Fusobacteria (3.4%). Other constituents included Planctomycetes, Verrucomicrobia, SR1, TM7, Acidobacteria, Spirochaetes, OD1, Chloroflexi, Cyanobacteria, Nitrospirae, Tenericutes, and Chlorobi, to name some (**Figure 8A**). Seed-embryos also displayed a high bacterial diversity with 24 phyla for both 'RG' and 'BB' and identical distribution pattern in respect of major phyla in both instances. Seed samples showed 28 phyla common to both which constituted 99.9%–100% OTUs, whereas the two embryo samples exhibited 21 phyla common to both cultivars (99.8%–99.9% OTUs) out of a total of 26 phyla documented. The main differences between the seed and embryo samples included relatively more diversity in the whole seeds (35 phyla) than in the embryos (30) considering both the cultivars, and lower levels of Proteobacteria and Bacteroidetes (phylum level) for embryos than whole seeds. One Archaeal phylum (Crenarchaeota) was also documented across the four samples although in minor shares.

At the Class level, a high amount of endophytic bacterial diversity was documented with 108 constituents in seed samples and 71 for embryos, with 10–12 phyla accounting for nearly 95% OTUs. The major ones across the four tissue samples included γ-proteobacteria, Bacteroidia, Clostridia, Actinobacteria, Bacilli, α-proteobacteria, β-proteobacteria, Fusobacteriia, and Flavobacteria (data not shown). While the seeds harbored more of γ-Proteobacteria and α-Proteobacteria which probably arose from the seed-coat, seed-embryos showed more of Bacteroidia, Bacilli, and Fusobacteriia. Family level distribution endorsed the prevalence of a high amount of bacterial diversity spanning over 330 constituents across the four samples. Embryos showed a high share of Prevotellaceae (15%–16%) than seeds (10%–10.5%) followed by Nocardiaceae (9.3%–10.4%), Clostridiaceae, Pseudomonadaceae, Moraxellaceae, Neisseriaceae, Streptococcaceae, and others.

Genus level distribution brought to light an unprecedented taxonomic diversity prevailing in embryos to the tune of about 350 genera (293 in 'RG' and 279 in 'BB') and about 570 genera for whole seeds (443 in 'RG' and 465 in 'BB') (**Supplementary Dataset 3**). The embryos showed *Prevotella* as the most dominant constituent (15.1% in 'RG' and 16.1% in 'BB') followed by *Rhodococcus* (9.3%–9.7%), *Clostridium, Streptococcus, Neisseria, Fusobacterium, Pseudomonas, Porphyromonas, [Prevotella],* and *Lactobacillus*; the seeds showed similar amounts of *Prevotella* and *Rhodococcus* (10.0%–10.5%) followed by *Clostridium, Pseudomonas, Acinetobacter, Streptococcus, Neisseria, Sphingomonas, Fusobacterium, Porphyromonas,* and *Veillonella* (**Figure 8B**). Thus, a high diversity of seed-associated and vertically transmissible endophytic bacteria were documented but sharing similar taxonomic profiles for the two cultivars which were growing in two different parts of the world.

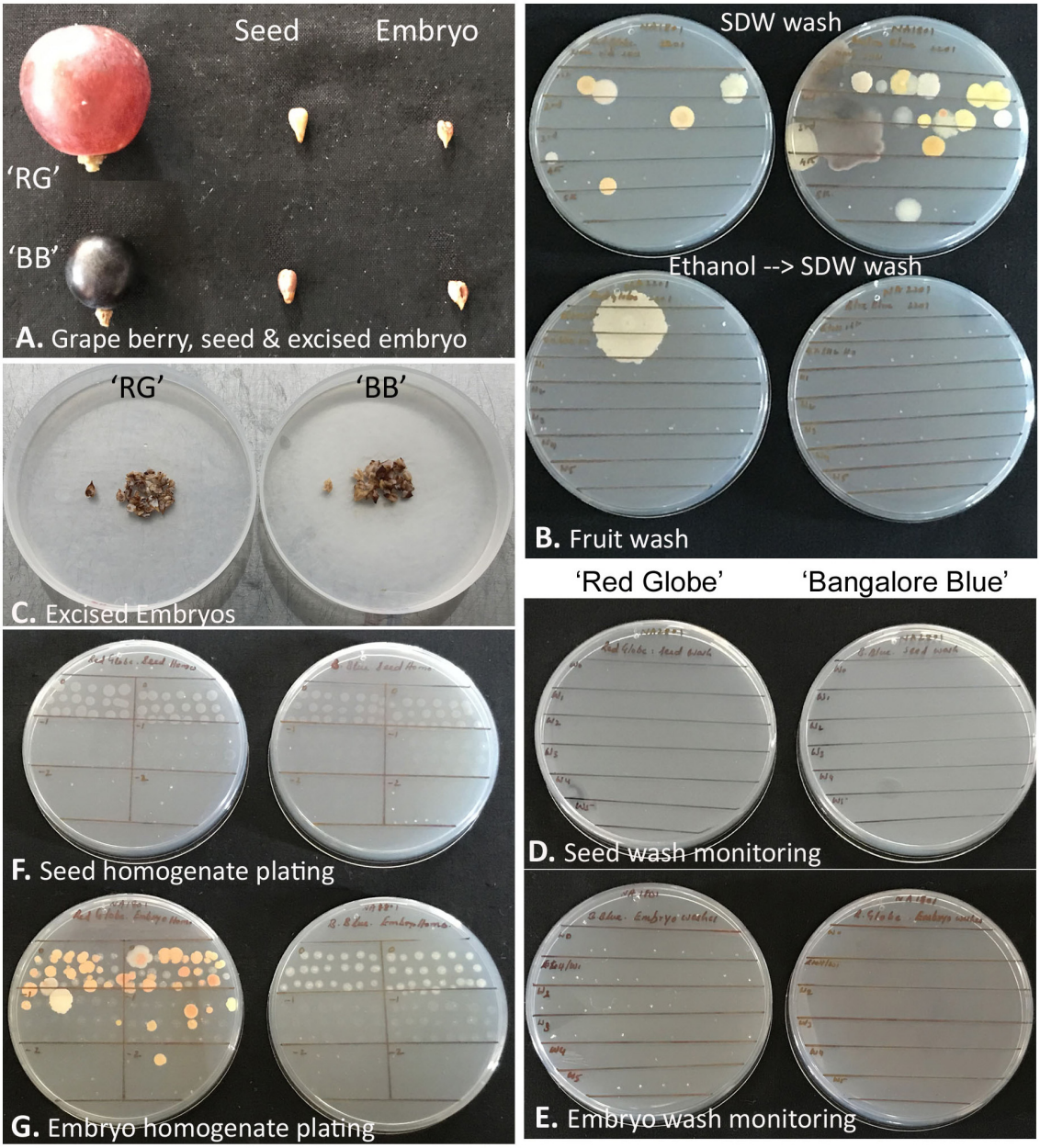
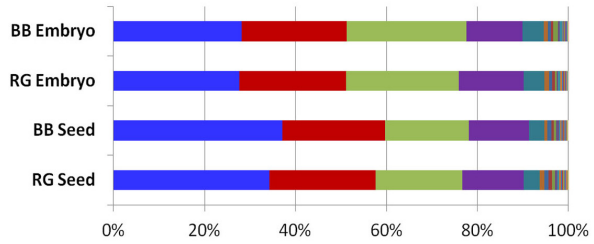
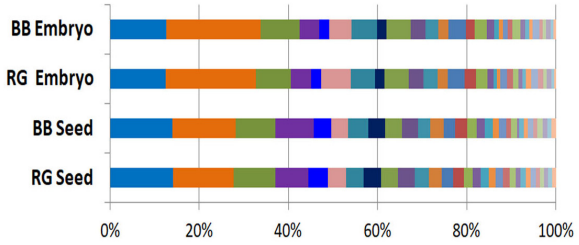


FIGURE 7 | Description of grapevine seed *versus* excised seed-embryo 16S rRNA V3–V4 amplicon-based taxonomic profiling. **(A)** Berry, seed, and excised seed-embryo in 'Red Globe' ('RG') and 'Bangalore Blue' ('BB'). **(B)** Monitoring the fruit wash solutions for the microbial load by spotting on nutrient agar, the upper panel showing results after six sterile distilled water (SDW) washes, and the lower panel showing the outcome after one ethanol wash followed by six FDW washes. **(C)** Excised embryos from the seeds of 'RG' and 'BB.' **(D)** Monitoring seed-wash solutions. **(E)** Monitoring embryo-wash solutions. **(F)** Testing the seed-tissue homogenate for cultivable bacteria through SP-SDS of serial dilutions with no bacterial CFU for 2–4 weeks. **(G)** Seed-embryo homogenate tested for cultivable bacteria through SP-SDS showing bacterial CFU after 2–4 weeks.

DISCUSSION

The study elucidates intracellular bacteria 'Cytobacts' as a ubiquitous phenomenon across vascular plants enduring perennially through embryo-mediated vertical transmission with considerable structural, functional, and genomic significance. As per the preexisting information, all plant species and plant organs are known to harbor endophytic bacteria, and substantial information has been emerging on the utility and application of such plant intimately associated microorganisms in crop husbandry toward plant growth promotion, bio-control of pathogens and pests, alleviation of abiotic stress, etc. (Hardoim et al., 2015; Liu et al., 2017; Araujo et al., 2020; Vandana et al., 2021). Initial studies on bacterial endophytes





	RG Seed	BB Seed	RG Embryo	BB Embryo
Proteobacteria	34.263	37.094	27.712	28.202
Firmicutes	23.399	22.652	23.376	23.097
Bacteroidetes	18.966	18.418	24.777	26.319
Actinobacteria	13.530	13.190	14.249	12.276
Fusobacteria	3.478	3.298	4.616	4.729
SR1	1.097	0.759	0.961	0.805
Planctomycetes	0.896	0.893	0.707	0.688
Verrucomicrobia	0.711	0.446	0.614	0.572
TM7	0.661	0.560	0.387	0.948
Acidobacteria	0.600	0.674	0.240	0.455
Spirochaetes	0.379	0.265	0.627	0.572
OD1	0.304	0.183	0.374	0.195
Chloroflexi	0.294	0.237	0.200	0.156
Cyanobacteria	0.258	0.260	0.213	0.078
Nitrospirae	0.256	0.187	0.147	0.208
Euryarchaeota	0.220	0.255	0.267	0.351
Tenericutes	0.164	0.083	0.040	0.091
Chlorobi	0.121	0.075	0.160	0.026
[Thermi]	0.091	0.013	0.040	0.000
AD3	0.081	0.091	0.053	0.026
Gemmatimonadetes	0.068	0.096	0.080	0.052
Elusimicrobia	0.037	0.012	0.000	0.000
Armatimonadetes	0.024	0.021	0.000	0.039
WPS-2	0.021	0.002	0.000	0.000
SBR1093	0.021	0.021	0.000	0.026
Synergistetes	0.013	0.028	0.067	0.000
OP3	0.013	0.030	0.000	0.026
NC10	0.013	0.001	0.000	0.000
WWE1	0.000	0.004	0.000	0.000
TM6	0.000	0.020	0.000	0.000
OP11	0.000	0.007	0.000	0.000
Lentisphaerae	0.000	0.052	0.027	0.026
GAL15	0.000	0.014	0.000	0.000
Fibrobacteres	0.000	0.012	0.000	0.000
Chlamydiae	0.000	0.000	0.027	0.000
Crenarchaeota	0.023	0.047	0.040	0.039

	RG Seed	BB Seed	RG Embryo	BB Embryo
01. Rhodococcus (Actinobacteria)	10.323	10.377	9.326	9.678
02. Prevotella (Bacteroidetes)	10.010	10.469	15.050	16.160
03. Clostridium (Firmicutes)	6.945	6.590	5.857	6.664
04. Pseudomonas (Proteobacteria)	5.433	6.383	3.376	3.274
05. Acinetobacter (Proteobacteria)	3.172	2.881	1.614	1.741
06. Streptococcus (Firmicutes)	2.971	2.829	5.030	3.806
07. Neisseria (Proteobacteria	2.957	3.316	4.016	4.404
08. Sphingomonas (Proteobacteria)	2.835	2.789	1.548	1.598
09. Fusobacterium (Fusobacteria)	2.821	2.844	4.003	4.157
10. Unclassified (Proteobacteria)	2.724	2.598	2.575	2.572
11. Porphyromonas (Bacteroidetes)	2.298	2.058	2.322	2.169
12. Veillonella (Firmicutes)	2.133	2.187	1.641	1.754
13. [Prevotella] (Bacteroidetes)	1.899	1.917	2.802	3.014
14. Haemophilus (Proteobacteria)	1.796	2.024	1.975	1.546
15. Lactobacillus (Firmicutes)	1.395	1.523	1.935	2.014
16. Unclassified (Firmicutes)	1.388	1.389	1.014	1.247
17. Unclassified (Proteobacteria)	1.270	1.383	0.494	0.831
18. Staphylococcus (Firmicutes)	1.201	0.921	0.600	0.714
19. Unclassified (Proteobacteria)	1.196	1.312	1.121	0.844
20. Unclassified (SR1)	1.097	0.759	0.961	0.805
21. Sediminibacterium (Bacteroidetes)	0.942	0.942	0.934	1.338
22. Bacteroides (Bacteroidetes)	0.864	0.365	0.654	0.779
23. Unclassified (Firmicutes)	0.858	0.777	0.587	0.676
24. Unclassified (Proteobacteria)	0.811	0.680	0.881	0.637
25. Enterococcus (Firmicutes)	0.799	0.916	1.201	1.156
26. Chryseobacterium (Bacteroidetes)	0.718	0.675	0.814	0.585
27. Unclassified (Proteobacteria)	0.692	0.878	0.600	0.611
28. Klebsiella (Proteobacteria)	0.675	0.780	0.761	0.701
29. Psychrobacter (Proteobacteria)	0.658	0.693	0.467	0.507
30. Unclassified (Proteobacteria)	0.581	0.709	0.294	0.416

FIGURE 8 | 16S rRNA metagene V3–V4 region profiling with Illumina MiSeq NGS platform on grapevine seeds and embryos. **(A)** Distribution of OTUs (%) under different phyla. **(B)** Major genera (OTU %) in the four samples.

had adopted cultivation-based approaches for assessing the organismal diversity and toward the exploitation of beneficial organisms in agriculture and allied sectors (Hallmann et al., 1997; Rosenblueth and Martínez-Romero, 2006). Such studies have often brought out notable bacterial diversity in root tissues. Conversely, the shoot portions were not investigated proportionately or often showed relatively low CFU and less taxonomic diversity. Cultivation-independent molecular studies such as metagenomics or 16S rRNA gene amplicon profiling have revealed high amounts of bacterial taxonomic and functional diversity for root tissues (Bulgarelli et al., 2012; Sessitsch et al., 2012). Similar studies targeting shoot tissues, although relatively limited, have given a similar picture revealing a vast bacterial diversity (Dos-Santos et al., 2017; Thomas and Sekhar, 2017).

While cultivation-based and molecular-based explorations bring out the diversity data, they do not provide information about the abundance of microorganisms and the niches of colonization which are attained through microscopy. Although a number of publications on endophytic microorganisms are available giving the impression about such associations as ubiquitous even to the extent of stating that microbe-free plants are rather impossible (Partida-Martinez and Heil, 2011; Heil, 2015), they do not make mention about visualizing them inside the tissues. Studies giving microscopic evidence on tissue colonization by bacterial endophytes have often encompassed on root tissues showing both intercellular and intracellular colonization (Compant et al., 2005, 2008; White et al., 2014, 2018; Shehata et al., 2017). Thus, the current understanding suggests bacterial entry through root hairs or other root openings, traversing the root cortex with intracellular accommodation, xylem immigration, and subsequent vascular transmittance (Compant et al., 2011, 2021; Prieto et al., 2011). Microscopic studies on shoot tissue colonization by bacterial endophytes have been very few considering the large volume of literature on endophytic bacteria, which often give the impression of intercellular colonization with occasional reference to intracellular presence (Compant et al., 2005, 2008, 2011). This statement does not cover the nitrogen fixing genera which show intracellular colonization in root nodules. The general understanding about endophytic bacterial association

in shoot tissues is that they are apoplastic or intercellular colonizers comprising of xylem, intercellular spaces, and vascular interconnections (Hallmann et al., 1997; Sattelmacher, 2001; Hardoim et al., 2015; Liu et al., 2017). On the other hand, some dedicated studies targeting the shoot system have shown intracellular colonization in field plants (Pirttilä et al., 2000; Thomas and Reddy, 2013; Koskimäki et al., 2015; Thomas et al., 2019) and also in tissue culture systems which are established from surface-sterilized tissues (de Almeida et al., 2009; Thomas and Sekhar, 2014; Esposito-Polesi et al., 2017).

Despite these established reports on intracellular bacterial colonization, which may constitute a negligible share considering the whole gamut of endophytic microbiome research, there seems to be not much research exploring deeper into tissue colonization aspects and clarifying the intercellular *versus* intracellular nature of associations which has significant implications for plant biology. A clearer picture on endophytic bacteria as intracellular inhabitants in healthy plant cells has emerged with the recent microscopic explorations of long-term actively maintained or freshly established, supposedly aseptic and axenic tissue cultures of grapevine, periwinkle, tobacco, *Arabidopsis*, and certain medicinal plant species. This showed the prevalence of an enormity of 'Cytobacts' in terms of both abundance and taxonomic diversity in cell and callus cultures (Thomas and Franco, 2021). The organisms proved to be largely uncultivable although a micro-share of such organisms could be activated to cultivation with specific treatments such as the use of host-tissue-extract in a low nutrient environment. *In vitro* activation of CREB is also a common feature in plant tissue cultures which generally emerge as microbial contaminations (Shaik and Thomas, 2019; Thomas et al., 2019). The present study brings to light 'Cytobacts' as a ubiquitous entity in vascular plants.

Intracellular presence of diverse 'Cytobacts' in shoot-tip tissues makes us infer that these organisms would move to the daughter cells during mitosis and thus reach all plant tissues. This also implies that there is possible gametic transmission through meiosis that brings in vertical movement to the next generation. This is evident from the molecular explorations reported in this study on watermelon and grape seed-embryos. Recent reports on endophytic bacteria in pollen grains (Maniranjan et al., 2017), excised seed-embryos in wheat (Kuźniar et al., 2020a,b), and in the *in vitro* raised tomato seedlings after seed-coat removal post-seed germination illustrate this significant result (Shaik and Thomas, 2019). Very recent studies employing excised seed-embryos, different parts of embryos, and embryo-derived *in vitro* seedlings of watermelon have clearly established vertical transmission of abundant and diverse bacteria prevailing as CREBs (Thomas and Sahu, 2021). Intracellular colonization facilitates the diverse organisms to reach the embryo and in turn all the cells of the zygote-derived new plant allowing a concoction of the organisms from either parent. Microbial association with the seed as determinants of seed quality, seedling vigor, and even in conferring disease resistance is greatly appreciated in plant biology (Nelson, 2018; Matsumoto et al., 2021). The observation on embryo-colonization by abundant and diverse bacteria adds a new insight in plant microbiology at variance from the general perception that endophytes are acquired by plants primarily from soil through roots, and from atmosphere through natural openings and other vectors (Frank et al., 2017; Kandel et al., 2017).

The endophytic bacterial diversity documented for shoot-tip tissues and seed-embryos in this study appeared quite vast, which included mainly Proteobacteria and varying shares of Firmicutes, Actinobacteria, and Bacteroidetes as the next major phyla and minor shares of other diverse phyla including several candidate phyla and some Euryarchaeota. It is to be borne in mind that orthodox seeds go through extreme drying which could affect the survival of the organisms. This necessitates the use of dried seeds to assess if the organisms survive the desiccation. Observations with watermelon seeds which were in storage for 6–12 months indicated the prevalence of high bacterial diversity as CREBs with the dominance of Firmicutes barring spore formers, with the activation of more organisms and a modified diversity profile in favor of Proteobacteria with seedling development (Thomas and Sahu, 2021). The extent of taxonomic diversity documented with the seed-embryos could be even more considering the effects due to the DNA extraction kit or the metagenomics approach on phylogenetic variability recorded in this study and in other reports. The bacterial diversity appeared to be masked initially with the predominance of chloroplast and mitochondrial sequences, removal of which helped in unraveling the phylogenetic variability as is the case in other cultivation-independent studies (Thomas and Sekhar, 2017; Thomas and Franco, 2021). Embryo excision from grape seeds warranted testa softening with concentrated alkali which additionally ensured the elimination of all seed external bacteria. It is worth noting that the two grape cultivars that were grown in two different continents shared identical taxonomic profiles for both seeds and seed-embryos. Seed/embryo colonization by endophytic bacteria has been demonstrated in *Vitis* sp. (Compant et al., 2011), *Cucumis melo* (Glassner et al., 2018), and in a few other instances (Berg and Raaijmakers, 2018; Nelson, 2018). The present study targeting embryo *per se* further strengthens such reports. A clear understanding of the cell constituents and their functioning is essential in plant cell biology. The elucidation of the ubiquitous existence of diverse microorganisms in live plant cells with their continuous vertical transmission opens up scope for further research on spin-off study areas in plant biology similar to the interdependent human- and animal-associated microbiota (Engel and Moran, 2013; Almeida et al., 2019). It might be possible to bring out more diversity adopting alternate methods to block the amplification of mitochondrial and plastid sequences, such as the design and the use of PNA PCR clamps (Lundberg et al., 2013; Lefèvre et al., 2020). This warrants a comparative study to assess if the two-step QIIME analysis adopted in this study, or the use of PNA clamps harnesses more taxonomic diversity.

It is indeed inexplicable as to how such ubiquitous intracellular bacterial association of a wide array of prokaryotes in live plant cells went unnoticed by plant biologists and microbiologists. The present 'discovery' was facilitated with follow-up research on intracellular micro-particle motility with live-cell imaging. It is possible that such observations in the past were overlooked,

possibly as Brownian motion (Brown, 1828), or micro-organelle movement, cytoplasmic streaming, etc., as discussed elsewhere (Thomas and Franco, 2021). This may also be confounded by the cultivation-recalcitrance of associated organisms. Large shares of environmental organisms are known to defy cultivation for lack of clear information about the cultivation requirements (Colwell, 2009) and on account of their smaller genomes (Kantor et al., 2013). Bacteria are known to occur in viable but non-culturable (VBNC) state and they could switch between VBNC and cultivable states, which is reported as a common feature with endophytic bacteria (Podolich et al., 2015; Thomas et al., 2019). It might not be an exaggeration to state that the CFU formed from the shoot-tip, seed- or embryo-homogenates during the cultivation-based part of this investigation constituted just one-millionth to one-billionth of motile micro-particles noticed in the microscopic fields that were construed as bacteria. It perhaps needs the intervention of researchers from molecular physics to unravel this mystery.

With their ubiquitous and integral cellular associations, 'Cytobacts' have significant functional roles in native plants such as improved plant fitness, growth promotion, activating the plant immune system, or governing the responses to biotic and abiotic stresses (Podolich et al., 2015; Thomas et al., 2017). PICRUSt functional analysis provided certain indications in regard to plant cell-energetics, plant defense, and phyto-physiology. Endophytic bacteria have been alluded to be involved in plant growth promotion, plant defense, biocontrol of pathogens/pests, bioremediation, and as sources of novel biomolecules (Conn et al., 2008; Hardoim et al., 2015; Podolich et al., 2015; Liu et al., 2017) and possibly as the plant immune system (Thomas et al., 2017). Several metabolic pathways that are observed in plants could be aided by these microorganisms (Holland, 1997; Thomas and Franco, 2021). It was also significant to document a notable share of Archaea in the shoot-tip tissues of field plants, particularly for maize and tomato under the phylum Euryarchaeota and the class Methanobacteria. Archaea has also been documented in seed-embryos of watermelon and grapes albeit in minor shares, but a more significant share (5%) was documented earlier with tomato seeds (Thomas and Shaik, 2020). Archaeome is now receiving increasing attention as plant and seed associated beneficial organisms (Taffner et al., 2018; Wassermann et al., 2019).

The endophytic microbiome within the plant holobiome in a holistic consideration of the plant and of hologenome in plant breeding and functional hologenomics is gaining significance now (Vandenkoornhuyse et al., 2015; Khare et al., 2018). Next-generation plant breeding strategies could be significantly linked to microbiome selection (Gopal and Gupta, 2016). Pollen-mediated microbial integration (Mitter et al., 2017) and seed-microbiome-targeted progeny selections (Matsumoto et al., 2021) are emerging plant breeding strategies. The endophytic microbiome perhaps needs to be viewed as a complex plant trait (Wagner et al., 2016). The unearthing of the ubiquitous existence of abundant and diverse bacteria in field plants and the long-term actively maintained live cell cultures (Thomas and Franco, 2021) with their integral and vertical transmission across generations has far-reaching implications opening the gateway for research on various interrelated aspects. Their functional roles in plants need more discussion as to how plants have accommodated such a huge variety of endophytic bacteria in the different phenology of plants. The observations also assume significance in environmental microbiology since the organisms invariably return to the environment at the termination of the life-term of plants/tissues.

The high amounts of taxonomic diversity and the organismal abundance also assume significance in genomics considering the possibility of co-extraction of microbial DNA with the plant DNA. For instance, 500 bacterial species with an average genome size of 5 Mb could constitute about 2,500 Mb genome data compared with a plant genome of ∼500 Mb (Thomas et al., 2017). However, the possibility of contamination of host genetic material by bacterial DNA appeared low considering the poor DNA recovery from bacterial cells. Further, a high share of 16S rRNA sequences corresponded to plant sequences as documented in other studies (Thomas et al., 2017, 2019). Such bacterial sequences are easily removed with bioinformatics tools during genome sequencing projects. However, it is not possible to exclude or ignore the functional contributions of these organisms in native plants and the *in vitro* cultures.

The current observations also assume evolutionary significance in the context of serial endosymbiont theory (Margulis and Bermudes, 1985; Margulis, 2004), reviewed in detail by Martin et al. (2015). Plastids and mitochondria are known to be of prokaryotic origin. The high share of 16S rRNA amplicon reads that matched Cyanobacteria at the phylum level (chloroplasts at the class level) and Proteobacteria at the phylum level (Rickettsiales at the order level and mitochondria at the family level) was a common observation for most of the plant samples. During the course of evolution, the functional autotrophic plant cells possibly emerged by the integration of free-living bacteria as mitochondria and photosynthetic cyanobacteria as chloroplasts that somehow survived the endocytosis into the cytoplasm in an amoeba-like eukaryotic protist, losing their independent nature and becoming cell organelles during the course of time. Chloroplasts and mitochondria are double-walled, containing their own circular DNA, as in the case of bacteria, along with their own transcriptional and translational machinery and self-replication. The long-term association of diverse organisms in the cytoplasmic niche could possibly pave the way for future plant evolutions.

This study targeted mainly Phanerogamae covering angiosperms and one representative of gymnosperms, namely, pine. Preliminary microscopic observations on members of Cryptogamae such as Thallophytes (algae, fungi), Bryophytes (mosses), and Pteridophytes (ferns) through bright-field microscopy showed abundant intracellular bacteria suggesting 'Cytobacts' as a ubiquitous phenomenon in the plant kingdom. Fungal–bacterial endosymbiosis has been well studied with different systems such as *Burkholderia* sp. and *Rhizopus microspores*, *Nostoc punctiforme*, and *Geosiphon pyriforme*, etc., which share a symbiotic relationship (Pawlowska et al., 2018; Bastías et al., 2020). Bacterial endosymbionts associated with insects has been well documented

and has been a topic of extensive research where the associations ranged from obligate mutualism to facultative parasitism with a large taxonomic diversity (Moran and Baumann, 2000; Kikuchi, 2009). The term Cytobacts was coined to describe the abundant cytoplasmic bacteria first documented in banana (Thomas and Sekhar, 2014). Subsequent molecular diversity analyses indicated that the population could include Archaea as well. It warrants further microscopic explorations targeting Archaea to verify if they also are cytoplasmic inhabitants.

In conclusion, all plants and plant organs ubiquitously harbor endophytic bacteria, and the microscopic observations in this study on shoot-tip tissues and the mature seed-embryos leads to the conclusion that all vascular plants bear abundant and diverse intracellular bacteria, and their numbers far exceed the amount of host cells considering that each host cell harbors an abundant amount of Cytobacts. The observations on shoot tissue indicated that Cytobacts could be distributed to all daughter cells through mitosis and to the gametes through meiosis on account of their presence in the cytoplasm. Observations on mature seed embryos of representative plants indicate that the organisms are transmitted vertically to the next generation with a high amount of taxonomic diversity. This way, the endophytic bacterial continuity is maintained in seed-propagated as well as clonally perpetuated plants, making 'Cytobacts' an integral part of plant cell biology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/ (SAMN18092256, SAMN18092258, SAMN18092402, SAMN18092404, SAMN12726312, SAMN12726313, SAMN17848617, SAMN17848618, SAMN17848891, and SAMN17848989).

AUTHOR CONTRIBUTIONS

PT and CF conceived the idea and co-wrote the manuscript. PT undertook the microscopic and genomics studies in discussion with TR. TR critically edited the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.806222/full#supplementary-material

Supplementary Figure 1 | Fresh tissue sections of tender shoot or petiole tissue from different plant species under bright-field (1000×). These tissue sections show no obvious intercellular spaces, or limited space between cells at the junction of multiple cells, where no bacterial cells are generally observed, but display abundant bacteria in the cytoplasmic niche. Bacterial cells (indicated by arrow-head) are better viewed with zoom-out option. **(A)** Banana corm tissue. **(B)** Banana pseudostem tissue. **(C)** Papaya flower stalk. **(D)** Okra petiole tissue. **(E)** Hibiscus petiole tissue. **(F)** Hydrangea petiole tissue.

Supplementary Figure 2 | A view of watermelon fruit, seeds, and seed-embryos used for embryo-microbiome studies. **(A)** Cut fruit used as source of seeds. **(B)** Aseptically gathered seeds. **(C)** De-coated seeds. **(D)** Seed-embryos after surface sterilization.

Supplementary Figure 3 | A view of the cultivation-based assessment of watermelon seed-embryos for endophytic bacteria by plating the embryo-homogenate at different concentrations documented after 2–3 weeks of plating. **(A)** Original homogenate at the rate of one seed-embryo of about 25 mg in 1 ml sterile water, **(B)** after 1:10 dilution, and **(C)** after 1:100 dilution. No cultivable bacteria were observed in most instances except for isolated cases 1–2 weeks after the plating.

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Symbiotic Modulation as a Driver of Niche Expansion of Coastal Plants in the San Juan Archipelago of Washington State

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Modern evolutionary theory and population genetics posit that adaptation and habitat expansion of plants result from processes exclusive to their genomes. Here, we present studies showing that plants can grow across complex habitat gradients by modulating symbiotic associations with Class 2 fungal endophytes. Endophyte analysis of three native (*Leymus mollis, Distichlis spicata, and Salicornia pacifica*) and one invasive (*Spartina anglica*) plant growing across adjacent microhabitats in the San Juan Archipelago altered associations with Class 2 fungal endophytes in response to soil salinity levels. At the microhabitat interfaces where the gradation of salinity varied, the plants were colonized by endophytes from both microhabitats. A reciprocal transplant study along a salt gradient demonstrated that *Leymus mollis* (dunegrass) required endophytes indigenous to each microhabitat for optimal fitness and/or survival. In contrast, when dunegrass and *Grindelia integrifolia* (gumweed) were found growing in low salinity, but high drought habitats, these plant species had their own unique dominant endophyte association regardless of geographic proximity and conferred drought but not high salt stress tolerance. Modulation of endophyte abundance occurred *in planta* based on the ability of the symbiont to confer tolerance to the stress imposed on plants. The ability of an endophyte to confer appropriate stress tolerance resulted in a significant increase of *in planta* fungal abundance. Conversely, the inability of an endophyte to confer stress tolerance resulted in a decrease of *in planta* fungal abundance. Our studies indicate that Class 2 fungal endophytes can provide a symbiotic mechanism for niche expansion and phenotypic plasticity across environmental gradients.

Keywords: fungal endophytes, stress tolerance, symbiosis, plant-fungal interactions, Class 2 endophytes

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INTRODUCTION

The geographic pattern and distribution of plants across complex habitats have been extensively studied and well-documented (Martyn, 1729; Bradshaw, 1965; Crisci, 2001). The cellular and mechanistic processes responsible for the adaptive potential and phenotypic plasticity of plants are still largely undefined but thought to involve processes exclusive to the plant's genome and considered as the primary factor responsible for plant distribution patterns and biogeography

(Chevin et al., 2010; Matesanz et al., 2010; Nicotra et al., 2010; Zhang et al., 2013; Gratani, 2014; Zhou et al., 2019; Liu et al., 2020; Monforte, 2020; Abady et al., 2021; Klupczyńska and Ratajczak, 2021; Stotz et al., 2021; Syngelaki et al., 2021; Yang et al., 2021; Yu et al., 2021; Wang et al., 2022). For example, some plants are adapted to the presence of selenium enabling them to grow in soils with high concentrations of the element that limit the distribution of the plant's competitors (El Mehdawi et al., 2011a,b). The biochemical basis for this adaptation is thought to involve particular proteins involved in sulfur and selenium uptake and transport, coded by genes such as SHST1, SHST2, and SHST3 (Terry et al., 2000). Since these genes are found in the plant's nuclear genome, their expression is presumed to be responsible for the adaptation. However, the first report of habitat-adapted symbiosis 20 years ago revealed that plant adaptation in high-stress habitats (Redman et al., 2002b) can occur on an intergenomic level via symbiosis with Class 2 fungal endophytes (Rodriguez et al., 2009; Goh et al., 2013). Class 2 endophytes can be transmitted vertically or horizontally, confer habitat-specific stress tolerances, can grow asymptomatically throughout the plant vegetative tissue from roots to leaves and seed coats, but not the embryo seed, and can have specific and profound effects on plant physiology (Redman et al., 2002a). For example, plants growing in geothermal soils are symbiotic with fungal endophytes that adapt the plants to heat stress but the same endophytic species growing in plants from temperate soils do not adapt plants to heat stress (Rodriguez et al., 2008). Studies have also demonstrated that without the appropriate fungal endophyte, plants are unable to compete and/or survive in stressful habitats to which they appear adapted, irrespective of the stress (Miglia et al., 2007; Kim et al., 2008; Rodriguez et al., 2008).

Over the last several decades, there have been numerous studies assessing the potential of fungal endophytes for conferring stress tolerance to plants (Redman et al., 2001; Hamilton and Bauerle, 2012; Ravindran et al., 2012; Azad and Kaminskyj, 2015; Dastogeer, 2018; Giauque et al., 2019; González-Teuber et al., 2019; Gonzalez Mateu et al., 2020; Kaur, 2020; Morsy et al., 2020). It is now clear that the ecology and adaptive potential of plants is driven, at least in part, by microbial symbionts. This is best represented by the symbiotic dynamics observed in plants growing across environmental gradients (Maciá-Vicente et al., 2012; Ranelli et al., 2015; Glynou et al., 2016; Hammami et al., 2016; Kia et al., 2018).

In plant ecology, the mechanisms responsible for the ability of plants to grow across microhabitats imposing different abiotic stresses (niche expansion) have yet to be elucidated. We hypothesized that niche expansion required either that plants associate with: (1) individual fungal endophytes that adapt to edaphic differences between adjacent microhabitats and confer appropriate stress tolerances, or (2) different endophytes specific to each microhabitat. To test these hypotheses, we assessed the symbiotic dynamics and ecological significance of Class 2 endophytes in native plant species growing across salinity gradients and in uniform low salt habitats on two islands in the San Juan Archipelago of Washington State, USA. Here, we describe a newly observed ecological phenomenon that provides a symbiotic mechanism for niche expansion and distribution of plants across complex landscapes.

MATERIALS AND METHODS

Habitat Locations and Descriptions

Five native and one invasive (*Spartina anglica*) coastal plant were collected from several locations of Shaw, Waldron, and Camano islands in the San Juan Island Archipelago, WA. Plant species were found growing along costal beach [*Salicornia pacifica* (pickleweed), *Leymus mollis* (dunegrass), *Distichlis spicata* (saltgrass), *Spartina anglica* (spartina)] and rocky cliff [*Grindelia integrifolia* (gumweed)] habitats exposed to either low or high salinity and drought conditions during summer months. Beach substrate ranged from dirt and sand to cobble and salt gradients driven by inundation from high tides and salt spray. Prior to plant sampling, the relative salinity levels surrounding the roots of plants were measured and recorded using a Stevens HydraProbe (USA) conductivity meter to delineate salt gradients. The experimental field site was located at the University of Washington's Cedar Rocks Biological Preserve on Shaw Island. At one location, dune grass had colonized beach cobble and grew up a slope (10 m) and began colonizing an upper grassland meadow (**Figure 1**). Slope and meadow substrate consisted of loamy soil, and the beach was cobble ranging in size from 1 cm to 3 cm.

Endophyte Isolation

The plant species analyzed in this study and collection locations are listed in **Table 1**. Plants were washed to remove soil debris and any dead or decaying pant matter, placed into plastic ziplock bags, and surface-sterilized as previously described (Redman et al., 2001, 2002a). Using aseptic technique, roots, rhizomes, stems, and seed coats were separated, plated on fungal growth media (see below), and incubated at ambient temperature for 10–14 days under cool fluorescent lights until fungi emerged. Dominant endophytes were defined based on the number of fungi that emerged from surface-sterilized plant tissue sections. Endophytes found in >70% of plant samples and represented 70% of all fungi isolated/plant species/microhabitat were classified as the dominant endophytes (**Figure 1**, **Tables 1**–**4**). Ten representative isolates of the dominant fungal endophytes from each plant species were sub-cultured and single spore derived cultures generated (Redman et al., 1999). Agar plugs of mycelia from each culture were placed under sterile water supplemented with 50–100 ug/ml of ampicillin in sterile 1.5-ml screw-cap tubes and stored at 4°C for long-term storage. Taxonomic identification of the isolates is described below. The effectiveness of surface sterilization was verified using the imprint technique (Schulz et al., 1999).

Fungal Cultures

Fungal species were cultured on 0.1x potato dextrose agar (PDA) medium, supplemented with 50–100 ug/ml of ampicillin, tetracycline, and streptomycin, and grown at 22–25°C with a 12-hr cool fluorescent light regime. After 5–14 days of growth, conidia were harvested from plates by adding 10 ml of sterile

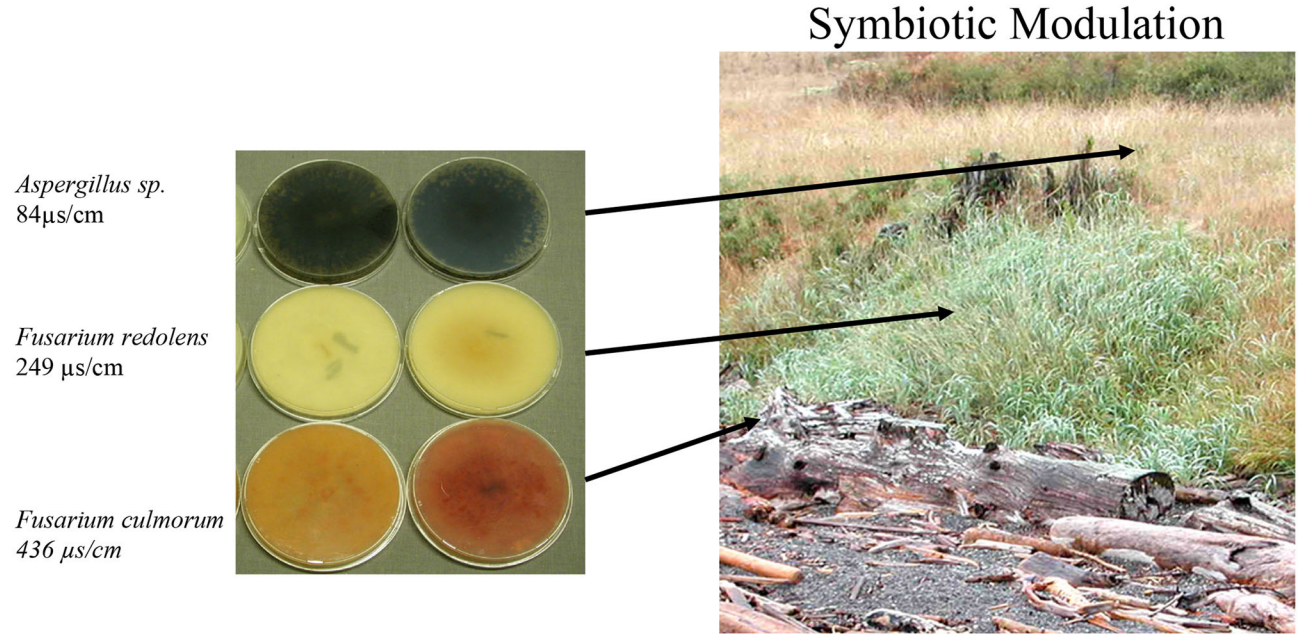

FIGURE 1 | Endophyte distribution across a salt gradient. Endophytes were isolated from plants ($N = 30$/location) collected from beach, slope, and upper meadow habitats within a contiguous population of *Leymus mollis* (dunegrass). Fungal growth media plates (0.1x PDA) on the left represent the dominant endophyte (<70%) identified from plants in each location. Soil conductivity measurements were taken and the highest salinity levels found to occur on the beach, moderate levels on the slope, and low salinity levels in the upper meadow.

water, gently scraping off spores with a sterile glass slide, and filtered through four layers of sterile cotton cheesecloth gauze. Spore concentrations were adjusted to 10^2-10^5 spores/ml with sterile water.

Fungal Identification

The 10 single-spored isolates representing each dominant endophyte were identified using conidiophore and conidial morphology (Arx, 1981; Barnett and Hunter, 1998; Leslie and Summerell, 2005). DNA was isolated from three isolates of each morphotype for sequence analysis of the variable ITS1 and ITS2 sequences of rDNA [ITS4 (5'-tcctccgcttattgatatgc-3')/ITS5 (5'-ggaagtaaaagtcgtaacaagg-3') primers] and translation elongation factor [EF1T (5'-atgggtaaggaggacaagac-3')/EF2T (5'-ggaagtaccagtgatcatgtt-3') primers] (White et al., 1990; O'Donnell et al., 2000). DNA was extracted from mycelia and PCR amplified as previously described (Rodriguez and Yoder, 1991; Rodriguez, 1993). Purification and sequencing of PCR products were performed at the High-Throughput Genomics Unit, Department of Genome Sciences, University of Washington. Sequences were compared using Sequencher and BLAST searched against the GenBank database. Although genus designations were possible for all endophytes analyzed, species designations could not be made for all isolates.

Plant Colonization

Seeds of *L. mollis* (dunegrass) obtained from their native habitats were surface-sterilized in 0.5–1.0% (v/v) sodium hypochlorite for 15–20 min with moderate agitation and rinsed with 10–20 volumes of sterile distilled water and seeds allowed to air-dry under sterile conditions. Dunegrass seeds were germinated on 1% agar medium supplemented with 1x Hoagland's solution or 0.1x PDA medium and maintained at 22–25°C and exposed to a 12-hr fluorescent light regime. To ensure that our studies began only with uninoculated (mock-inoculated with sterile water, no fungal endophytes) plants, seedlings that showed no outgrowth of fungi into the surrounding media were chosen for field and laboratory salt stress studies (see below).

Lolium perenne (perennial ryegrass) seeds commonly used in the Pacific Northwest were commercially purchased (Seed Factory NW, Puyallup, WA, USA), processed in a similar manner as described above for dunegrass (see above), and used for laboratory drought stress studies (see below). However, due to the small size of perennial ryegrass seeds and the high seeding rates required, a random selection of 100 seeds was chosen from the sterilized batch of seeds and plated onto fungal growth media in a similar manner as the dunegrass seeds (described above) to ensure seeds were uninoculated.

Salt Stress

Propagation for Field Studies

Dunegrass seeds were transplanted into cell packs containing soil (Sunshine Mix #4). Plants were watered by filling the lower tray with 1x Hoagland's solution supplemented with 5 mM $CaCl_2$. After 2 weeks, plants were either mock-inoculated with water (uninoculated) or inoculated with *Fusarium* or *Alternaria* species by pipetting 1 ml of spores (10^4-10^5/ml) at the base of the stems. Plants were grown under a 12-hr light regime at 25°C for 2 weeks

TABLE 1 | Dominant endophytes from plants growing across salinity gradients.

Plant species and location	Soil conductivity*	Endophyte morphotype**	Salt stress category***	Endophyte identity****
Pickleweed (Salicornia pacifica), Picnic Cove, Shaw Island				
Low Tide Zone	2,750 uS/cm	A	Extreme	*Phoma* sp.
Mid Tide Zone	1,282 uS/cm	A/B	High	*Phoma* sp./*F. culmorum*
High Tide Zone	701 uS/cm	B	Moderate	*F. culmorum*
Dunegrass (*Leymus mollis*), Cedar Rocks, Shaw Island				
Beach	436 μS/cm	B	Moderate	*F. culmorum*
Slope	249 μS/cm	C	Low	*Fusarium redolens*
Upper Meadow	84 μS/cm	D	Low	*Alternaria* sp.
Dunegrass (*Leymus mollis*), Cowlitz Bay, Waldron Island				
Beach	364 μS/cm	B	Moderate	*F. culmorum*
Slope	32 μS/cm	E	Low	*Fusarium* sp. *1*
Upper	43 μS/cm	F	Low	*Fusarium* sp. *2*
Saltgrass (*Distichlis spicata*), Cowlitz Bay, Waldron Island				
Low Tide Zone	23,70 μS/cm	A	Extreme	*Phoma* sp.
Mid Tide Zone	1,801 μS/cm	A/B	High	*Phoma* sp./*F. culmorum*
High Tide Zone	702 μS/cm	B	Moderate/High	*Fusarium culmorum*
Spartina (*Spartina anglica*), Camano Island				
Tidal Channel	4,000 μS/cm	A	Extreme	*Phoma* sp.
Slope	3,628 μS/cm	A/B	Extreme	*Phoma* sp./*F. culmorum*
Mud Flat	1,600 μS/cm	B	Moderate/High	*F. culmorum*

Salinity measurements were taken using a conductivity meter from soils in a central point where plants grew in abundance (*) and 10–30 plants were collected from each population for endophyte analysis. Endophytes were first placed into morphotype groups after microscopic analysis (**). Deionized water equates to 1.0 μS/cm, rainwater average is 50–100 μS/cm, and seawater is approximately 50,000 μS/cm. Salt stress habitats were categorized as extreme at conductivity reads <2,500 μS/cm, high at 700–2,499 μS/cm, moderate at 200–699 μS/cm, and low at a range of 0–199 μS/cm (***). Isolates were then further identified using DNA sequence analysis (****).

TABLE 2 | Reciprocal transplant study on Cedar Rocks Preserve.

	Beach habitat**		Slope habitat**		Meadow habitat**	
Treatment*	**#Plants**	**Biomass**	**#Plants**	**Biomass**	**#Plants**	**Biomass**
Uninoculated control	7/20	10.2 ± 0.33^{d}	20/20	9.2 ± 0.68^{c}	11/20	11.1 ± 0.41^{c}
Beach endophyte *F. culmorum*	**20/20**[a]	**19.9 ± 1.21**[a]	18/20	15.1 ± 072^{b}	15/20	11.4 ± 0.74^{c}
Slope endophyte *F. redolens*	16/20	17.0 ± 0.61^{b}	**20/20**	**18.9 ± 1.67**[a]	18/20	13.0 ± 016^{b}
Meadow endophyte *Alternaria sp.*	12/20	13.3 ± 1.06^{c}	18/20	15.0 ± 0.67^{b}	**20/20**	**16.9 ± 0.84**[a]

Seeds isolated from dunegrass plants growing across a salinity gradient were used to generate dunegrass plants under laboratory and greenhouse conditions that were either used as uninoculated controls or colonized with endophytes from the beach, slope, or upper meadow habitats (*). After 4 months of growth, 20 dunegrass plants for each treatment were transplanted into each of the habitats and allowed to establish for 3 months prior to assessment of plant survival, wet biomass, and endophyte profiles. The number of surviving plants at the end of the study/number of plants at the beginning of the study (**). Biomass is the mean number of wet grams/surviving plants at the end of the study ± standard deviation. Data were analyzed by Duncan's multiple range test ($P < 0.0001$). Means with the same superscripted letters (a,b,c,d) are not statistically different. Numbers in bold represent plants colonized with the endophyte indigenous to the specific niche.

and transferred to a cold frame greenhouse exposed to ambient temperature and light for 3 months prior to transplanting at field sites in early summer (May 2005) (**Figure 1**). A replicate set of one-month-old plants (20/treatment) were processed for endophyte analysis to ensure that control plants were free of fungi and that symbiotic plants contained the appropriate endophyte [*Fusarium culmorum, Fusarium redolens,* or *Alternaria sp.* (see **Figure 1**)]. Plants colonized with each of the three endophytes were transplanted into the beach, slope, and meadow habitats located at the University of Washington's Cedar Rocks Preserve (Shaw Island, WA). Three months after transplanting, plants were removed with root systems intact and transported back to the laboratory to assess plant viability and biomass (described below), and Class 2 fungal colonization (described above) (**Figure 1**, **Table 2**).

Laboratory Salt Stress Studies

Experiments were performed with dunegrass plants grown in double-decker magenta boxes (Rodriguez et al., 2008) containing sand as the growth matrix and kept at 25–28°C with a 16-hr fluorescent light regime (**Figure 2**). Magenta boxes were randomly placed in different locations on shelves, and each

TABLE 3 | Plant metrics and endophyte function across a salt gradient on Cedar Rocks Preserve.

Microhabitat	Endophyte ID*	Plant size**	Inflorescences**	Field site soil conductivity***
Beach	*F. culmorum*	6"-2'	0%	436 μS/cm
Slope	*F. redolens*	2'-4'	70–80%	249 μS/cm
Meadow	*Alternaria sp.*	2'-4'	40–50%	84 μS/cm

*Endophytes were identified using microscopic and ITS sequences analysis.
**Plant heights and %inflorescences were determined in three random sites 3' in diameter for each microhabitat.
***Conductivity of microhabitats was taken in a central point in each microhabitat.

TABLE 4 | Plant endophyte analysis in low salt (dunegrass) and high drought (gumweed) stress habitats at Cedar Rocks Preserve.

	Plant species	Sampling site	Endophyte ID*	% In plant tissues**	GPS Coordinate range***
Leymus mollis	x4 costal beach	*Cladosporium*	Roots/Stems	80%	48.54939 N,−122.95419 W
Dunegrass (N = 30)		*cladosporioides*	Seed Coat	70%	48.56431N,−122.93583 W
Grindellia integrifolia	x4 costal cliff	*Alternaria*	Roots/Stems	93%	48.54997 N,−122.95584 W
Gumweed (N = 42)		*alternata*	Seed Coat	95%	48.55006 N,−122.95603 W

*Dominant endophyte analyzed by classic microbiological and DNA sequence analysis.
**N = 30 dunegrass plants and 576 seed coats, and N = 42 gumweed plants and 181 seed coats were analyzed for the presence of endophytes.
***Plants with seed heads (approx. 70% of plants had inflorescence) were collected along a linear gradient encompassing the GPS coordinates.

experiment was repeated three times. Magenta boxes contained five dunegrass plants, and there were five replications for each treatment. Two-week-old dunegrass plants were mock-inoculated or symbiotically colonized in the same manner as described above with the three endophytes (*F. culmorum, F. redolens, and Alternaria sp.*) from the Cedar Rocks Preserve site. One-month-old plants were exposed to either no salt (1x Hoagland's solution supplemented with 5 mM $CaCl_2$) or salt stress (1x Hoagland's solution supplemented with 5 mM $CaCl_2$ and 300 mM NaCl) for 2 weeks by filling the lower chamber of the double-decker magenta boxes with 200 ml of one of these solutions. After plants were showing symptoms (i.e., uninoculated plants dead or severely wilted) approximately 2 weeks later, plants were re-hydrated in sterile water devoid of NaCl for 3–4 days, plant health assessed, and plants photographed (**Figure 3**). Plant health was assessed on a scale of 1–5 (1 = dead, 2 = severely wilted and chlorotic, 3 = wilted ± chlorosis, 4 = slightly wilted, and 5 = healthy w/o lesions or wilting). All assays were repeated a minimum of three times.

Drought Stress

Laboratory Studies

Experiments were performed with perennial ryegrass plants grown in double-decker magenta boxes using sand as the growth matrix and incubated at 25–28°C with a 16-hr fluorescent light regime. Magenta boxes were randomly placed in different locations on shelves, and each experiment was repeated three times. Seven-day-old ryegrass seeds were either mock-inoculated with water (uninoculated) or inoculated with 16 spores/seed by vortexing seeds in a sterile 50-ml tube and pipetting the appropriate volume of diluted spores onto seeds. Each magenta box was seeded with 545 turfgrass seeds (1 gram), and there were four replications for each treatment. Control plants were hydrated throughout the experiment with sterile water or 1x Hoagland's solution supplemented with 5 mM $CaCl_2$. Ryegrass plants were colonized with two endophytes. Both endophytes were isolated from plants growing in low salt but high drought stress sites. The first endophyte was *Cladosporium cladosporioides* from costal beach dunegrass plants, and the second endophyte was *Alternaria alternata* from gumweed plants growing on the costal rocky cliff habitats of the Cedar Rocks Preserve site (**Table 4**). Plants in magenta boxes (x8 boxes/treatment) were allowed to grow for 12 days, and the lower chamber fluid was removed from x4 boxes/treatment to begin drought conditions for 9 days; then, 200 mls fluid was added back to the lower chambers of all treatments and plants were allowed to rehydrate for 7 days. Twenty-eight-day-old ryegrass plants were photographed, shoots were harvested, and wet biomass was measured (**Figure 3**). All assays were repeated a minimum of three times.

Colony-Forming Units (CFU) to Assess in Planta Modulation

Symbiotic dunegrass plants were generated in double-decker magenta boxes in the same manner as described above. There were five dunegrass plants/magenta box and three magenta boxes per symbiotic treatments. Two-week-old plants were inoculated with *Fusarium culmorum* beach (imparts habitat-adapted salt tolerance) or *Fusarium culmorum* ATCC (from nonsalt habitat and does not impart salt stress) isolates by pipetting 1 ml of spores (10^4/ml) at the base of the stems to generate symbiotic plants (**Figure 4**). One-month-old plants were exposed to 0 mM and 300 mM NaCl in 1x Hoagland's solution supplemented

Figure 2: Plant health rating and biomass of laboratory grown *Leymus mollis* (dunegrass) in the absence and presence of salt stress

	Without salt stress (0 mM NaCl)		With salt stress (300 mM NaCl)	
Treatment	**Plant health***	**Biomass (g)****	**Plant health ***	**Biomass (g)****
Non-inoculated	5	3.81±0.40	1.5	2.20±0.29
Beach endophyte (*F. culmorum*)	5	3.85±0.18 (P=0.874)	4.5	3.68±0.36 (P=0.0003)
Slope endophyte (*F. redolens*)	5	3.92±0.46 (P=0.727)	2.5	2.96±0.22 (P=0.004)
Upper grassland endophyte (*Alternaria sp.*)	5	3.57±0.18 (P=0.361)	1.5	1.93±0.18 (P=0.171)

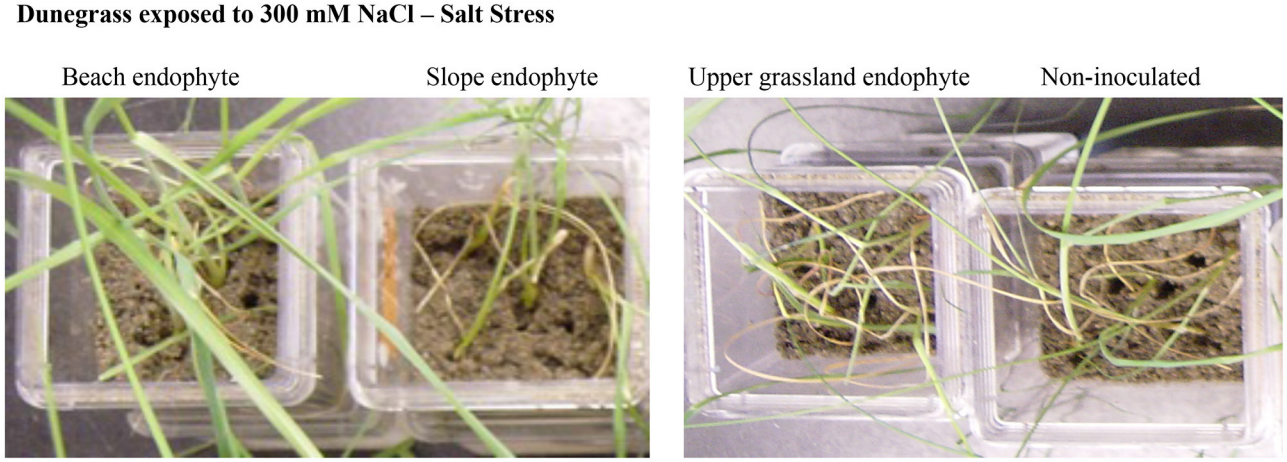

FIGURE 2 | Laboratory studies were conducted in double-decker magenta boxes with four-week-old dunegrass plants (5 plants /magenta box, x10 reps of each treatment). Treatments were uninoculated controls, or symbiotically colonized *F. culmorum, F. redolens,* or *Alternaria sp.* plants. The average plant health score (*) and wet biomass (**) of five magenta boxes/treatment exposed to 0 mM or 300 mM NaCl after 2 weeks ± standard deviation values were recorded (table on top). The plant health score was assessed on a scale of 1–5 (1=dead, 2=severely wilted and chlorotic, 3=wilted +/- chlorosis, 4=slightly wilted, and 5=healthy w/o lesions or wilting). The highest salt tolerance was found with *F. culmorum,* and moderate and no salt tolerance were observed with *F. redolens* and *Alternaria sp.* plants, respectively, when compared to uninoculated controls. No differences in plant health or biomass were observed in treatments in the absence of salt stress. In the presence of 300 mM NaCl stress, differences in plant health and biomass were observed in *F. culmorum* and *F. redolens* treatments, and no plant health or biomass differences were observed in *Alternaria sp*. treatments when compared to uninoculated controls. A representative photograph showing health differences in dunegrass treatments (lower panel) exposed to salt stress (300 mM NaCl). Statistical analysis was performed using Student's *t*-test. $P < 0.05$ values were determined to be significant.

with 5 mM $CaCl_2$ by filling the lower chamber of the double-decker magenta boxes with 200 ml with these solutions. After 7 days, when *Fusarium culmorum* ATCC symbiotic plants started showing salt stress symptoms (wilting and some chlorosis), plants were surface-sterilized (described above) and five plants were pooled with equal amounts of roots and lower stems collected and a subset of this sample equating to 0.5 g of tissue was used for the assay. Plant tissues were homogenized (Tekmar tissue homogenizer) in 10 ml of STC osmotic buffer (1M Sorbitol, 10 mM TRIS-HCl, 50 mM $CaCl_2$, pH 7.5) on ice and 100 µl plated onto multiple 0.1xPDA fungal growth medium (see above). Fungal colonies (CFU) were quantified after 5–7 days and normalized to equate to CFU/g of tissue. All assays were repeated a minimum of three times.

Statistical Analysis

Depending upon the study, *P*-values were determined by either Duncan's multiple range test, ANOVA single-factor analysis and data analyzed using SAS (SAS Institute, 2000), or Student's *t*-test.

RESULTS

Coastal Plants

Four coastal plant species, growing across salt gradients on two islands of the San Juan Archipelago, changed fungal endophytes at discreet microhabitats that differed in salt concentrations determined by soil conductivity measurements (**Table 1**). In all plants tested, the endophytes appearing in different microhabitats represented different fungal species with typically one dominant Class 2 fungal endophyte present in >70% of plants sampled/microhabitat. None of the plants analyzed had one endophyte dominant throughout the salinity gradient. However, in microhabitat transition zones, defined by salinity levels, plants typically contained endophytes from the adjacent microhabitats (**Table 1**). The distribution patterns of endophytes suggested ecological function (salinity adaptation) rather than taxon-specific interactions, with endophytic species correlating to soil salinity levels, irrespective of the plant species. For example, *Fusarium culmorum* was isolated from native dunegrass (*Leymus mollis*) plants in beach habitats with similar levels of salinity irrespective of geographic location.

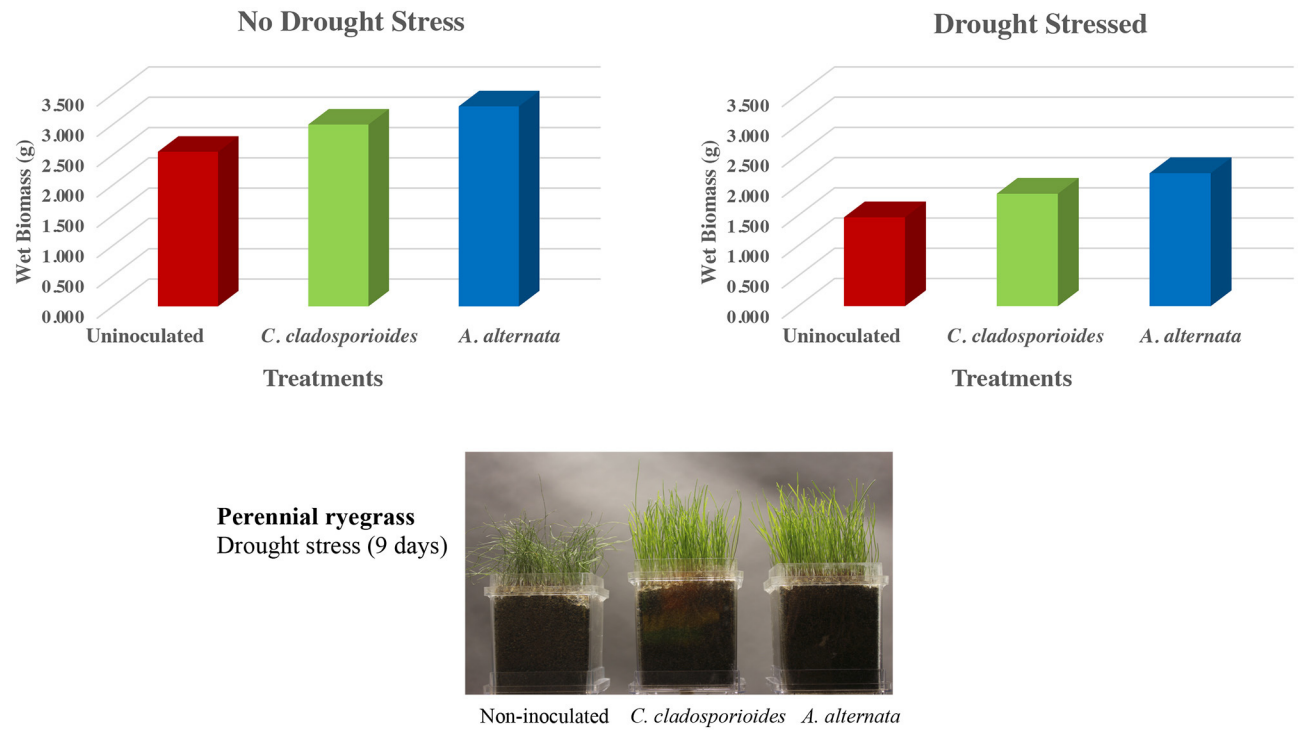

FIGURE 3 | Drought stress studies in double-decker magenta boxes containing uninoculated or symbiotic (*C. cladosporioides* or *A. alternata*) treated ryegrass seeds (545 seeds/box) grown for 12 days when fluid was removed from the lower chamber of ($N = 4$ magenta boxes) to begin drought stress. Four additional magenta boxes remained as hydrated controls. After 9 days in the absence of fluids, 200 mls of fluid was added back to all the boxes and plants were allowed to recover for 7 days and the wet biomass (g) of ryegrass shoots were assessed. No significant differences in biomass were observed in the absence of stress (SD = 0.39–0.79; P = 0.209–0.394) and statistically significant differences were observed in both symbiotic plants (indicated with *; SD = 0.12–0.28; $P = 0.014$–0.016) when compared to uninoculated plants in the presence of drought stress. A representative photograph of ryegrass plants prior to shoot harvests after 9 days of drought stress (bottom panel). Statistical analysis was conducted with Student's *t*-test to determine significance between two groups (i.e., symbiotically treated vs. uninoculated controls). Values of $P < 0.05$ were considered to be statistically significant.

Dunegrass was chosen for more detailed analysis due to its importance in coastal ecosystems and distribution patterns along salt gradients (Greipsson and Davy, 1997; Houle, 2002). In one location at the University of Washington's Cedar Rocks Biological Preserve on Shaw Island, WA, dunegrass was found growing along a salinity gradient stretching 20 meters from the beach up a slope and onto an upper grassland meadow (**Figure 1**). Salinity levels across the gradient were 436 μS/cm (beach), 249 μS/cm (slope), and 84μS/cm (upper meadow), and one dominant endophyte was isolated from plants in each salinity microhabitat (**Figure 1**). Plants along the gradient differed in size and sexual reproduction. Plants growing in saline habitats were smaller, lacked plant inflorescences, and spread via, rhizomes while plants on the slope and upper meadow were large, more than twice the size of plants growing on the beach habitat. Plants growing on the slope were densely populated and robust with 70–80% inflorescences, while plants in the upper meadow grow sparsely spread out among other plant species with 40–50% of plants having inflorescences (**Figure 1**, **Table 3**). Laboratory experiments with endophytes from each microhabitat conferred levels of salt tolerance to dunegrass similar to field site salinity levels with the beach, slope, and upper meadow endophytes imparting high, moderate, and low salt tolerance, respectively, under field (**Tables 2**, **3**) and laboratory conditions (**Figure 2**).

The ecological significance of the observed endophyte patterns was demonstrated at the Cedar Rocks site through a reciprocal transplant study of plants colonized with each dominant endophyte from each microhabitat in a factorial design where plants colonized with each of the microhabitat-specific endophytes were transplanted into each of the three microhabitats (**Table 2**) (Miglia et al., 2007). In every microhabitat, the highest fitness was observed by plants colonized with the endophyte indigenous to that microhabitat. Some percentage of plants colonized with nonindigenous endophytes survived in each microhabitat but achieved lower biomasses than plants with indigenous endophytes. Plant endophyte analysis revealed that during the growing season: (1) all of the plants became colonized by indigenous endophytes and (2) the indigenous endophytes became the dominant endophytes in the plants, regardless of the original colonizing endophyte or microhabitat of transplantation (**Table 2**). We surmise that the

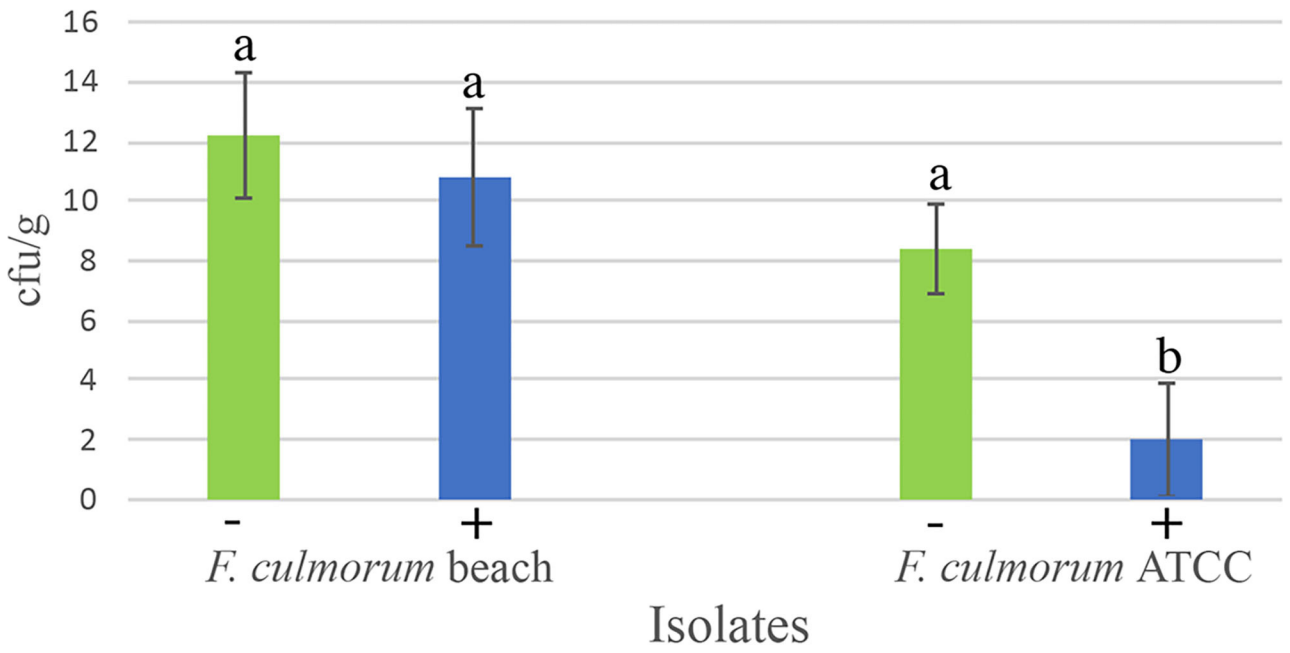

FIGURE 4 | Modulation of endophytes *in planta* dunegrass (*Leymus mollis*). Laboratory studies were conducted in double-decker magenta boxes with four-week-old dunegrass plants (x5 plants/magenta box, x3 reps of each treatment) colonized with *F. culmorum* beach isolate that does impart salt tolerance or *F. culmorum* ATCC isolate that does not impart salt tolerance, exposed either to no (0 mM NaCl; green bars) or high salt stress (300 mM NaCl, blue bars) for 7 days. When visible stress symptoms were observed in the *F. culmorum* ATCC isolate, the lower stem and roots of five plants were pooled and processed for cfu analysis by grinding plant tissues in an osmotic solution and plating aliquots onto fungal growth media (0.1x PDA). The *in planta* cfu abundance of the *F. culmorum* beach isolate remained the same in the presence and absence of salt stress. In contrast, in the presence of salt stress, the abundance of *F. culmorum* ATCC isolate decreased significantly. In each graph, ± standard deviation bars are indicated and the different lowercase letters above the bars indicate significant differences (Duncan's Grouping; $P < 0.001$).

survival and final biomass were proportional to the timing of colonization with indigenous endophyte post-establishment.

Endophyte Transmission

The colonization of endophyte-free plants in the transplantation study indicated soil transmission of endophytes, so it was of interest to assess potential seed transmission. Unfortunately, we could not determine seed transmission of plants in the transplant study because the site was destroyed by a violent storm. Instead, seed transmission of endophytes was assessed for two plant species: *Grindelia integrifolia* (gumweed) which commonly occurs in rocky cliff outcroppings that become very dry in summer months and *Leymus mollis* (dunegrass) which commonly occurs on the beach habitat and can be found from below the intermittent high tide line to the tops of bluffs. Endophytes were analyzed from seed coats and in roots of plants from four populations growing along a beach transect above the high tide delineation on Shaw Island. In both species, the dominant root endophytes occurred in 70–95% of all seed coats indicating very effective seed transmission (**Table 4**).

Endophyte Modulation *In planta*

To gain insight into the mechanisms responsible for endophyte distribution patterns, we examined the relationship between *in planta* endophyte abundance in the presence and absence of salt stress of plants symbiotic with endophytes that do or do not impart salt stress tolerance. Dunegrass plants were colonized either with habitat-adapted *Fusarium culmorum* beach isolate that confers salt stress or an isolate acquired from the American type culture collection described here as *Fusarium culmorum* ATCC obtained from a habitat devoid of salt stress (**Figure 4**). In the absence of stress, there were no significant differences in the *in planta* colony-forming units (cfu) of endophytes recovered from plants regardless of the endophytes ability to confer stress tolerance. However, when symbiotic plants were exposed to salt stress, the fungal abundance of *F. culmorum* beach isolate remained the same while the abundance of *F. culmorum* ATCC decreased significantly. Interestingly, previous studies indicated that axenically cultured endophytes, grown on fungal growth media incubated at elevated temperatures or addition of NaCl in the medium, showed that non-habitat-adapted isolates were

more tolerant of heat and salt stress *in vitro* than the habitat-adapted isolates. It appears that the stress alone does not account for the change in the *in planta* cfu (Rodriguez et al., 2008).

DISCUSSION

Analysis of four plant species growing across environmental gradients in different geographic locations revealed a dynamic ecological process that occurs between plants and fungal symbionts based on soil chemistry and plant fitness needs (**Figure 1**, **Table 1**). This is in line with other studies indicating that changes in soil chemistry along a chemical gradient can alter fungal endophyte associations within a single plant species (Maciá-Vicente et al., 2012; Glynou et al., 2016; Hammami et al., 2016; Kia et al., 2018). The interplay between endophyte conferred benefits and plant fitness appears to be a significant driver in plant niche expansion (**Table 2**). For example, the growth and development of dunegrass along the salt gradient varied significantly with regard to biomass and reproduction. None of the plants growing on the beach produced inflorescences and achieved lower biomasses than plants on the slope or upper grassland meadow (**Table 3**). The absence of sexual reproduction by beach plants could be viewed as decreased fitness. However, the trade-off for meiotic reproduction is salt stress tolerance and a lack of competition from other plant species (dunegrass was the solitary species in the beach cobble exposed to seawater at high tide). Moreover, dunegrass is a rhizomatous species that can grow upslope from the beach and colonize a less saline microhabitat (**Figure 1**). In the slope microhabitat, the plants modulate their endophytic associations to achieve optimal fitness and are able to reproduce. Although *L. mollis* (dunegrass) is known to express differential growth responses along salt gradients in costal habitats, the basis of this plasticity has not been defined (Imbert and Houle, 2000; Houle, 2002). Based on laboratory and field transplant studies, we propose that fungal endophytes are largely responsible for the plasticity of dunegrass (**Figure 2**, **Table 2**).

The interaction between imposed stress and abundance levels of endophytes *in planta* (**Figure 4**) suggests that endophyte composition is regulated by the host. Host regulation may explain why so few species of Class 2 endophytes are dominant in plants adapted to high-stress habitats (**Table 1**). Moreover, the *in planta* endophyte ecology varies between plant species which likely reflects the importance of symbiotic communication in plant biogeographical patterns. Our studies indicate that either plants communicate optimally with indigenous endophytes or exhibit decreased fitness in any specific habitat (**Figure 4**, **Table 2**).

Plant seeds can be dispersed locally or over distances into habitats to which plants are not adapted. The transmission of endophytes via seed coats ensures that newly establishing plants will emerge symbiotic but does not guarantee that the plants will be adapted for optimal fitness in distant habitats. However, as long as there are plants in the new habitats, dispersed seeds will be exposed to new fungal endophytes in the soil that can adapt germinated seeds for better fitness. This is easily envisioned along stress gradients where seed dispersal can be over very short or long distances. It is tempting to hypothesize that this type of symbiotic flexibility has allowed for the adaptive capabilities of plants to colonize complex habitats around the world.

Symbiotic associations between fungi and plants have been in existence since plants moved onto land >400 MYA and all plants are thought to be symbiotic with fungal endophytes and other microbes (Pirozynski and Malloch, 1975; Redecker et al., 2000; Krings et al., 2007). The ability of a single fungal endophyte to confer the same stress tolerance to monocots and eudicots suggests that the symbiotic communication responsible for stress tolerance is conserved and predates the divergence of these lineages (130-320 MYA (Chernikova et al., 2011; Hertweck et al., 2015). The discovery of habitat-adapted symbiosis (HAS) demonstrates that fungal endophytes can be responsible for the adaptation of plants to high-stress habitats (Rodriguez et al., 2008). The fact that Class 2 endophytes can be transmitted both vertically via seed coats and are not themselves stress-tolerant, indicates that endophyte conferred stress tolerance is an epigenetic phenomenon. Here, we demonstrate that as plants are exposed to changing environmental conditions, they modulate associations with fungal endophytes to optimize fitness. This "symbiotic modulation" is a dynamic aspect of HAS allowing individual plant species to grow as continuous populations across chemical gradients. We hypothesize that the ability of plants to modulate symbiotic associations provides a mechanistic basis for phenotypic plasticity, adaptation, niche expansion, and the biogeography of plants.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GeneBank - OM816650, OM816673, OM812680, OM826983, OM826984, OM826979, OM826980, and OM812800.

AUTHOR CONTRIBUTIONS

RSR and RJR conceived and designed the experiments, performed the experiments, contributed reagents/materials/analysis tools, and wrote the paper. RSR, JA, TB, KM, MR, and JW analyzed the data. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: RSR, JA, TB, KM, MR, JW, and RJR were employed by Adaptive Symbiotic Technologies.

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