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Heritability and Functional Importance of the *Setaria viridis* Bacterial Seed Microbiome

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ABSTRACT

Seed-associated bacteria represent an important reservoir of microorganisms passed onto progeny plants and have been postulated to be important for early plant development and early plant vigor. According to a few reports, some bacterial taxa seem to be transferred from seed to seed and some seed-associated microorganisms may derive from insect visits during flowering; however, the origin of seed endophytes is poorly understood. To better understand the origin, ecology, and functional role of seed bacterial endophytes, we planted *Setaria viridis* seeds over several generations in a sterile growth substrate. Seed microbiota of each generation were analyzed by next generation sequencing of 16S rRNA genes and seeds were characterized regarding to their germination and plant growth. Growing plants in a sterile (or highly depleted) substrate resulted in seed microbiota, which were largely less diverse and which had altered community composition,

particularly at later generations, indicating that soil is an important reservoir of seed microbiota. Some taxa were inherited to the next generations seeds; however, different subsets of taxa were inherited in different seeds/seed batches and across different generations. This suggests that other factors than the host control the establishment of most seed endophytes and only few, e.g., obligate endophytes, might be consistently inherited. Furthermore, we observed a drastic decline in seed vigor and later generations were particularly affected. Overall, our results demonstrated that the supply of endophytes from external sources such as the soil/rhizosphere environment is highly important for the build-up of a healthy seed microbiome warranting early plant establishment and vigor of next generation plants.

Keywords: bacterial microbiota, diversity, endophytes, heritability, microbiome, seed germination, seed microbiota, *Setaria*

Plants are associated with highly diverse microbiota, which have important functions for their host and are responsible for certain plant traits. Both, external (e.g., rhizosphere, phyllosphere) and internal plant habitats (e.g., endosphere) host microorganisms, and microbial diversity may greatly differ depending on the plant habitat, but also on the vegetation stage, the plant genotype, as well

as on environmental factors (Hardoim et al. 2015; Vorholt 2012). Generally, most plant tissues are colonized by bacterial, archaeal and/or fungal endophytes, microorganisms, which spend at least part of their life cycle inside plants (Hardoim et al. 2015). Endophytes have a highly intimate association with plants, and therefore have been reported to have important functions for their host (Berg et al. 2016; Hardoim et al. 2015). Many bacterial endophytes derive from the soil and rhizosphere environment, representing a subset of rhizosphere microorganisms, which establish first on roots and then enter roots colonizing either the epidermis or cortical cell layers. From there, bacteria may spread to above-ground tissues by migrating via the xylem or intracellular spaces. Whereas colonization of stems and leaves is well known, endophytic colonization of reproductive organs like flowers, fruits, and seeds has been investigated only recently (Bergna et al. 2018; Compant et al. 2011; Escobar Rodríguez et al. 2018; Rezki et al. 2018; Shade et al. 2013; Truyens et al. 2015, 2016).

The consideration of microorganisms colonizing reproductive organs, most importantly seeds, is highly relevant, as these microbiota represent a reservoir of microorganisms passed on to progeny plants (reviewed by López-López et al. 2010; Mitter et al. 2017; Truyens et al. 2013, 2015) and thus an additional important source of endophytic bacteria. Heritability (i.e., transmission from seed to seed) of bacterial endophytes has been rarely reported

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(Bergna et al. 2018; Escobar Rodríguez et al. 2018; Johnston-Monje and Raizada 2011; Rezki et al. 2018) and is better known for certain fungal endophytes such as *Epichloë* members (*Neotyphodium* for anamorphs) (Saikkonen et al. 2002). Seed endophytes have been postulated to play an important role for germination and early plant development (Cope-Selby et al. 2016; Goggin et al. 2015; Sánchez-López et al. 2018); however, only few studies have addressed specific functions of seed microbiota on plant function (Goggin et al. 2015; Johnston-Monje and Raizada 2011; Puente et al. 2009). It has been shown that seed endophytes may also be released into the rhizosphere of juvenile plants (Hardoim et al. 2012; Johnston-Monje et al. 2016). Based on these findings as well as on their own results, Cope-Selby et al. (2016) suggested that seed endophytes are dormant until the seed matures and then germinate along with the seed. They found that active seed-derived bacteria were concentrated at the root tip of developing plants providing a rich and active inoculum, and that the mucilage produced by the root tip provides a rich source of nutrients for the multiplication of seed-derived bacteria. At the same time the mucilage helps to establish the rhizosphere environment supporting the colonization of bacteria released by roots as well as those deriving from the surrounding soil. Barret et al. (2015) reported a major decrease in endophyte diversity during the transmission from germinating seeds to seedlings; however, the mechanisms of this selection exerted by young plants on seed-derived microbiota are unknown.

Bacterial seed endophytes have shown plant growth-promoting activities (Chimwamurombe et al. 2016; Johnston-Monje and Raizada 2011; Puente et al. 2009). Furthermore, Rybakova et al. (2017) found a relationship between the diversity and abundance of seed microbiota and pathogen susceptibility. Therefore, seed endophytes are generally considered as highly important for their host (Berg and Raaijmakers 2018; Cope-Selby et al. 2016; Truyens et al. 2015); however, the specific role of seed microbiota for plant development and vitality has been rarely addressed. The major objective of this study was to test the role of bacteria associated with seeds for early plant growth and vitality. Our understanding is that many (but not all) seedborne bacterial endophytes derive from the soil environment, whereas some endophytes may be inherited or derive from other sources (e.g., transmission via insects, water splashing, etc.). This implies that if plants are grown in a substrate with reduced microbial complexity over several generations, their seed microbiota become less diverse. Our hypothesis is that seed microbiota are important for plant development, and thus the offspring plants emerging from seeds harboring a microbial community with limited diversity are expected to have deficiencies and lose certain characteristics such as germination capacity. To test our hypothesis, we used *Setaria viridis* L. as a model plant and collected seeds of plants, grown for several generations in a sterile substrate, and assessed the bacterial community composition in seeds as well as seed germination and plant growth characteristics.

MATERIALS AND METHODS

Source of generation zero (G0) seeds. Seeds of *Setaria viridis* reference accession A10.1 (PI 669942) were kindly provided by colleagues of the Brutnell Lab (St. Louis, MO). To obtain sufficient amounts of seeds they were propagated in the greenhouse at Austrian Institute of Technology (AIT) using a 3:5 mixture of perlite and clay substrate SP ED63 (Einheitserde Werkverband, Germany). Plants were grown for 60 days with 12 h of daylight at $25 \pm 5^\circ\text{C}$ (relative humidity of 40%) and 12 h of no light at $20 \pm 5^\circ\text{C}$ (relative humidity 20%). After harvest, these generation zero (G0) seeds were stored in paper bags and dried at room temperature until

further use. Dead or aborted seeds were manually discarded and excluded from any experiments.

Promotion of seed germination and seed surface sterilization. To interrupt dormancy of harvested seeds, a total of 25 seeds were incubated overnight in 1 ml of a 2.89 mM gibberellic acid solution supplemented with 30 mM KNO_3 (Sebastian et al. 2014). The following day, seeds were surface-sterilized by treatment with a 0.5% NaOCl solution containing 0.2% K_2HPO_4 and 0.1% Tween 20 for 20 min with occasional shaking, followed by placing seeds for 5 min in 70% ethanol and washing them three times with sterile distilled water. One hundred microliters of water from the last washing step was plated on a 10% TSA (tryptic soy agar, Sigma Aldrich) plate and incubated for 5 days at 28°C to check for microbial presence. No colonies were obtained.

Production of successive generation seeds (G1, G2, and G3). Surface-sterilized G0 seeds were placed on the surface of 0.5× Murashige-Skoog (MS) phytagel plates, pH 5.7, and incubated close to a window for 14 days with natural light/dark cycles. Seedlings at the Z12 Zadoks scale (two leaves unfolded) (Zadoks et al. 1974) were carefully transferred to a jar consisting of a sterile double-decker magenta box containing 380 ± 5 g of sterile sand (Fig. 1A). The lower chamber was filled with 200 ml of a sterile filtered 0.05% Wuxal Super solution (Kwizda Agro, Austria). One seedling was grown per jar in a containment covered with a double-layered insect net in the greenhouse at AIT for 60 days (conditions as described above) or until grains were ripe and easily dented from the panicles (Zadoks scale of Z93 to Z94) (Zadoks et al. 1974) (Fig. 1B, C, and D). After harvest, these first generation seeds (G1) were stored in paper bags and dried at room temperature until further use. Dead or aborted seeds were manually discarded and excluded from any experiments. G1 seeds were further sown and cultivated in sterile substrate as described above, resulting in a total of nine seed lines that yielded “good” or “poor” performing second generation seeds (G2). While good performing seedlings from G2 seeds resulted in plants that yielded plenty of G3 seeds, their poor performing counterparts were characterized by low germination rates and high seedling mortality rates (Supplementary Data S1). Cultivation in sterile substrate was repeated for each seed line to produce a total of three consecutive generations (G1, G2, and G3, respectively). Seeds of each generation were treated to interrupt seed dormancy and to disinfect their surfaces as described above.

Assessment of seed vigor and plant fitness traits. Seeds from a total of 24 plants from all four generations (G0 to G3) of three chosen seed lines (A, E, and H) were used for the assessment of seed vigor and fitness traits of their seedlings. This choice was made to include “good” as well as “poor” performing seeds (Fig. 2). To this end, 48 surface-sterilized seeds obtained from each chosen plant were sowed separately on two different substrates (24 seeds/substrate), namely MS and a 3:5 mixture of sand and clay substrate SP ED63 (CSM). Germination percentages were monitored and documented each third day for 30 days. Watering was performed daily by gently spraying with sterile tap water. It is noteworthy that in order to obtain unaffected seed germination rates, no treatment to break seed dormancy was performed. To assess plant fitness traits, up to five seedlings at the Z12 Zadoks scale (two leaves unfolded) (Zadoks et al. 1974) were carefully transferred to a multipot tray containing either sterile sand (for seedlings germinated on MS) or CSM. Plants were grown for 60 days in the greenhouse (conditions as described above) and watered with 0.05% Wuxal Super (Kwizda Agro, Austria). After 60 days, main tiller height (TH), number and length of panicles as well as fresh weight of each plant were recorded. Total germination percentages after 30 days and the averages of fitness traits of seedlings grown for 60 days on CSM were used for correlation analyses with their

corresponding seed microbiota based on the relative abundances of bacterial operational taxonomic units (OTUs).

16S rRNA library generation for assessment of seed endophytic bacterial microbiota. For total DNA extraction, a total of three replicates per harvested plant, each consisting of 20 surface-sterilized seeds were transferred to a 2-ml tube equipped with two sterile stainless-steel beads (5 mm) and immediately frozen in liquid nitrogen. Pulverization of the frozen seed material was achieved employing a mixer mill (Type MM301, Retsch) at a speed of 20 Hz for 30 s. The obtained frozen powder was immediately suspended in the Matrix E solution of the FastDNA SPIN Kit for Soil and the DNA extraction was performed following the manufacturer's indications (MP Biomedicals). Quality and quantity of the isolated total DNA was confirmed by gel electrophoresis. Bacterial communities within seeds of each generation as well as those in negative

controls from DNA extraction procedures and no-template PCRs were assessed by sequencing amplicons of the V5 to V7 region of the 16S rRNA gene, obtained by two rounds of PCR amplification with a high-fidelity polymerase (KAPAHiFi PCR Kit, KAPA Biosystems) and employing a strategy to overcome mitochondrial DNA interference described in a related study (Escobar Rodríguez et al. 2018). Briefly, two rounds of PCR were performed for each sample. Round one included a modified 799F primer, a 1392R primer and a blocking primer designed to bind specifically to chloroplast DNA and block further amplification through a phosphoryl-group at the 3'-end. The resulting amplicons were purified after excision of the 593-bp band from the gel and the second PCR round was performed employing these amplicons as template. Round two PCR was performed with unmodified 799F/1175R primers carrying sample-specific barcodes to generate

Fig. 1. Production of seeds by growing seedlings in a semisterile sand substrate for 60 days. **A**, Seedlings in 0.5x Murashige-Skoog phytagel plates are carefully transferred to a sterile double-decker magenta box. The upper chamber was filled with sterile sand and the seedlings were provided with a nutrient solution with a wick from the lower chamber. **B**, Each box was placed in a protected zone in the greenhouse where plants grew for 60 days. **C**, Plant after 60 days (one single box per plant was used). **D**, Panicle after 60 days of cultivation: seeds are mature and are easily dented by hand. After seed harvesting, this procedure was repeated for a total of three consecutive generations.

Fig. 2. Overview of seed samples studied in this experiment. Bubbles indicate individual seed batches. *Setaria viridis* seeds were provided by the Brutnell Lab (St. Louis, MO) and propagated in the greenhouse at the Austrian Institute of Technology to obtain G0 seeds. Subsequent seed generations were obtained by cultivating seedlings in sterile sand for 60 days as indicated above. Green and red arrows point toward the progeny seeds of good and poor performing G2 batches, respectively.

376-bp-long fragments. Primer names and sequences are available (Escobar Rodríguez et al. 2018). All amplicons were purified with the Agencourt AMPure XP system and quantified with Quant-iT PicoGreen following the indications of the manufacturers. Purified amplicons were subsequently pooled in equimolar amounts and the quality of the library was assessed by 2100 Bioanalyzer (Agilent Technologies). Libraries were subjected to Illumina-adapter ligation and sequencing using 2×250 bp MiSeq v2 sequencing (Illumina Inc. San Diego, CA) at LGC Group (Berlin, Germany).

Raw sequence data processing. Raw reads were filtered with Bowtie2 v.2.3.4.3 (Langmead and Salzberg 2012) to avoid the presence of Illumina's phi X contamination and quality was preliminarily checked with FastQC v.0.11.8 (Andrews 2010). Samples were demultiplexed, adapters and primers were stripped using Cutadapt v.1.18 (Martin 2011). Paired-end reads of each sample were merged with PEAR v.0.9.8 (Zhang et al. 2013). The quality of merged reads was accurately assessed and then sequences filtered accordingly using VSEARCH v.2.9.1 (Rognes et al. 2016) with a maximum expected error threshold of 1. Filtered reads were screened with Metaxa2 v.2.2 (Bengtsson-Palme et al. 2015) to target the presence of the V5 to V7 region of the 16S rRNA gene and to discard sequences other than archaea or bacteria. Targeted reads were dereplicated and then denoised and chimeras were removed following this approach reimplemented in VSEARCH: denoising was performed with the UNOISE3 method (Edgar 2018) and sequence variants checked for the presence of chimeras with UCHIME3 de-novo detection, which is an improved version of the UCHIME2 algorithm (Edgar 2016). Zero-radius OTUs (ZOTUs) obtained were then mapped against previous targeted reads with a global pairwise alignment and an OTU-like count table generated. For simplification purposes, we will further refer to all ZOTUs as "OTUs". Taxonomic assignment was performed with the RDP classifier (Wang et al. 2007) against the SILVA SSU 132 database (Quast et al. 2012). Prior to any further analyses, OTUs from negative controls were removed from the rest of the data set.

Sequence data are available at NCBI SRA database under the accession SRR8940706 and BioProject number PRJNA534478.

Analyses of seed endophytic bacterial microbiota. Alpha-diversity values were calculated using the rtk R package (Forslund et al. 2017) after multiple rarefactions, averaging the results of 999 iterations. The rarefaction minimum depth was 1,030. For beta-diversity purposes, a cumulative sum scaling (CSS) normalization (Paulson et al. 2013) was applied. Microbial richness was assessed by counting observed OTUs and diversity was quantified using the Simpson's index. We explored the differences in richness, diversity, and composition across generations including the seed line factor as a random effect component in the model as well as for each seed line (A, E, and H), separately. Both alpha- and beta-diversity values were calculated using absolute OTU counts. Indicator genera for G0, G1, G2, and G3 seeds were assessed by fitting a multivariate generalized linear model to the CSS normalized data using the mvabund::manyglm R function. The top 15 most abundant significantly different genera across the generations were depicted as an area plot using the ggplot2 R package. Representative bacterial members in each set of triplicates were targeted by only considering reproducibly occurring OTUs (rOTUs), namely those OTUs present in at least two of three replicates. Presence and absence of rOTUs in each generation across seed batches were tracked using the VENN v2.1 online tool (Oliveros 2007). Furthermore, differential OTU abundances in G1, G2, and G3 seeds were identified employing the DESeq function of the DESeq2 R-package (Love et al. 2014) using raw OTU counts of G0 seeds as reference. To this end, samples with less than 500 OTU counts were removed from the data set and the adjusted *P* value was set to a

cutoff of 0.01. Spearman correlations of relative rOTU abundances with seed germination percentages and plant fitness traits were performed using CSS normalized rOTU read counts as well as by scaling germination and trait values using z-scores. This was achieved by employing the microbiome R package (Leo et al. 2017) using adjusted *P* values of 0.01 as threshold for statistical significance (see below).

Statistical methods. Processed sequence data were analyzed in R v3.5.1 software environment using the Phyloseq (McMurdie and Holmes 2013) and vegan (Oksanen et al. 2017) packages. We assessed statistical significance at $\alpha = 0.05$ (unless indicated otherwise) and, whenever necessary, adjusted *P* values for multiple comparisons using false discovery rate controlling procedures. Differences across generations (G0, G1, G2, and G3) were investigated on richness and diversity values, respectively, considering the seed lines as a random component in linear mixed-effect models followed by analysis of variance (ANOVA). The homogeneity assumption for the applicability of the ANOVA was checked with a graphical verification of residuals versus fitted values. A confirmatory test based on a nonparametric recursive partitioning analysis (Monte Carlo random sampling, 9,999 iterations) was also applied on a model including both richness and diversity values, together with the generation and seed line factors (partykit R package). Post hoc pairwise comparisons were carried out employing an estimated marginal mean (EMM) approach (Lenth 2018).

Additionally, differences among seed batches of each generation were further investigated with EMMs on linear models in which seed lines were treated separately. Differences in bacterial community composition (beta-diversity) within seeds across generations were tested with a multivariate generalized linear model function implemented in the mvabund R package (Wang et al. 2019), assuming a negative binomial distribution of the data and reshuffling samples with 999 permutations considering the seed line as a random component in the model. Significant differently abundant OTUs through generations ($P < 0.05$, adjusted with a step-down resampling procedure) were extracted and grouped at genus level using the RAM R package (Chen et al. 2018).

RESULTS

Seed vigor and plant fitness traits across generations. Overall, the number of seed batches with germination percentages greater than 0% was higher in medium agar plates (MS, Murashige-Skoog phytagel) than in the clay-sand mixture (CSM, sand and clay substrate SP ED63) (Fig. 3). While G0 seeds reached similar germination percentages in both substrates after 30 days (67% in MS versus 71% in CSM), germination was faster in MS than in CSM, maybe due to the higher humidity on MS plates. Moreover, when planted in CSM, germination percentages of seeds from all subsequent generations decreased dramatically, reaching a maximum of 21% (G1/A) (Fig. 3A). In MS, germination percentages were relatively stable in G1 compared with G0 seeds. However, germination was mostly inhibited in G2 and remained low in G3 seeds, with few exceptions (Fig. 3B). In terms of plant performance, G0 seedlings grew into mature plants that showed comparable biomass production in both substrates, and G1 seedlings resulted in plants with similar characteristics when grown in CSM (Fig. 4). In contrast, seedlings of generations G2 and G3 failed to establish in CSM and grew into plants with reduced biomass, plant height, panicle numbers, and panicle length when compared with G0 seedlings grown in MS (Fig. 4B, C, and D). Additionally, seedlings showed increasing and/or more variable mortality rates with each generation (Fig. 4E).

Seed endophytic bacterial communities across generations.

Sequences of the V5 to V7 region of the 16S rRNA gene present in seed samples of all generations were processed and data were used to generate an OTU table to be further analyzed in R 3.5.1. OTUs were filtered keeping only those with a maximum relative abundance greater than 0.01% in at least one sample, ending up with a total of 797 unique OTUs. For statistical purposes, a total of 552,997 high-quality merged reads corresponding to an average of $8,132.31 \pm 5,183.47$ reads per sample and a mean length of 375.81 bp were considered. Number of reads per generation as well as average sequences and OTUs per sample are displayed in Table 1.

Despite not displaying significant differences ($P > 0.05$), bacterial richness showed a decreasing trend with each generation (Fig. 5A). The steepest decline in bacterial richness was observed for seed line A, which remained consistent throughout generations. Richness in seed line E decreased dramatically from G1 to G2 and was less abrupt from G2 to G3. In contrast, richness values for seed line H remained virtually unaffected. In terms of diversity (Simpson's index), G3 batches from seed line A showed significantly lower values than those from preceding generations ($P < 0.05$). Diversity values for seed batches in line E significantly declined with each generation. No loss in diversity was observed throughout generations for seed line H (Table 2). Overall, the analysis conducted on diversity values (Fig. 5B) showed a significant effect of the

generation ($F = 12.56$, $P < 0.0001$) and EMM comparisons reported significant differences between the first three generations against the last one. The decision tree generated by recursive partitioning analysis (Supplementary Fig. S1) confirmed that differences must be imputed mainly to the generation ($P < 0.001$) and only marginally to variations in the seed line ($P = 0.045$).

In all seed lines, predominant endophytic bacterial colonists were members of *Proteobacteria* (44%), *Actinobacteria* (27%), *Bacteroidetes* (11%), and *Firmicutes* (7%). However, there were significant differences in the bacterial communities among generations ($\text{Dev} = 5,205.65$, $P < 0.001$). These differences were also shown in a nonmetric multidimensional scaling ordination plot ($R^2 = 0.992$, stress = 0.089) (Fig. 6A). While G0 and G1 seeds hosted similar assemblages, abrupt shifts in the abundance of indicator genera in generations G2 and G3 were responsible for these differences). G2 seeds, for instance, hosted higher abundances of *Aliihoeflea*, *Lactobacillus*, *Longispora*, and *Nocardioopsis* compared with G0 and G1 seeds, while *Microbacterium*, *Flavobacterium*, and *Pedobacter* were eradicated. *Burkholderia-Paraburkholderia* and *Ralstonia* became dominant in G3 seeds, which also showed enrichment of *Aquabacterium*, *Acidocella*, and *Bradyrhizobium*, while *Stenotrophomonas* and *Arthrobacter* perished (Fig. 6B).

Further, there were increases in the number of differentially abundant OTUs with each generation compared with G0 communities (Fig. 7A).

Fig. 3. A, Germination curves of different *Setaria viridis* seed lines and batches throughout generations G0, G1, G2, and G3 in a 3:5 clay/soil mixture (CSM) and in **B**, 0.5x Murashige-Skoog (MS) phytagel.

When comparing G1 to G0 seeds, seven different OTUs were significantly depleted in G1, while the other two were enriched. Intriguingly, the numbers of differentially abundant OTUs increased as we compared G2 seeds with those of G0, namely 49

OTUs were found depleted and 28 enriched in G2. Similarly, G3 showed 48 significantly depleted and a total of 14 OTUs enriched OTUs compared with the original G0 seeds. Investigation of those OTUs that were depleted or enriched for each generation when

Fig. 4. Fitness traits recorded for seedlings and plants of all three generations grown in both clay/soil mixture (CSM) and Murashige-Skoog (MS) phytagel for 60 days in the greenhouse. No G2 plants were obtained after 60 days of cultivation in either CSM or MS and G3 seedlings failed to establish in CSM. **A**, Fresh weight declined in G1 and G3 plants grown in MS compared with G0. **B**, Main tiller height was comparable for plants grown in the same substrate. **C**, Number of panicles per plant increased in plants grown in MS. **D**, The first panicles of each plant decreased with each generation. **E**, Seedling mortality rates became higher and/or more variable with each generation. A zero-mortality percentage for G2 seeds in CSM is displayed as no germination occurred for these seeds in this substrate.

compared with G0 seeds (Fig. 7B) revealed seven OTUs whose abundances consistently declined from G1 to G3 seeds. Furthermore, abundances of 30 OTUs of 21 different genera declined in both G2 and G3 seeds when compared with G0, whereas 11 OTUs were depleted specifically in G2 as well as G3 seeds. In contrast, a trend of generation-specific enrichment of OTUs was observed in each G1, G2, and G3, while no OTUs showed consistent increase in abundance across generations. Additional information of differentially abundant OTUs and taxa can be found in Supplementary Data 2.

Seed endophytic bacterial microbiota, seed vigor, and plant performance. In order to identify representative communities from each seed batch, we used a subset of the data set containing only rOTUs (Escobar Rodríguez et al. 2018; Pfeiffer et al. 2017). We identified a total of 41 rOTUs whose relative abundances correlated significantly to seed germination percentages at 30 days postsowing (GP) (Fig. 8A). Relative abundances of 10 of these rOTUs correlated positively to the GP in both substrates and were members of four genera: *Flavobacterium*, *Microbacterium*, *Pedobacter*, and *Sphingomonas*. Additionally, relative abundances of six further OTUs were positively correlated with germination in CSM and

belonged to the *Acidimicrobiales*, *Bacillus*, *Oxalobacteraceae*, *Propionibacterium*, *Psychrobacter*, and *Sphingomonas*. Interestingly, eight of these rOTUs were found to be depleted in G2 seeds after comparison with G0 or G1. Relative abundance of one single rOTU (*Aliihoeflea*) showed negative correlation with GP on this substrate and was significantly enriched in G2 and G3 seeds. Moreover, relative abundance of three rOTUs (OTU_776, OTU_8, and OTU_61 classified as *Burkholderiaceae*, *Burkholderia-Paraburkholderia*, and *Dyella*, respectively) showed positive correlation with GP only in MS and were identified among the depleted OTUs in G2 seeds, while being enriched in G3. Finally, relative abundances of a large group of 21 OTUs of 16 different taxa were negatively correlated with GP in the same medium and were mostly enriched in G2 seeds, while depleted in G3.

In terms of plant fitness traits (Fig. 8B), relative abundances of six rOTUs were correlated negatively with obtained fresh plant weight, main tiller height and panicle length in plants grown in CSM and were significantly depleted in G2 and G3 seeds. An additional single rOTU (OTU_208) classified as *Burkholderia-Paraburkholderia* showed positive correlation with panicle numbers and was found to be enriched in G3 seeds.

TABLE 1 Number of high-quality merged reads (mean length = 375.81 bp) per generation and average number of sequences per sample used for statistical tests				
Generation	Number of samples (n)	Number of high-quality merged reads	Average number of sequences per sample	Average of observed operational taxonomic units per sample
G0	n = 3	19,752	6,584 ± 2,263.8	58.55 ± 15.3
G1	n = 9	61,020	6,780 ± 3,515.5	52.38 ± 12.5
G2	n = 27	305,997	11,769.12 ± 5,812.8	48.19 ± 11.2
G3	n = 33	166,228	5,540.93 ± 3,092.5	45.36 ± 11.4
Total	n = 72	552,997	8,132.31 ± 5,183.4	797

Fig. 5. Boxplots of **A**, microbial richness and **B**, diversity throughout generations (G0, G1, G2, and G3). Seed lines (A, E, and H) are shown as colors.

We further traced the transmission/loss of rOTUs from G0 to G2 and G3 seeds to identify consistent presence/absence of taxa among G2 seed batches with good and bad viability (Fig. 9). Good performing G2 seeds (those which resulted in plenty of healthy seedlings and adult plants during the seed production stage of this experiment) were characterized by conserving the most amount of rOTUs (i.e., conserved overall bacterial assemblages) from G0 through G1 to G2 and G3, while poor performing G2 seed batches showed minimal overlap with the microbiota of G0 and G1 seeds. In

seed line A, for instance, 23 different rOTUs were transmitted from G0 to good performing G2 and G3 seed batches, while only five persisted in the poor performers (Fig. 9A). This observation was similar in seed line E: 29 versus three rOTUs were conserved across generations in good and poor performing G2 and G3 seed batches, respectively (Fig. 9B). In seed line H, however, the overlap of inherited rOTUs between good and poor performing seed batches comprised five rOTUs, while three were specific to good performing seed batches (Fig. 9C). Interestingly, seven rOTUs of five

TABLE 2
Diversity values for seed batches of all three seed lines based on Simpson's index

Seed line	Role of generation in diversity (Simpson's index)	Generation/seed batch	Estimated marginal means for diversity values	Contrasts in diversity (alpha = 0.05)	Role of seed batch in beta diversity values
A	$F = 15.24, P < 0.001$	G1/A	0.87	a	$P = 0.001$
		G2/A1	0.90	a	
		G2/A2	0.88	a	
		G2/A5	0.85	a	
		G3/A1/1	0.81	ab	
		G3/A1/2	0.74	b	
		G3/A1/3	0.74	b	
		G3/A2/2	0.72	b	
E	$F = 20.59, P < 0.001$	G1/E	0.90	a	$P < 0.01$
		G2/E1	0.85	ab	
		G2/E2	0.89	a	
		G2/E4	0.87	ab	
		G3/E1/2	0.73	c	
		G3/E4/1	0.80	bc	
		G3/E4/2	0.76	c	
H	$F = 1.37, P > 0.05$	–	–	–	$P < 0.01$

Fig. 6. A, Nonmetric multidimensional scaling (NMDS) plot depicts differences among all generations. **B,** Area plot represents the first 15 most abundant genera resulting in significant differences across generations after analysis of deviance on a multivariate generalized linear model. CSS, cumulative sum scaling.

genera were conserved across all generations of good performing seed batches and were shared among the three seed lines, namely *Agromyces* (OTU_238), *Burkholderia-Paraburkholderia* (OTU_8, OTU_31, and OTU_273), *Mycobacterium* (OTU_246), *Propionibacterium* (OTU_28), and *Ralstonia* (OTU_34), together with two rOTUs of uncultured candidate classes (OTU_788 and OTU_3258, classified as MB-A2-108 of the *Actinobacteria* and OPB35 soil group of the *Verrucomicrobia*, respectively). A complete list of overall conserved rOTUs across generations, their taxonomic classification, and representative 16S rRNA gene sequences is presented in Supplementary Data 3.

Finally, poor performing G2 seed batches were also characterized by loss of inherited rOTUs after G1 and included taxa with a potentially positive effect on germination (Fig. 6), namely *Bacillus* (OTU_68), *Flavobacterium* (OTU_63 and OTU_2845), *Microbacterium* (OTU_1348, OTU_1710, and OTU_3040), *Acidimicrobiales* (OTU_235), *Pedobacter* (OTU_1368, OTU_2354, and OTU_2698), and *Sphingomonas*

(OTU_81 and OTU_179). Additional information on taxa in good and poor performers can be found in Supplementary Data 4.

DISCUSSION

For a long time, healthy seeds have been considered as “sterile” and chemical treatments for seed cleaning have been developed with the aim to avoid any pathogen contamination (Berg and Raaijmakers 2018). However, in the last years it has become evident that different niches within seeds are colonized by endophytes (Glassner et al. 2017) and that seeds are host to rather diverse microbial communities (Truyens et al. 2015). Several studies report that seed endophytes derive from the soil environment, colonize roots, and then translocate to above-ground plant tissues (Compant et al. 2010; Escobar Rodríguez et al. 2017; Truyens et al. 2015); however, few studies also indicate heritability of some endophytes, i.e., transmission from seed to seed (Escobar Rodríguez et al. 2018;

Fig. 7. A, Volcano plots display the amount of differentially abundant operational taxonomic units (OTUs) for each generation compared with G0. **B**, Number and taxonomic classifications of depleted and enriched OTUs across generations.

Johnston-Monje and Raizada 2011; Rezki et al. 2018). In addition, seed endophytes may derive from insect visits, particularly phloem-feeding insects may transfer microbiota from plant to plant (López-Fernández et al. 2017). Our results confirm that the soil is an important reservoir of seed microbiota, as seeds of plants grown over several generations in a sterile substrate generally showed a decline in bacterial richness and diversity. Similarly, it is likely that in our experimental set-up phyllosphere microbiota were depleted, which might also be a source of seed microbiota. However, the soil

environment is generally considered the major reservoir of bacterial endophytes, which potentially could further translocate to seeds. Furthermore, our results indicated heritability of seed endophytes, but only a restricted set of taxa was transmitted to the successive plant generation, which is in line with the observations of Rezki et al. (2018). However, strain-specific identification would be required to confirm seed to seed transmission.

We obtained different results for seeds derived from different mother seeds (i.e., seed lines A, E, and H). Line A showed a

Fig. 8. A, Correlations between reproducibly occurring operational taxonomic units (rOTUs) and germination percentages after 30 days (GP) in clay/soil mixture (CSM) and Murashige-Skoog (MS) phytigel. **B**, Correlations between rOTUs and the recorded plant fitness traits grown in CSM. Significant correlations are indicated with a plus sign (+). Circles at the right of the plot indicate whether an rOTU was found depleted or enriched. Text inside the circle specify the seed generation where this effect was observed. Lanes indicate the generation used as reference to compare differences in abundance.

declining bacterial richness throughout generations, whereas line E showed a drastic decline from G1 to G2 and a less drastic decline from G2 to G3, and no decline was observed for line H. Nevertheless, all seed lines showed generation-specific community structures, i.e., also seeds derived from line H having similar richness and Simpson's diversity across generations showed different community structures. As plants were grown in sterile sand and were protected from insect visits, we expect only limited colonization from the outside environment and most or at least many of the endophytes seem to derive from seeds. The fact that different seeds (obtained from the same seed batch) showed different diversities as well as different patterns in regard to changes over generations indicate that different subsets of endophytes are inherited to the next generation, potentially depending on the set of bacteria residing in the original (G0) seed. This is in line with previous reports suggesting that ecological drift is most important for structuring bacterial seed microbiota (Klaedtke et al. 2016; Rezki et al. 2018). Furthermore, various parameters such as soil fertility, plant stress conditions or more generally the physiological state of the plant or even microbial interactions may have contributed to the different sets of inherited bacteria. Overall, the fact that only subsets of bacteria are transmitted from seed to seed confirm the filtering process during seed to seed transmission. Similar effects were also observed by Vannier et al. (2018) when analyzing transmission of plant endophytes to clonal progeny.

Each generation of *Setaria* seeds hosted specific microbiomes; however, G0 and G1 seeds hosted similar assemblages and drastic shifts were observed in the later generations when compared with G0. Differences in seed microbial composition at different generations may be partly explained with the observed depletion of seed microbiomes, at least of lines A and E, but might also be more

generally related to the absence (or drastically reduced abundance and diversity) of microorganisms in the substrate (sterilized sand) and rhizosphere environment. Also, altered seed morphology might have contributed to the observed differences. Even if richness of Simpson's diversity values remained similar over generations, as it was the case of line H, the lack of microorganisms derived from the rhizosphere could have led to the formation of different communities, e.g., providing an opportunity for seed-derived microbiota (e.g., in low abundance) to establish, which would have been outcompeted by soil-derived microorganisms. It is furthermore well known, that stress conditions greatly influence plant-associated microbiota via modulating plant physiology (Berendsen et al. 2018; Naylor et al. 2017). In our experimental set-up it was evident that plants grown in sterile conditions showed low fitness indicating stressed plants. The lack or limited availability of microorganisms in the rhizosphere as well as the altered or depleted seed microbiota may be associated with plant (seed) physiological changes (Goggin et al. 2015), also contributing to the establishment of different microbial communities.

A number of taxa diminished at later generations, particularly starting with G2. A wide range of taxa were depleted including genera well known to comprise strains with plant beneficial effects such as *Pseudomonas*, *Kocuria*, *Bacillus*, *Paenibacillus*, *Microbacterium*, *Pedobacter*, *Burkholderia/Paraburkholderia*, *Flavobacterium*, and others. Some taxa were enriched, generally comprising different taxa than those depleted with few exceptions, and generally the enriched taxa comprised less well known for plant growth promotion than the depleted taxa. Overall, we observed a drastic decline in seed vigor from G0 to G3, and G2 and G3 seeds were particularly affected. Seeds obtained from later generations in many cases failed to produce seedlings, produced less biomass,

Fig. 9. Transmission or loss of reproducibly occurring operational taxonomic units (rOTUs) throughout generations (A, seed line A; B, seed line E; and C, seed line H). Blue numbers indicate the amount of rOTUs inherited from G0 to G1 and lost before G2. Green and red numbers indicate the amount of rOTUs inherited from G0 through G1 to good and poor performing G2 and G3 seeds, respectively.

height, and panicle length compared with G0 seeds. Considering that seeds are an important reservoir of microorganisms supporting early plant development (Cope-Selby et al. 2016; Goggin et al. 2015), it seems that inherited bacteria from G0 to G1 still maintain beneficial functions to a certain extent. However, due to the constantly missing supply of bacteria from external sources as well as the lack of consistently inherited bacterial taxa from G0 to the later generations, it seems that seed microbiota get depleted and presumably lose important supportive functions. Also, those seeds, which could maintain similar microbiota as in G0 showed better performance compared with seeds that had minimal overlap with G0 or depleted seed microbiota. These results indicate the high importance of seed microbiota for plant growth and vitality, at least at early stages. Additionally, the depleted rhizosphere and phyllosphere microbiota may have also been responsible for the declined seed vigor, however, do not necessarily explain the increasing decline of seed vigor at later generations.

We found few core taxa, which were shared among the three seed lines and which were also conserved across generations of good-performing seeds, including *Agromyces*, *Burkholderia/Paraburkholderia*, *Mycobacterium*, *Propionibacterium*, *Ralstonia*, and two uncultured taxa belonging to the *Actinobacteria* and the *Verrucomicrobia*. Further, the abundance of various taxa such as *Microbacterium*, *Flavobacterium*, *Pedobacter*, *Sphingomonas*, *Bacillus*, *Propionibacterium*, and others correlated with germination capability (seed germination percentage). These taxa were also typically depleted in poorly performing seeds of the later generations. Many of these taxa are well known for plant growth promotion. However, others like *Ralstonia* are better known as plant pathogens or do not represent typical plant-associated bacteria (like *Mycobacterium* or *Propionibacterium*). Recently, similar taxa including *Ralstonia* were reported to colonize tomato seeds (Bergna et al. 2018). More detailed classification, beyond a partial 16S rRNA gene sequence, would be needed for a more elaborate phylogenetic assignment. Furthermore, beneficial functions might not only depend on individual members but may also be a result of microbial interactions involving microorganisms less known for plant beneficial effects (Sessitsch et al. 2019). In addition, the dynamics of soil-derived microbiota in plants and their transmission to seeds should be further investigated using various comparative treatments such as using soils with defined soil microbiota of different complexities or different stress conditions.

In conclusion, our results demonstrate the huge importance of seed microbiota for plant establishment and growth. It can be expected that seed microbiota do not only play a role at early stages, but act also as reservoir of microorganisms systematically colonizing plants and exhibiting important functions at later stages by, for instance, facilitating nutrient uptake or suppressing pathogens (Bergna et al. 2018; Rybakova et al. 2017). We could also demonstrate that some taxa are inherited to the next generation seeds; however, different subsets of taxa were inherited in different seeds/seed batches and across different generations. This suggests that factors other than the host control the establishment of most seed endophytes and only few, e.g., obligate endophytes, might be consistently inherited. Nevertheless, seed microbiota have been reported to be highly cultivar-dependent (Rybakova et al. 2017). This might be related to different physiological characteristics of different cultivars resulting in the establishment of different plant- and seed-associated microbiota. Our results also demonstrated that the supply of bacterial endophytes from external sources such as the soil/rhizosphere/phyllosphere environment is highly important for the build-up of a healthy seed microbiome warranting early plant establishment and vigor of next generation plants. In addition, seed endophytic fungi as well as interactions between different

microbiota members are likely to affect seed quality and plant performance and merit further investigation.

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LITERATURE CITED

- Andrews, S. 2010. FastQC: A quality control tool for high throughput sequence data. Babraham Bioinformatics. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
- Barret, M., Briand, M., Bonneau, S., Prévieux, A., Valière, S., Bouchez, O., Hunault, G., Simoneau, P., and Jaques, M.-A. 2015. Emergence shapes the structure of the seed microbiota. *Appl. Environ. Microbiol.* 81:1257-1266.
- Bengtsson-Palme, J., Hartmann, M., Eriksson, K. M., Pal, C., Thorell, K., Larsson, D. G. J., and Nilsson, R. H. 2015. metaxa2: Improved identification and taxonomic classification of small and large subunit rRNA in metagenomic data. *Mol. Ecol. Resour.* 15:1403-1414.
- Berendsen, R. L., Vismans, G., Yu, K., Song, Y., de Jonge, R., Burgman, W. P., Burmølle, M., Herschend, J., Bakker, P. A. H. M., and Pieterse, C. M. J. 2018. Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME J.* 12:1496-1507.
- Berg, G., and Raaijmakers, J. M. 2018. Saving seed microbiomes. *ISME J.* 12:1167-1170.
- Berg, G., Rybakova, D., Grube, M., and Köberl, M. 2016. The plant microbiome explored: implications for experimental botany. *J. Exp. Bot.* 67:995-1002.
- Bergna, A., Cernava, T., Rändler, M., Grosch, R., Zachow, C., and Berg, G. 2018. Tomato seeds preferably transmit plant beneficial endophytes. *Phytobiomes J.* 2:183-193.
- Chen, W., Simpson, J., and Levesque, C. A. 2018. RAM: R for amplicon-sequencing-based microbial-ecology. R package version 1.2.1.7. <https://cran.r-project.org/web/packages/RAM/index.html>
- Chimwamurombe, P. M., Grönemeyer, J. L., and Reinhold-Hurek, B. 2016. Isolation and characterization of culturable seed-associated bacterial endophytes from gnotobiotically grown Maramba bean seedlings. *FEMS Microbiol. Ecol.* 92:fiw083.
- Compant, S., Clément, C., and Sessitsch, A. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42:669-678.
- Compant, S., Mitter, B., Colli-Mull, J. G., Gangl, H., and Sessitsch, A. 2011. Endophytes of grapevine flowers, berries, and seeds: Identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microbiol. Ecol.* 62:188-197.
- Cope-Selby, N., Cookson, A., Squance, M., Donnison, I., Flavell, R., and Farrar, K. 2016. Endophytic bacteria in *Miscanthus* seed: Implications for germination, vertical inheritance of endophytes, plant evolution and breeding. *GBC Bioenergy* 9:57-77.
- Edgar, R. C. 2016. UCHIME2: Improved chimera prediction for amplicon sequencing. *bioRxiv* 074252.
- Edgar, R. C. 2018. Updating the 97% identity threshold for 16S ribosomal RNA OTUs. *Bioinformatics* 34:2371-2375.
- Escobar Rodríguez, C., Mitter, B., Antonielli, L., Trognitz, F., Compant, S., and Sessitsch, A. 2018. Roots and panicles of the C4 model grasses *Setaria viridis* (L.) and *S. pumila* host distinct bacterial assemblages with core taxa conserved across host genotypes and sampling sites. *Front. Microbiol.* 9:2708.
- Escobar Rodríguez, C., Mitter, B., Barret, M., Sessitsch, A., and Compant, S. 2017. Commentary: Seed bacterial inhabitants and their routes of colonization. *Plant Soil* 422:129-134.
- Forslund, K., Saary, P., Hildebrand, F., and Bork, P. 2017. RTK: Efficient rarefaction analysis of large datasets. *Bioinformatics* 33:2594-2595.
- Glassner, H., Zchori-Fein, E., Yaron, S., Sessitsch, A., Sauer, U., and Compant, S. 2017. Bacterial niches inside seeds of *Cucumis melo* L. *Plant Soil* 422:1-13.
- Goggin, D. E., Neil Emery, R. J., Kurepin, L. V., and Powles, S. B. 2015. A potential role for endogenous microflora in dormancy release, cytokinin

- metabolism and the response to fluridone in *Lolium rigidum* seeds. *Ann. Bot.* 115:293-301.
- Hardoim, P. R., Hardoim, C. C. P., van Overbeek, L. S., van Elsas, J. D., Overbeek, L. S., van Elsas, J. D., et al. 2012. Dynamics of seed-borne rice endophytes on early plant growth stages. *PLoS One* 7:e30438.
- Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Döring, M., and Sessitsch, A. 2015. The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.* 79:293-320.
- Johnston-Monje, D., Lundberg, D. S., Lazarovits, G., Reis, V. M., and Raizada, M. N. 2016. Bacterial populations in juvenile maize rhizospheres originate both from seed and soil. *Plant Soil* 405:337-355.
- Johnston-Monje, D., and Raizada, M. N. 2011. Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. *PLoS One* 6:e20396.
- Klaedtke, S., Jaques, M.-A., Raggi, L., Préveaux, A., Bonneau, S., Negri, V., Chable, V., and Barret, M. 2016. Terroir is a key driver of seed-associated microbial assemblages. *Environ. Microbiol.* 18:1792-1804.
- Langmead, B., and Salzberg, S. 2012. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9:357-359.
- Lenth, R. 2018. emmeans: Estimated marginal means, aka least-squares means. R package version 1.2.3. <https://cran.r-project.org/web/packages/emmeans/index.html>
- Leo, L., Sudarshan, S., Klake, T., and Salojärvi, J. 2017. Microbiome R package. <http://microbiome.github.io>
- López-Fernández, S., Mazzoni, V., Pedrazzoli, F., Pertot, I., and Campisano, A. 2017. A phloem-feeding insect transfers bacterial endophytic communities between grapevine plants. *Front. Microbiol.* 8:834.
- López-López, A., Rogel, M. A., Ormeno-Orrillo, E., Martínez-Romero, J., and Martínez-Romero, E. 2010. *Phaseolus vulgaris* seed-borne endophytic community with novel bacterial species such as *Rhizobium endophyticum* sp. nov. *Syst. Appl. Microbiol.* 33:322-327.
- Love, M. I., Huber, W., and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550.
- Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17:10-12.
- McMurdie, P. J., and Holmes, S. 2013. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8: e61217.
- Mitter, B., Pfaffenbichler, N., Flavell, R., Compant, S., Antonielli, L., Petric, A., Berninger, T., Naveed, M., Sheibani-Tezerji, R., von Maltzahn, G., and Sessitsch, A. 2017. A new approach to modify plant microbiomes and traits by introducing beneficial bacteria at flowering into progeny seeds. *Front. Microbiol.* 8:11.
- Naylor, D., DeGraaf, S., Purdom, E., and Coleman-Derr, D. 2017. Drought and host selection influence bacterial community dynamics in the grass root microbiome. *ISME J.* 11:2691-2704.
- Oksanen, F. J., Blanchet, G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P., O'Hara, R. B., Simpson, G., Solymos, P., et al. 2017. vegan: Community Ecology Package. R package version 2.4-4. <https://cran.r-project.org/web/packages/vegan/index.html>
- Oliveros, J. C. 2007. VENN. An interactive tool for comparing lists with Venn diagrams. <https://bioinfo.cnb.csic.es/tools/venny/index.html>
- Paulson, J. N., Stine, O. C., Bravo, H. C., and Pop, M. 2013. Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* 10: 1200-1202.
- Pfeiffer, S., Mitter, B., Oswald, A., Schlöter-Hai, B., Schlöter, M., Declerck, S., and Sessitsch, A. 2017. Rhizosphere microbiomes of potato cultivated in the High Andes show stable and dynamic core microbiomes with different responses to plant development. *FEMS Microbiol. Ecol.* 93:fiw242.
- Puente, M. E., Li, C. Y., and Bashan, Y. 2009. Endophytic bacteria in cacti seeds can improve the development of cactus seedlings. *Environ. Exp. Bot.* 66:402-408.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O. 2012. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* 41: D590-D596.
- Rezki, S., Campion, C., Simoneau, P., Jaques, M.-A., Shade, A., and Barret, M. 2018. Assembly of seed-associated microbial communities within and across successive plant generations. *Plant Soil* 422:67-79.
- Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. 2016. VSEARCH: A versatile open source tool for metagenomics. *PeerJ* 4:e2584.
- Rybakova, D., Mancinelli, R., Wikström, M., Birch-Jensen, A.-S., Postma, J., Ehlers, R.-U., Goertz, S., and Berg, G. 2017. The structure of the *Brassica napus* seed microbiome is cultivar-dependent and affects the interactions of symbionts and pathogens. *Microbiome* 5:104.
- Saikkonen, K., Ion, D., and Gyllenberg, M. 2002. The persistence of vertically transmitted fungi in grass metapopulations. *Proc. Biol. Sci.* 269:1397-1403.
- Sánchez-López, A. S., Pintelon, I., Stevens, V., Imperato, V., Timmermans, J.-P., González-Chávez, C., Carrillo-González, R., Van Hamme, J., Vangronsveld, J., and Thijs, S. 2018. Seed endophyte microbiome of *Crotalaria pumila* unpeeled: Identification of plant-beneficial *Methylobacteria*. *Int. J. Mol. Sci.* 19:291.
- Sebastian, J., Wong, M. K., Tang, E., and Dinneny, J. R. 2014. Methods to promote germination of dormant *Setaria viridis* seeds. *PLoS One* 9:e95109.
- Sessitsch, A., Pfaffenbichler, N., and Mitter, B. 2019. Microbiome applications from lab to field: Facing complexity. *Trends Plant Sci.* 24:194-198.
- Shade, A., McManus, P. S., and Handelsman, J. 2013. Unexpected diversity during community succession in the apple flower microbiome. *MBio* 4: e00602-12.
- Truyens, S., Beckers, B., Thijs, S., Weyens, N., Cuypers, A., and Vangronsveld, J. 2016. Cadmium-induced and trans-generational changes in the cultivable and total seed endophytic community of *Arabidopsis thaliana*. *Plant Biol. J.* 18:376-381.
- Truyens, S., Weyens, N., Cuypers, A., and Vangronsveld, J. 2013. Changes in the population of seed bacteria of transgenerationally Cd-exposed *Arabidopsis thaliana*. *Plant Biol. J.* 15:971-981.
- Truyens, S., Weyens, N., Cuypers, A., and Vangronsveld, J. 2015. Bacterial seed endophytes: Genera, vertical transmission and interaction with plants. *Environ. Microbiol. Rep.* 7:40-50.
- Vannier, N., Mony, C., Bittebiere, A.-K., Michon-Coudouel, S., Biget, M., and Vandenkoornhuysen, P. 2018. A microorganisms' journey between plant generations. *Microbiome* 6:79.
- Vorholt, J. A. 2012. Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* 10: 828-840.
- Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73:5261-5267.
- Wang, Y., Naumann, U., Eddelbuettel, D., Wilshire, J., and Warton, D. 2019. mvabund: Statistical Methods for Analyzing Multivariate Abundance Data. R package version 4.0.1. <https://cran.r-project.org/web/packages/mvabund/index.html>
- Zadoks, J. C., Chang, T. T., and Konzak, C. F. 1974. A decimal code for the growth stages of cereals. *Weed Res.* 14:415-421.
- Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. 2013. PEAR: A fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* 30: 614-620.