



Exploring the influence of seed bacteria and fungi on seed germination and seedling traits in *Brachypodium* congeners

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Abstract

Seed microbiomes represent the first colonization of the plant by microbes and influence community assembly of subsequent microbes. The role that parental genotype and species of origin play in structuring and sourcing the seed microbiome is crucial to understanding the seed microbiome and patterns in microbiome variation. Seed-borne microbes become part of the seedling microbiome and have the potential to influence seedling traits and whether variation in seedling traits can be explained by seed fungal and bacterial microbiome characteristics is important to improving our ability to predict seedling-microbiome trait responses *a priori* for application. Unfortunately, the relationship between seedling traits or germination response and seed microbiome characteristics have only been quantitatively linked in one study to our knowledge. So, here we grew individuals of a wild collection of the annual grass, *Brachypodium distachyon* and inbred lines of a congener, *Brachypodium hybridum*. We grew plants in a greenhouse for seed production and used high throughput amplicon sequencing to characterize offspring seed microbiome. We also characterized the growth of sibling seedlings to those sequenced. We found that for only one of the eight traits evaluated, seed germination during cold stratification, the abundance of the most prevalent microbial families had a marginal positive influence on whether germination happened during cold stratification ($R^2=0.29$, $p=0.07$). These results critically show that seed microbiomes are strongly influenced by parental genotype and species and seed microbiomes may have diminishing influences on seed and seedling traits. Thus, more work is needed to realize the goal of microbiome-trait prediction.

Keywords Bacteriome · Germination · Microbiome · Seedling · Traits · Variability

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1 Introduction

The importance of seed microbiomes cannot be understated as they represent the first colonization of the plant by microbes and influence community assembly of subsequent microbes (Shahzad et al. 2018; Garrido-Sanz & Keel 2025). Bacteria, fungi, viruses and oomycetes are key players in the seed microbiome (Nelson 2018) with most metagenomic research and knowledge focused on bacteria and fungi (Simonin et al. 2022). One source of the seed microbiome is from horizontal transmission from biotic and abiotic physical encounters in the environment, largely from soil, but also via rain and bioaerosols (Frank et al. 2017; Fort et al. 2021). Some seed endophytes can benefit plants through enhancing the plant's stress tolerance (Truyens et al. 2015; Nelson 2018) and by facilitating growth and development by improving nutrient availability and exuding growth hormones like cytokinins that break seed dormancy (Puente et al. 2009; Truyens et al. 2015). Beneficial microbes can

also antagonize phytopathogens and limit herbivore activity (Czarnoleski et al. 2010; Shahzad et al. 2018), therefore favoring key functions during germination.

Seed microbiota are also acquired via vertical transmission (Shade et al. 2017; Abdelfattah et al. 2023) from paternal plants through the pollen microbiome (Frank et al. 2017; Prado et al. 2020). However, most of the seed microbiome originates from the maternal plant, coming from reproductive structures, vascularization, and intercellular spaces (Truyens et al. 2015). Maternal genotype has similar or greater influences on the seed microbiome compared to environmental factors (Klaedtke et al. 2016; Fort et al. 2021; but see Hardoim et al. 2012) and the host species influences variation in seed microbiome (Johnston-Monje et al. 2022). Furthermore, the microbial taxa most prevalent in maternal tissues strongly appear in the seed microbiome, even after seeds fall to the ground where most horizontally transmitted microbes are sourced (Fort et al. 2021). In all, the role that the parental plant plays in structuring and sourcing the seed microbiome is crucial to understanding the seed microbiome and patterns in microbiome variation.

Seedling traits also vary by maternal plant genotype and maternal environment (Foxx et al. 2024) with consequences for plant growth, competition, and survival (Hintz et al. 2016; Foxx and Kramer 2020; Van Allen et al. 2021). Seed-borne microbes become part of the seedling microbiome and have the potential to influence seedling traits when seed endophytes can survive high osmotic pressure within seeds and persist during development to colonize seedling tissue (Truyens et al. 2015; Shade et al. 2017). However, whether variation in seedling traits can be explained by seed fungal and bacterial microbiome characteristics is important to improving our ability to predict seedling-microbiome trait responses a priori for both agriculture and restoration (Friesen et al. 2011; Herrera Paredes et al. 2018). Unfortunately, the relationship between seedling traits or germination response and seed microbiome characteristics have only been quantitatively linked in two other studies to our knowledge (Chen et al. 2020; Nanfack et al. 2024). For example, (Chen et al. (2020) found varying relationships between seed germination percentage and time to germination with bacterial taxa abundances in *Festuca* and *Lolium* grass species. The need for more research is clear, including expanding to seedling traits and fungal community contributions.

Whether bacterial and fungal taxa and their abundances differentially influence seedling growth remains unknown, given the limited evidence to date. However, this is a key knowledge gap to fill towards understanding how properties of seed microbiomes influence plant morphology and physiology. Both bacteria and fungi can act as pathogens or beneficial microbes to their host depending on the circumstances (Nelson 2018). Bacteria and fungi also interact in

microbial communities and can regulate each other in plants (Frank et al. 2017; Chen et al. 2020). For example, seed bacterial endophytes act as biocontrol through producing anti-fungal compounds (Truyens et al. 2015); in fact, some seeds produce chitinases to inhibit growth of chitin in fungal cell walls (Gomez et al. 2002), lending to the hypothesis that fungi tending to have pathogenic relationships with seeds and germination. The consequences bacteria and fungi have had on plant phenotype has been thoroughly explored in inoculation studies (Chandrasekaran 2022), but microbial community studies would help to understand the cumulative community influence as well as how bacteria and fungi influence seed germination and seedling traits.

Here we address whether seed microbiomes vary by accession and parental genotype (species) and ask whether bacteria and fungi influence seedling root and shoot traits. We grew accessions of annual grasses, *Brachypodium distachyon* and *B. hybridum* in a greenhouse for seed production and used high throughput amplicon sequencing to characterize its seed mycobiome and bacteriome. We also characterized the growth of sibling seedlings to those sequenced. *Brachypodium* is an ideal plant system as it has documented genotypic root and shoot trait variation (Filiz et al. 2009; Pacheco-Villalobos and Hardtke 2012; Chochois et al. 2015) and a previous characterization of seed and root microbiomes (Kawasaki et al. 2016; Johnston-Monje et al. 2021). We hypothesized that: 1) seed bacterial and fungal diversity would differ in predicting seedling traits; 2) abundance of the most prevalent bacterial and fungal families would differentially predict seed germination and seedling traits, with fungi being more negative; and 3) fungal and bacterial communities and plant genotype will explain differing amounts of variation in seedling traits.

2 Materials and methods

2.1 Target species

We used closely related *Brachypodium* congeners, *Brachypodium distachyon* and *B. hybridum*. *B. distachyon* is a small stature annual grass native to the Mediterranean region, southwestern Asia, and southern Europe (Filiz et al. 2009) and has been split into three species: *B. distachyon*, *B. hybridum*, and *B. stacei* (Catalán et al. 2012). We also used *B. hybridum* here, an allohexaploid resulting from *B. stacei* and *B. distachyon* crosses (Catalán et al. 2012), and made references to the biology of congeneric *B. distachyon* for its relevance. *B. distachyon* has been leveraged as a model organism in functional genomics research due to its small genome size and simplicity, and applicability to major cereals (Draper et al. 2001; Filiz et al. 2009). *B. distachyon* is

a self-fertilizing, cleistogamous plant with non-shattering seed heads (Draper et al. 2001; Filiz et al. 2009), and its reproductive characteristics make it an ideal plant for seed microbiome analysis. Due to self-fertilization, we refer to parental rather than maternal genotype as the maternal and paternal are one in the same.

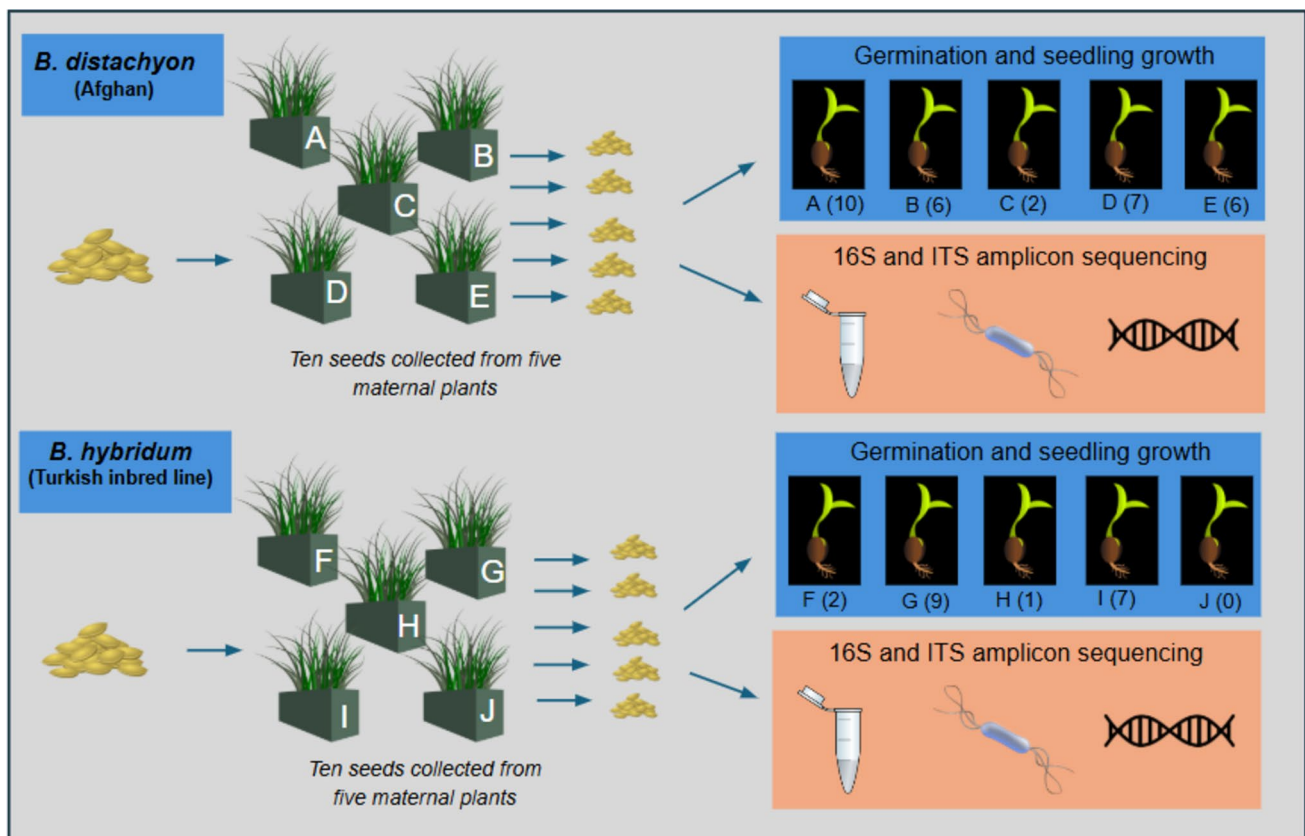
2.2 Plant material procurement

We requested *B. hybridum* and *B. distachyon* accessions from the Germplasm Resources Information Network (GRIN) from two accessions: an Afghan wild population collected in 1954 and an inbred line originating from Türkiye (Bal P6; Box 1). The storage conditions of the seeds are unknown prior to receipt in June 2021; however, we used standardized methods for both accessions and stored the seeds at 3°C with desiccation packs for one month in a refrigerator at the University of Florida (Gainesville, FL, USA) until seed processing. We first surface sterilized the seeds by submerging 50 seeds of each accession into a 6% bleach solution for 2 min followed by a 1-min rinse in deionized water. We placed these imbibed seeds in Ziplock

bags with paper towels and 5 mL of deionized water for cold moist stratification at 3°C for two weeks.

We planted germinated seeds in a greenhouse on August 8, 2021, at the Institute of Food and Agricultural Sciences (IFAS) (Citra, FL, USA). We planted germinants in 9.5 cm × 11.4 cm square pots containing a sphagnum peat, perlite, and vermiculite mixture (Jolly Gardener C/25, BWI company, TX, USA). The plants grew for three months at an average greenhouse temperature of 25 °C with ambient lighting. We grew plants in a common environment to help standardize maternal effects (Van Allen et al. 2021). We supplemented Lighting with lamps for a 16 h day/8 h night light cycle in October until the experiment's end. Pots were randomized into trays across two benchtops and were watered three times weekly and fertilized weekly (Miracle Gro, Marysville, OH, USA).

We collected seeds that adult plants produced on November 11, 2021, using forceps sterilized with a bleach solution and stored the seeds in separate Ziploc bags separated along maternal Lines. We collected seeds from five parental plants for each accession and immediately stored the seeds on ice for transport to the lab. To assess seed endophytic diversity, we then surface sterilized the seeds with a 6% bleach



Box 1 *Brachypodium* accession information and genotypes. *Brachypodium distachyon* seeds were collected in 1954 from a wild population in Afghanistan (GRIN PI 219971) and *Brachypodium hybridum*

originate from Turkish inbred Lines created ca. 2010 (Bal P6, GRIN PI 689090; Vogel et al. 2009). We collected seeds from five maternal plants and performed amplicon sequencing on seven samples

solution for 2 min followed by a 1-min deionized water rinse. We then stored the seeds in Eppendorf tubes at -80°C until DNA extraction and amplicon sequencing on March 18, 2022 (MR DNA, Shallowater, TX, USA).

2.3 Seed germination & seedling traits

We used a subset of the produced seeds to quantify seed germination and seedling traits. We cold-moist stratified the second generation of seeds for 17 days in November 2021 at 3°C by placing them in Ziploc bags with a sheet of paper and deionized water. By growing the seedlings on paper, we minimized confounding influences of microbes on the growth of the seedlings and thus, most microbiome influences on the seedlings originated from the seed microbiome. We then moved the sealed seeds to warm conditions on November 29, 2021, at a 12h day/12 h at 25°C for two weeks in a Percival incubator (Percival Scientific, IA, USA). We checked and recorded germination every two to three days and collected the following measures of the seeds and seedlings: germination status, number of days until germination occurred, seedling rooting depth (root length (cm)), number of primary roots, number of root tips, number of true leaves, and shoot height (cm).

2.4 DNA extraction and amplicon sequencing

Genomic bacterial DNA was isolated from the other subset of the produced seeds using the DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's instructions. Seed samples were cut into 5 to 6 pieces and added to the PowerBead Tube to undergo cell lysis. The purified DNA was eluted from the spin column using 100 μL of solution C6 and stored at -80°C until PCR amplification. The 16S PCR conditions included one μL of extracted DNA was used as template for PCR using UDI primers illcus515F (GTGY-CAGCMGCCGCGGTAA) and new806RB (GGACTAC-NVGGGTWTCTAAT). In addition, 16S peptide nucleic acid (PNA) blockers were used to reduce sequencing of plant sequences. The 16S rRNA gene V4 variable region PCR primers 515/806 with barcode on the forward (GTGY-CAGCMGCCGCGGTAA) and reverse (GGACTAC-NVGGGTWTCTAAT) primer were used in a 30 PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 30–35 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed.

A second PCR reaction to add i5 and i7 adapters was performed. One μL from the first PCR reaction was used as a template. Thermal cycling conditions were as follows: 95°C for 5 min, then 10 cycles at 95°C for 30 s, 55°C

for 30 s, 72°C for 30 s, 72°C for 5 min, then 4°C . The fungal PCR Conditions included one μL of extracted DNA used as a template for PCR using barcoded primers ITS7F (GTGARTCATCGAATCTTTG) and ITS4R (TCCTCCGCTTATTGATATGC). Thermal cycling conditions were as follows: 95°C for 5 min, 35 cycles at 95°C for 30 seconds, 53°C for 40 s, 72°C for 1 min, 72°C for 10 minutes, then 4°C .

Blanks were also sequenced to identify contaminants by using 100 μL DNA free water from Qiagen, the same water that is used for DNA extraction. After amplification, PCR products are checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product is used to prepare illumina DNA library (Illumina MiSeq, Illumina). Sequencing was performed at MR DNA (Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

2.5 Sequence data processing

The optimal parameters for read trimming by DADA2 (Callahan et al. 2016) were determined using FIGARO (Weinstein et al. 2019). Raw 16S reads were trimmed to 390 bps and shorter reads were discarded using Trimmomatic (Bolger et al. 2014). Reads were then imported into QIIME2 (Bolyen et al. 2019). The optimal parameters for DADA2 trimming were $\text{EE}=1,2$, and $\text{trimming}=246$, 203 for forward and reverse reads, respectively. Trimmed, demultiplexed raw 16S reads were imported into QIIME2 for visualization and further processing. The QIIME2 plugin cutadapt (Martin 2011), was used to remove primers while amplicon sequence variants (ASVs) were produced using the DADA2 plugin (Callahan et al. 2016) using the optimized parameters above. Taxonomy was determined using the QIIME2 classifier (Bokulich et al. 2018) which was trained on the SILVA 138 99% OTUs 515F/806 region pre-formatted reference sequences and taxonomy (QIIME2 page, (Robeson et al. 2021)). The 16S count table was then filtered using QIIME2 for taxa including "mitochondria" or "chloroplast", to remove host DNA from the table.

Raw demultiplexed ITS2 reads were trimmed using the QIIME2 plugin ITSxpress (v1.8.1, Rivers et al., 2018). Trimmed reads were denoised and ASVs were produced using the DADA2 plugin with default $\text{EE}(2,2)$ and no further trimming of the reads. A QIIME2 classifier was produced using the UNITE fungal database (Nilsson et al. 2019) with the feature-classifier QIIME2 plugin (Bokulich

et al. 2018). Classifier was developed using the 99% similarity in the 10/05/2021 UNITE release.

2.6 Seedling trait analyses

To address the hypothesis that seed bacterial and fungal microbial diversity would differ in their relationship with seedling traits and seed germination outcomes, we performed generalized linear models (*glm* function in the *stats* package; (R Core Team 2024)) to compare the means of microbial seed diversity generated with DivNet (Willis and Martin 2022) and mean seedling traits with a covariate “microbe” with two factor levels of “bacteria” and “fungi”. Tests were repeated independently for each trait (leaf height, rooting depth, number of primary roots, number of leaves, days to germinate, germination during cold stratification, germination proportion) and diversity measure (richness, Shannon diversity, and evenness).

We also used generalized linear models to test the hypothesis that the most prevalent bacterial and fungal families would differentially predict seed germination and seedling traits, with fungi being more negative. We used separate models to evaluate how the log abundance of the two most prevalent bacterial and fungal families affected mean trait values (leaf height, rooting depth, number of primary roots, number of leaves, days to germinate, germination during cold stratification, germination proportion) and whether the relationships differed for bacteria and fungi.

2.7 PERMANOVA comparing microbiomes along plant accessions

We next tested the hypothesis that fungal and bacterial diversity and individual genotype and accession will explain differing amounts of variation in seedling traits. The processed 16S and ITS reads were then analyzed using the R packages Breakaway (Willis and Bunge 2015) and DivNet (Willis and Martin 2022) to calculate similarity matrices (Bray–Curtis, (Bray and Curtis 1957); Shannon entropy, (Shannon 1948)), while accounting for the compositionality of the amplicon data. We used PERMANOVA tests (*adonis2* function, permutations=1000) from the *vegan* R package on the Bray–Curtis dissimilarity matrices to determine significance of host genotype (Afghanistan or Türkiye accessions). Further PERMANOVA tests were performed between parental plant genotypes and accession on the bacterial and fungal samples. Principal coordinate analysis of dissimilarity matrices figures were generated using R.

3 Results

3.1 Seed germination and seedling trait responses

Seeds varied in germination proportion along parental genotype with seeds from four parental plants from both *Brachypodium distachyon* and *B. hybridum* (Afghan and Turkish accessions, respectively) having low germination response, including no germination from the Afghan maternal genotype J (Fig. 1, Online Resource 1). Seedlings varied in trait values along parental genotypes; specifically, seedling rooting depth, root tip count and primary root count differed by accession (Fig. 1). The relationship between seed microbiome diversity and seed germination and seedling traits did not differ significantly between bacteria and fungi for all traits and for all diversity measures (richness, evenness, and Shannon diversity; Online Resource 1) (Fig. 2).

The evaluation that the most prevalent bacterial and fungal families would differentially predict seed germination and seedling traits showed that the relationship between the abundance of the most prevalent families and seed germination and seedling traits differed marginally only for whether the seed germinated during cold stratification ($R^2=0.29$, $p=0.07$, Residual deviance=4.2 on 38 df, AIC=29.1). As the abundance of the most prevalent family increased, so did the probability for germination during cold stratification (Fig. 3), and this did not differ for whether the microbe was a bacterium or a fungus.

3.2 Sequencing read outcomes

The amplicon sequencing of 16S DNA returned a total of 3,948,698 (min=14,69, max=55,696) paired-end reads, after filtering of host plant DNA the total number of ASVs was 1432. The sequencing of the ITS2 region had a total of 5,892,915 (min=27,072, max=302,445) paired-end reads. The total number of ASVs after trimming to the fungal ITS2 region, and denoising were 658.

The Bray–Curtis similarity from the DIVNET analysis on the 16S and ITS2 region is shown in Fig. 3 in NMDS ordination. A PERMANOVA analysis performed on the bacterial (16S) community similarity and host plant genotype (Afghanistan or Türkiye) indicated no significant difference between accessions. The same PERMANOVA analysis performed on the fungal (ITS2) community similarity shows differentiation in the non-metric multidimensional scaling (NMDS) plot between host plant accessions (Fig. 4). However, PERMANOVA analysis showed a non-significant correlation between host accession and the community similarity. Further PERMANOVA analysis on single parental genotype groups showed a significant difference between most groups of different genotypes (data not shown).

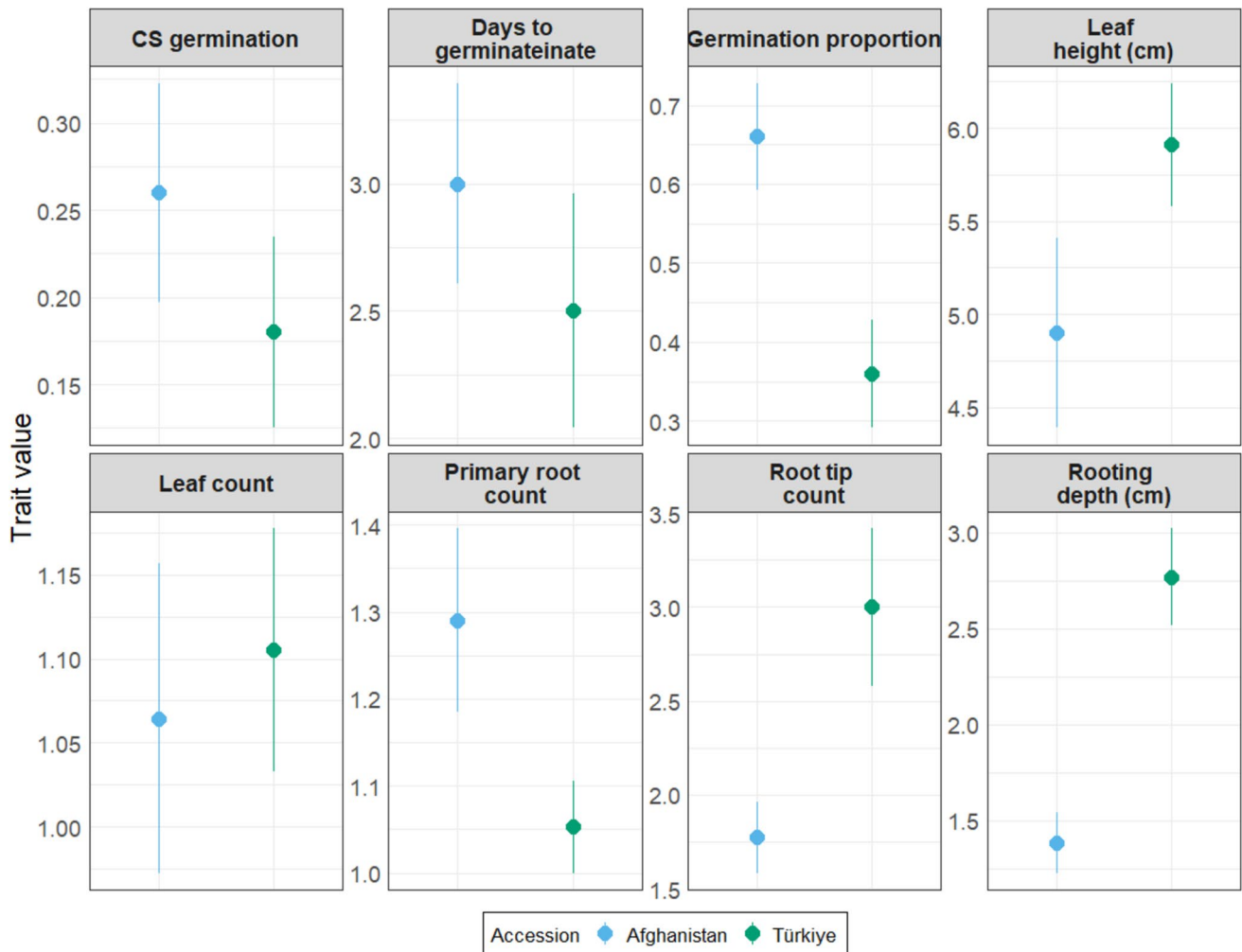


Fig. 1 *Brachypodium* congener germination and seedling morphological trait values along accessions (Afghan in blue and Turkish in green). Traits included: i) whether germination occurred in cold stratification (CS germination), ii) the number of days each individual seed took to germinate (days to germinate), iii) the proportion of individuals ger-

minating (germination proportion), iv) leaf length (leaf height (cm), v) the number of leaves (leaf count), vi) the number of primary roots (primary root count), vii) number of root tips (root tip count), and viii) rooting depth (cm). Mean and 95% CI are presented for each trait

4 Discussion

In this study, we examined the influences of seed fungal and bacterial communities on seed germination and seedling traits. This is one of only three studies to our knowledge to quantitatively Link the seed microbiome diversity to seed germination or seedling traits. We note three key findings: 1) we found no significant relationship between microbial diversity and seedling traits for both fungi and bacteria that we could identify, 2) the most prevalent microbial families positively predicted seed germination during cold stratification, but not other traits, and 3) while seedling and germination traits varied by accession source (species) and genotype, we only saw this pattern extend to seed fungal communities across plant accessions. These results suggest that seed microbiomes may have diminishing influences on

seed germination and seedling traits – with genotype having stronger influences – which has important implications for whether we can predict plant traits from attributes of seed microbiomes.

4.1 Weak influence between seed germination and seedling traits

We observed a limited relationship between traits and diversity measures and the abundance of prevalent microbial families suggesting a weakened influence of seed microbiome on seedling traits. However, we did see some influence on seed germination characteristics for whether the plant germinated during cold stratification. These results are expected since the seed is a reservoir for microbes and germination itself alters the microbial community (Nelson

Fig. 2 Evaluation of the relationship between the most prevalent bacterial and fungal families and seed germination during cold stratification. Families are for both bacteria and fungi and bacterial families are Lactobacillaceae and Corynebacteriaceae, and fungal families are Cladosporiaceae and Wallemiaceae. Fitted lines are from linear models, gray shading is the standard error of the fit

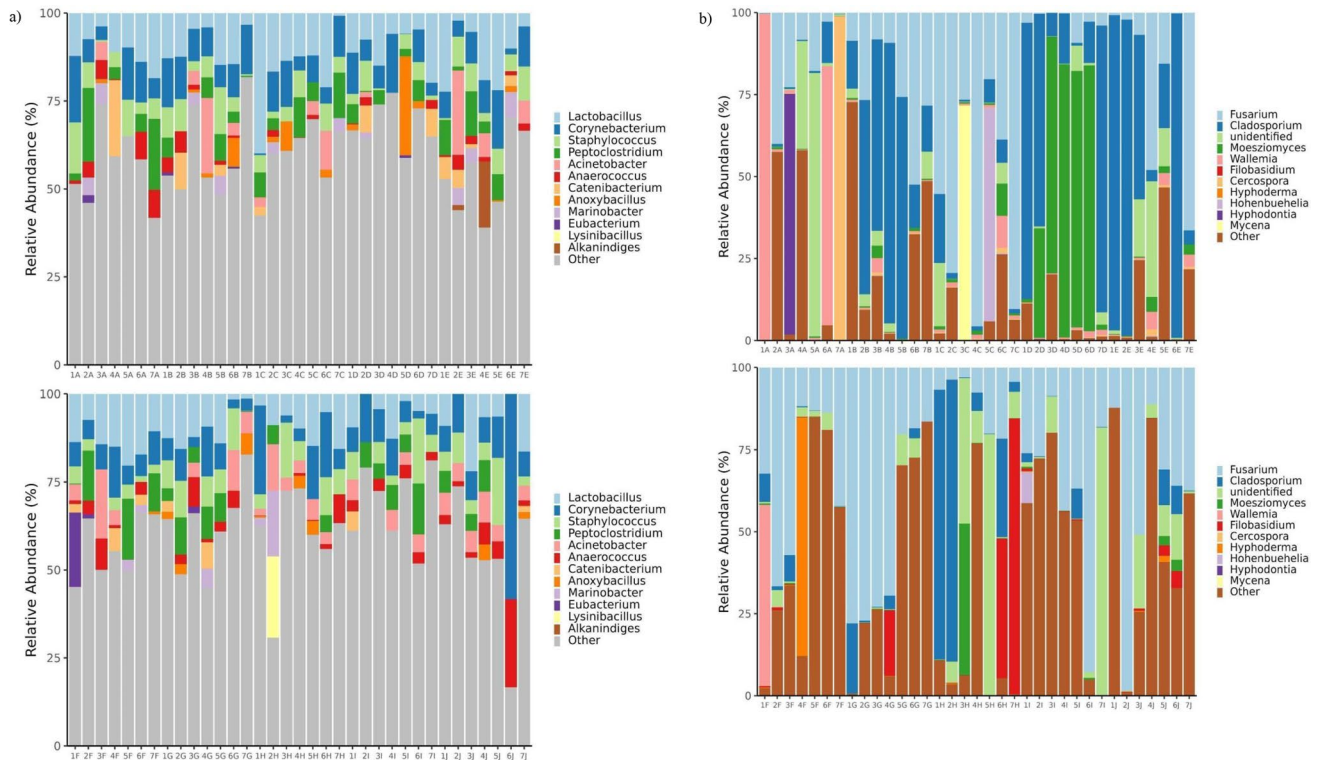
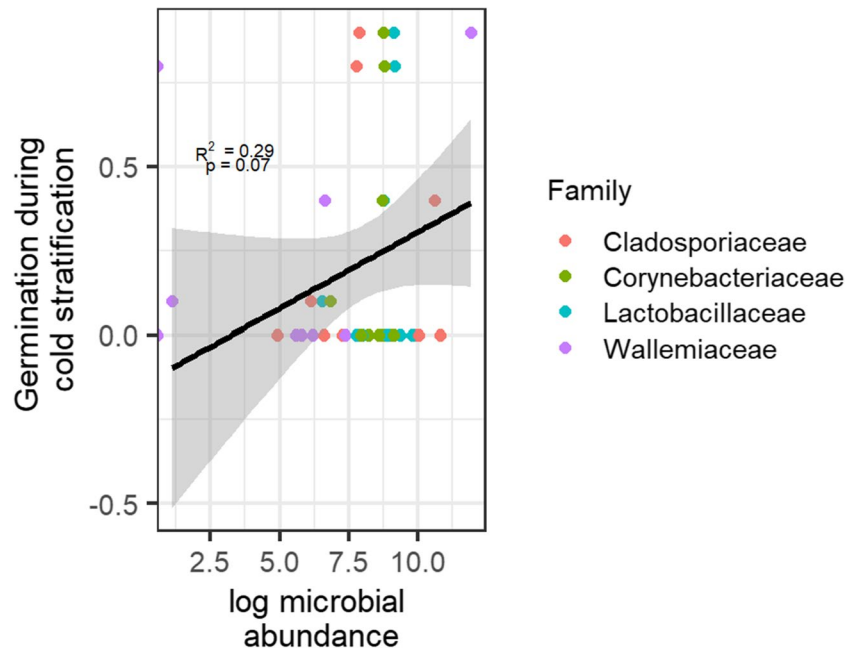


Fig. 3 Bar plots representing the mean relative abundances of **a)** bacterial or archaeal and **b)** fungal classes in seeds detected across plants. Each bar shows the average composition of parental lines. Each parental genotype consisted of seven seeds

2018) and may attenuate the impact of seed microbiome onto seedling traits. The resulting microbial community changes due to germination may lead to a different relationship between traits and the microbiome of the spermosphere that we did not evaluate here. However, Chesneau et al. (2022) found in *Phaseolus vulgaris* and *Raphanus*

sativus that the most prevalent seed microbiome taxa did not become the most prevalent taxa in seedlings, which should also weaken the plant's responses to taxa most prevalent in the seed. On the other hand, other studies differed in their findings of seed microbiome-trait relationships compared to our study. For example, Rodríguez et al. (2020) found

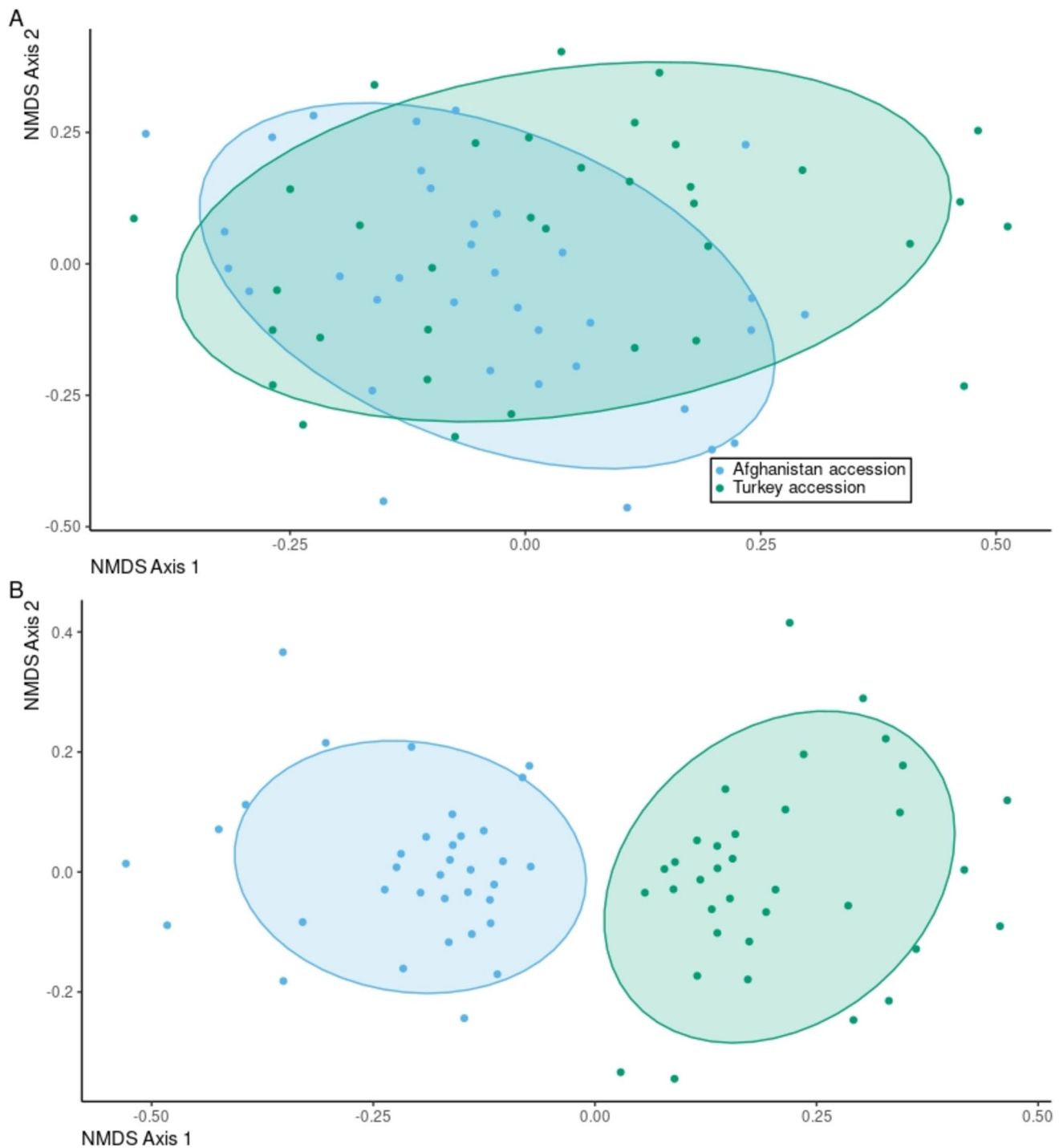


Fig. 4 Non-metric multidimensional scaling (NMDS) ordination of Bray–Curtis community similarity with host plant genotype (*Brachypodium distachyon* (Turkish) and *B. hybridum* (Afghan)) color differentiation. **a**) Bacterial (16S) Bray–Curtis community similarity using

DIVNET plotted in an NMDS ordination. **b**) Fungal (ITS2) Bray–Curtis community similarity using DIVNET plotted in an NMDS ordination

the relative abundance of 41 bacterial rOTUs correlated to seed germination percentage. Additionally, Chen et al. (2020) was the only other study to quantify the influence of seed microbiomes and germination. They found a negative relationship between seed germination percentage and the

abundance of 11 of 12 bacterial taxonomic groups assessed. Proteobacteria exhibited a positive relationship with germination percentage. Conversely, 12 of 21 bacterial taxonomic groups exhibited a positive relationship with time to germination (Chen et al. 2020). These results demonstrate the

variability in response of seed microbiome and germination and seedling traits.

The most prevalent bacterial and fungal families from the *Brachypodium* seed collections of this study differed from those of other studies (Simonin et al. 2022), which often reported Proteobacteria taxa as the most abundant. For example, Dai et al. (2019) demonstrated that Proteobacteria and Ascomycota were most prevalent in turfgrasses they evaluated. We found the probability of germination during cold stratification to be positively influenced by the abundance of the most prevalent bacterial and fungal families of Lactobacillaceae and Corynebacteriaceae, and Cladosporiaceae and Wallemiaceae, respectively. For bacterial families, Corynebacteriaceae has been found in the seed coat microbiome of *Amorphophallus muelleri* with *Corynebacterium* as a dominant genus. Corynebacteriaceae was most prevalent in the intermediate maturity stage of *A. muelleri* (Yang et al. 2022) and has been known to promote plant growth (Panke-Buisse et al. 2015). Taken together, *Corynebacterium* appears to be important in seed germination and seedling growth. Lactobacillaceae taxa are largely non-pathogenic, and they ferment simple carbohydrates and produce lactic acid (Walter and O'Toole 2023). Lactic acid fermentation can act to reduce pathogens including fungi on seeds and seedlings (Budryn et al. 2019). For fungal families, the significance of taxa from genera in the Cladosporiaceae (*Cladosporium*) on seed germination and seedling growth is varies as some have pathogenic effects resulting in leaf lesions (El-Dawy et al. 2021) and other taxa have been reported for stress alleviation and plant growth promotion (Räut et al. 2021). Whether the most prevalent bacteria and fungi interact to influence germination requires further study and inquiry but could fill knowledge gaps on taxa linkages and plant responses.

The lack of statistical significance in whether bacterial taxa and communities differ from fungal taxa and communities is surprising given the often-negative relationship between seed fungal communities and seed germination and seedling suppression (Perkins and Hatfield 2016). However, Kim et al. (2023) showed positive fungal effects on germination rate and the promotion of soybean plant growth for individual inoculating taxon. This raises the ongoing question of when and under which circumstances seed-associated communities favor seed health and germination or not. Additionally, the finding that some taxa promote germination under cold stratification supports the hypothesis that early germination, such as in cold stratification, allows for exuded nutrients from the seeds to become available to the microbes (Shade et al. 2017), thus making it beneficial to promote germination in the cold. The finding that fungal taxa promoted germination might be surprising, given that seeds and germinating seeds accumulate chitinases, which

can exert control over fungi, especially in late seed maturation stages when water content is highest (Gomez et al. 2002). These findings demonstrate complex community effects from fungal taxa in and on seeds that require further research.

4.2 Seedling root and shoot trait responses to seed endophytes

It is surprising that seedling root traits lacked a statistically significant response to the seed microbiome as all root functional traits are considered microbially mediated (Friesen et al. 2011). However, the influence of rhizosphere microbiota may be greater than that exerted by seed endophytes: indeed, evidence shows that rhizosphere microbiota have a stronger predictive ability for plant traits (Guo et al. 2021). One of the keyways in which seed endophytes influence seedling traits is through improved plant nutrition by solubilizing phosphates, nitrogen fixation, and growth hormone production (Pal et al. 2022). There were no nutrients added or microbes in the media in this experiment. Therefore, seed microbiome-seedling growth mediation in such a system would have to rely most on growth hormone production, though this was untested nor were growth impacts observed. Another important consideration for microbiome-trait prediction is that the host plant phenotype selects the seedling microbiome community membership (Matsumoto et al. 2021), and the seed microbiome is altered during germination resulting in the spermosphere microbiome that can differentially act upon the germinant (Shade et al. 2017; Nelson 2018). Both factors should influence whether and how microbes influence seedling traits.

Additional studies evaluating the contribution of seed microbiome to the community assembly of roots and shoots of seedlings may shed light on the patterns of seed microbiome and seed germination and seedling traits. Fungal communities in *Arabidopsis alpina* seeds were more similar between roots and shoots than with seeds (Sharma et al. 2019) - this assembly pattern may explain the lack of influence from fungal taxa on plant traits seen in our study. Furthermore, Rochefort et al. (2021) showed that seedling microbiota were more influenced by the soil microbiome than seed-borne taxa, and thus the seed microbiome may not be able to colonize seedlings in order to affect traits. Contrary to our results, other evidence suggests that the seed microbiome has stronger effects on shoot traits than roots (Hardoim et al. 2012). Interestingly, Johnston-Monje et al. (2022) assessed the seed microbiome of *Brachypodium distachyon* and found greater similarity between seed and rhizosphere bacteriome, and root and shoot bacteriome, compared to the mycobiome of seeds and rhizosphere, root, or shoot mycobiome. Furthermore, seed microbiome perturbations,

such as through seed surface sterilization, can affect both root and shoot microbiome colonization (Moroenyane et al. 2021) and the seed microbiome has different influences on the colonization propensities of seedling shoots (phyllosphere) and roots (rhizosphere, rhizoplane). Specifically, seed endophytes colonize shoots more readily as there is less microbial competition in comparison to the rhizosphere (Hardoim et al. 2012). This leads to the hypothesis that the seed microbiome differentially influences seedling root and shoot growth and traits but was not observed in this study.

Ultimately, genotype and accession source played a larger role in seed germination and seedling traits in *Brachypodium* congeners and is consistent with literature demonstrating that genotype and accession drive early plant traits and germination response (Galíndez et al. 2019; Foxx and Kramer 2020). This also indicates that the influence of the seed microbiome on seedling traits and seed germination is surpassed by genotypic influences in *B. distachyon* and *B. hybridum*. The parental genotype, including influences from the parental environment, affects seed germination (Rix et al. 2012) through seed size and other mechanisms (Herman and Sultan 2011). Previous research demonstrated variations in the seed microbiome by genotype or accession (e.g., Adam et al. 2018; Abdullaeva et al. 2020; Chen et al. 2020), thus illustrating that we cannot discount genotype influences; such studies should be incorporated in future seed microbiome research to deeper investigate microbiome-trait influences.

4.3 Microbial diversity and trait responses

Results of this study did not reveal statistically significant relationships between microbial diversity and seed germination and seedling traits. This may be due to the complex nature of microbial communities and microbial functions that make community-level summary measures like diversity estimates less clear. For instance, the application of *Bacillus subtilis* and *Pantoea agglomerans* isolates on surface-sterilized grass and legume seeds had both positive, negative, or no effects on their germination rate (Dai et al. 2019). Variation in seed germination response to individual taxon and taxon in seed microbiomes highlight that the next frontier of seed microbiome research requires evaluating the functions of seed endophytes on seed performance. Unfortunately, there are limited published examples quantifying whole genomes of seed microbiomes (Tshisekedi et al. 2024). One key issue of WGS studies is the large quantity of plant DNA compared to microbial DNA. Wassermann et al. (2019) developed a protocol to deplete the host DNA to support future metagenomic analyses of seed microbiomes.

4.4 Fungal communities are more differentiated along accessions than bacterial communities

The fungal communities were more differentiated by host plant accession than bacterial communities. Our results agree with Dai et al. (2019) who showed fungal communities were differentiated than bacteria communities in seeds and seedlings by host genotype. This may suggest different inheritance strategies for fungi through vertical transmission that limit which taxa are passed, thus resulting in differentiated communities. It is also possible that the more rapid reproduction of bacteria, compared to fungi, helps them to become more cosmopolitan or ubiquitous in the environment and explains dissimilarity patterns in this study. Previous research suggests that vector pollination influences the mycobiome of seeds in which seed microbiome from self-pollinated *Brassica napus* had reduced fungal heterogeneity compared to bee-pollinated plants (Prado et al. 2020). Therefore, it is possible that self-pollination in *Brachypodium* in the greenhouse of this study also promoted distinct fungal communities. We did not observe statistically significant parental genotype variation for seed bacteriomes and mycobiomes. This differs from (Fort et al. 2021) who found the maternal genotype to explain more variation in seed fungal community composition than did maternal environment, further identifying host plant genotype as a driver for microbial community dynamics.

4.5 Parental transmission of the seed microbiome

A portion of the seed microbiome in seeds in this study were vertically transmitted from the parental plant because we included the seed embryo and endosperm in microbiome quantification, for which microbes of this tissue type are more likely to be transmitted vertically than those associated with the seed coat (Barret et al. 2016). The seed embryo contains microbes (Fort et al. 2021) which should be directly transmitted from the maternal plant – strengthening the maternal microbial influence and transmission to the next plant generations (Barret et al. 2016; Nelson 2018). Vertical transmission of microbiota does not inherently indicate a benefit (Nelson 2018), which may also explain the lack of relationship between diversity or microbial families with traits in this study – this should populate seeds with microbes of various functions, if any. Additionally, the seed itself acts as an important filter in which microbiota must survive high osmotic pressures within the seed and persist during development to colonize seedling tissue, and thus further structures the seed microbiome (Truyens et al. 2015; Shade et al. 2017).

4.6 Study limitations and future work for seed microbiome and seedling biology

Our study aimed to quantitatively link the seed microbiome characteristics to seed germination and seedling traits; however, our study had limitations. One question is whether the differences in the structure of variation for the accessions affected the relationship between seed microbiome and traits. Specifically, the Turkish accession is an inbred line with little genotypic variation whereas the Afghan accession likely had more genotypic variation and this imbalanced genotypic structure could have influenced the resulting variation in seed microbiomes. Additionally, quantifying the seed microbiome precludes us from evaluating germination and seedling traits on the same seed as the entire seed sample was destroyed for microbial DNA extraction. It is not feasible to remove microbiota from the seed then germinate the seed as the seed microbiome is now significantly disrupted by its collection and any influences on germination and seedling traits are altered. Thus, here we used mean trait values for each parental genotype to correspond to the summarized microbiome characteristics from each parental plant. This sample mismatch problem is widespread in microbiome research (e.g., Li et al. 2022), ranging from longitudinal and cross-sectional studies that often must evaluate the phenotype of a matched pair, or a separate organism to compare an intervention (Sui et al. 2024). This means that inference cannot be directly made between the organism (seedlings, in our case) and its microbiome (the seed microbiome here) through conventional methods. Future work should quantitatively link organism attributes to its microbiome, as reported by Silverman et al. (2018), who summarized existing approaches to address statistical issues in microbiome data of longitudinal studies, ranging from design considerations to model choice and evaluation.

Surface seed sterilization functions to remove seed coat microbes that are more likely to be horizontally transferred through incidental contact and to leave a majority of internal microbiota that are more likely to be vertically transmitted. However, seed surface and endocarp microbiota can influence plant growth (Berg and Raaijmakers 2018) and studies should next assess if seed sterilization influences the relationship between seed microbiome and seedling traits as surface sterilization can slightly reduce bacterial ASV richness and significantly reduce fungal ASV richness (Simonin et al. 2022). Another consideration for this study is that seeds used here were sourced from a seed repository (GRIN) which limited our control and knowledge of the seed procurement and handling approaches before we received the seeds. This matters because the storage timing and temperature can affect bacterial communities of seeds (Dutta et al. 2022). Our approach of growing the parental plants in

a common environment for seed production helps to mitigate maternal effects from the maternal environment. This putatively includes differences in seed handling, allowing us to better highlight plant genotype effects, where they exist. Furthermore, whole genome sequencing and functional profiling through proteomics and metabolomics will allow the field to significantly expand our understanding of the role of seed endophytes and this represents a rich path to advance our knowledge. This should also help to vastly improve our understanding of the influences of oomycetes and viruses in seed microbial communities as well.

5 Conclusions

This is one of only two studies to our knowledge to quantitatively link the seed microbiome to seed germination or seedling traits. A major finding of this study is that the abundance of most prevalent bacterial and fungal families positively influenced germination during cold stratification for *Brachypodium* accessions and that the seed microbiome likely has a diminished influence on seedling traits. Whether and how the parental genotype and the seed microbiome mediate seedling traits requires further study. This is an area of interest in both agriculture towards trait prediction (Friesen et al. 2011) and similarly in restoration towards inoculation of arbuscular mycorrhizal fungi in native plant restoration (Kozioł et al. 2018) and requires future work to support in its application.

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Data availability The sequences are available on NCBI under the project number, PRJNA887430, and the code underlying R analyses is available on GitHub (https://github.com/aliciafoxx/BRDI_Seed_m_b_exp/tree/main).

Declarations

Competing interests The authors declare no competing interests.

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