



# Pathogen infection influences a distinct microbial community composition in sorghum RILs

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## Abstract

**Aims** The rhizosphere microbiome substantially affects plant health, yet comparatively little is known regarding the foliar community dynamics. Here, we examine the relationship between the microbiota and their response to natural infection by pathogens.

**Methods** We established an experimental system using a set of sorghum recombinant inbred lines (RILs). These RILs included four models denoted as resistant, moderately resistant, susceptible and highly susceptible. A combination of 16S rRNA and ITS gene amplicon approaches was used to assess bacteria and fungi, respectively, in foliar samples.

**Results** We show that the foliar microbiome differs substantially in asymptomatic and symptomatic RILs subsequent to natural infection by pathogens. A significant association was found between plant health and microbial community structure. Our analyses revealed several distinct fungal and bacterial pathogens. These pathogens included *Gibberella* and *Pantoea* genera, which were associated with the highly susceptible group. In addition to these pathogens, we also found signatures for *Ascochyta*, a known plant pathogenic genus. Members of the bacterial genus *Methylobacterium* and the fungal genus *Hannaella*, both known to exhibit plant growth-promoting (PGP) traits, were associated with the resistant and moderately resistant groups. These data also reveal numerous highly diverse fungal and bacterial taxa in RILs that did not show symptoms. We also found taxonomic differences between the microbiota hosted by the symptomatic and asymptomatic RILs.

**Conclusions** Together, these data suggest that pathogen infection may result in distinct microbiota. These results suggest that highly diverse microbiome may promote the plants ability to resist the effects of pathogens potentially contributing to plant health.

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## Introduction

The past three decades of research using model systems (*Nicotiana tabacum* and *Arabidopsis thaliana*) have revealed a variety of plant adaptations (Chang et al.

2016; Ritpitakphong et al. 2016; Xu et al. 2017). These studies have provided clear evidence that these traits have evolved in response to both biotic and abiotic environmental stressors (Lundberg et al. 2012; Ritpitakphong et al. 2016; Ryu et al. 2007). There is also evidence that healthy and asymptomatic plants co-exist with diverse assemblages of microorganisms, including protists, archaea, bacteria and fungi (Hassani et al. 2018; Lebreton et al. 2019). These microorganisms have collectively been shown to influence plant growth and productivity (Almario et al. 2017; Buée et al. 2009; Lindow and Brandl 2003; Stone et al. 2018). Plant-associated microorganisms positively influence plant health by increasing nutrient acquisition, stress tolerance and pathogen resistance (del Carmen Orozco-Mosqueda et al. 2020; Finkel et al. 2019; Jones et al. 2019; Mia et al. 2014; Schirawski and Perlin 2018; Tsolakidou et al. 2019). However, our understanding of the interplay between microbiomes and plants remains rudimentary and has largely focused on model plant species (Alekkett et al. 2014; Berendsen et al. 2018; Edwards et al. 2015). Understanding the interaction between microbiomes and plants is central to the elucidation of the response to biotic and abiotic stress in agriculturally important crops (Jones et al. 2019). To ensure food security, it is essential to optimize the reliability of production pipelines by minimizing environmental impacts (Saad et al. 2020; Wille et al. 2019). Integrating insights regarding beneficial plant microbiomes to enhance plant growth and disease resistance will contribute to increased agricultural production which will ultimately contribute to food security (Busby et al. 2017; Mounde 2015; Pascale et al. 2020; Sivakumar et al. 2020).

Sorghum (*Sorghum bicolor*) is a robust species with a high tolerance to drought, altitude and a wide range of temperatures (Medraoui et al. 2007). This species is a versatile crop which may be grown as a grain or sweet-stem. Sweet-stem sorghum is similar to grain (same species), however, it has a higher concentration of sugar in the stalks (Vanamala et al. 2018). Grain sorghum is an important staple food crop grown globally and sweet-stem sorghum is considered a promising biofuel feedstock (Mengistu et al. 2016). Yet, the full potential of sorghum productivity has not been realised due in part to an array of biotic and abiotic constraints (Savary et al. 2019). Biotic constraints may include weeds, animal pests, biological interactions and plant pathogens (Donatelli et al. 2017; Ghersa 2012). Plant pathogens represent a constant and

major food production constraint, with global crop losses estimated to be 20%–30%, principally in areas with food shortages (Bandara et al. 2017).

While the vast majority of microorganisms are beneficial to plants, pathogens may colonize leaves and overwhelm the innate defence mechanisms causing plant diseases (Bandara et al. 2017; Chala et al. 2019; Kelly et al. 2017). The colonization by bacterial and fungal pathogens is a direct threat to the productivity and sustainability of sorghum production (Mihajlovski et al. 2015; Sanmartín et al. 2018; Tripathi et al. 2018). Currently, little is known regarding the diversity of microbiomes in sorghum crops and the interplay between associated bacteria and fungi remains unexamined. Additionally, there are no reported microbial community studies on sorghum recombinant inbred lines (RILs) in response to biotic stressors. Elucidating the composition of the sorghum microbiome and relating this to its effects on plant health may provide important cues on pathogen management. The limited studies available on the sorghum microbiome have been based on culture-dependent methodologies, which are known to miss 99% of microbial communities (Naylor et al. 2017; Oberholster et al. 2018; Schlemper et al. 2017). Recent reports, using metagenomic analysis, have revealed potential key taxa associated with the rhizosphere and seed of sorghum (Guo 2016; Hara et al. 2019; Kinge et al. 2019; Kuramae et al. 2020; Xu et al. 2018). However, none of these studies assessed the aerial region of the plant, which is suggested to be one of the primary entry sites for pathogens (Cernava et al. 2019). This knowledge deficit is broadly true for plants where, in contrast to the rhizosphere, substantially less is known regarding the effects of plant-microbe associations on foliar diseases.

The phyllosphere, the leaf-dominated aerial part of plants, represents one of the most abundant habitats for microbiota colonization (Bodenhausen et al. 2013; Bulgarelli et al. 2013; Carlström et al. 2019; Vorholt 2012). Commensals or beneficial symbionts which affect plant fitness by providing pathogen protection are ubiquitous in the phyllosphere (Busby et al. 2017; Helfrich et al. 2018; Innerebner et al. 2011). Despite the short life span nature of the phyllosphere, bacterial communities dominated by the phyla Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes are found on leaves (Knief et al. 2010; Müller et al. 2016). Molecular studies suggest that yeast and fungal genera such as *Cryptococcus*, *Sporobolomyces*, *Rhodotorula* and

associated species largely inhabit plant leaves (Glushakova and Chernov 2004; Thompson et al. 1993). Genera such as *Cladosporium*, *Alternaria*, *Penicillium*, *Acremonium*, *Mucor*, and *Aspergillus* are the filamentous fungi frequently colonizing the phyllosphere as epiphytes and endophytes (Arnold et al. 2000; Inácio et al. 2002; Rana et al. 2019). However, such a phylogenetic conservation of community composition suggests that community assembly is not a random process but is instead governed by structuring principles, which are currently only poorly understood (Lafrest-Lapointe and Whitaker 2019; Vorholt et al. 2017). Additionally, in contrast to the intensively studied roles of root-colonizing microbiota in plant health (Durán et al. 2018; Finkel et al. 2017; Zhang et al. 2019), the collective community-level contribution of phyllosphere microbiota to plant growth, development and health is not well understood (Chen et al. 2020).

Sorghum RILs are useful models, produced for the population used in this work by inbreeding from individual F2 grain and sweet sorghum genotypes through single-seed descent procedure to the F9 generation (Shiringani et al. 2010). These lines have been used for quantitative genetic studies (Bekele et al. 2014; Shiringani et al. 2010). However, the microbiomes of these RILs and their relationship to natural pathogen infection has not been examined. To increase the understanding of the foliar microbiomes of sorghum RILs and its link to natural infection, we characterized bacteria and fungi associated with asymptomatic and symptomatic plants using 16S ribosomal ribonucleic acid (rRNA) gene and internal transcribed spacer (ITS) region sequencing, respectively. In addition to revealing the relative abundance patterns of bacteria and fungi, we assessed the significant differential abundance of taxa in asymptomatic and symptomatic sorghum RILs and studied fungal and bacterial diversity of sorghum RILs.

## Methods

### RIL material

The mapping population was originally derived by selfing a single F1 plant from *S. bicolor* grain (M71) and sweet sorghum (SS79) and advanced to the F9 generation by single seed descent (Shiringani et al. 2010) to produce a mapping population of 187 F9 recombinant inbred lines (RILs). These RILs were

mapped for quantitative traits such as grain yield and stem sugar-related traits for biofuel yield of sorghum. The F9 generation seeds used in this study were collected from the Agricultural Research Council (ARC) - Grain Crops Institute, Potchefstroom, South Africa (Table S1).

### Cultivation of sorghum RILs

The sorghum RIL seeds were cultivated in a mixture of autoclaved vermiculite and perlite medium in pots disinfected at the ARC - Biotechnology Platform (ARC-BTP), Onderstepoort, South Africa. A pot experiment was carried out in a net-house which was used to reduce the damage caused by insects, wind and the hail in the crop. The net-house was used to mimic nature and is naturally ventilated and climate controlled (natural temperature and light). The RILs were subjected to the same planting conditions and were left to grow until the matured grain filling developmental stage (120 days old plant) to allow the plants to be naturally colonized by pathogenic and commensal microbes (primarily from the environment). Moisture was maintained by watering to weight every 2–3 days. To assess the role of sorghum leaf microbial community structure in sorghum disease manifestation, 45 leaf samples (positioned higher on the plant) from individual RILs at the grain filling stage (maturity) were retrieved. The foliar symptoms (Table 1) were scored according to the method described by TeBeest et al. (2004) (Table S1). The pathogen susceptibility of the RILs was based on foliar symptoms after allowing for natural infection by pathogens. The scale used presented visual foliar symptoms of four models that denoted, resistant (R), moderately resistant (MR), susceptible (S) and highly susceptible (HS) disease groups under natural infection. The symptoms and lesions on the leaf area of the R group was (1–10%) with MR, S and HS symptoms and lesions represented by 11–30%, 31–> 50% and 51–> 75%, of the leaf area, respectively.

### Sampling

Leaf samples were collected from 45 individual RILs (foliar symptoms) across all disease groups. The number of samples collected for the resistant group (R) were ( $n = 11$ ), moderately resistant (MR) ( $n = 10$ ), susceptible (S) ( $n = 12$ ) and highly susceptible (HS) ( $n = 12$ ) Table 1. The leaves were harvested by hand (one

individual leaf per RIL using gloves and forceps which was pre-sterilized with 70% ethanol for each leaf sampled). The leaf samples were kept in sterile bags in a  $-4\text{ }^{\circ}\text{C}$  ice box and were put in a  $-80\text{ }^{\circ}\text{C}$  freezer until further processing (DNA extraction).

### Molecular ecology analyses

Leaf material, which was not disinfected to allow the identification of both the epiphytes and endophytes, was crushed using the Savant Fastprep™ FP120 Cell Disruptor (ThermoFisher Scientific), followed by total DNA extraction using the Chemagic DNA Plant Kit (Chemagen, Perkin Elmer) as detailed in the manufacturer's protocol. For bacterial amplification, primers with a PNA-PCR clamp added to block the amplification of host DNA were used to amplify the 16S rRNA gene V3-V4 region (Herlemann et al. 2011; Lundberg et al. 2012). For fungi taxon-specific primers, ITS1 and ITS4 regions were amplified as described previously (Gardes and Bruns 1993). Amplicons were purified using the MinElute® PCR Purification Kit (Qiagen). The concentration and quality of the purified PCR product was evaluated using the Qubit Fluorometer (Invitrogen). The amplicon library was normalized and prepared for sequencing following the Illumina MiSeq 16S rRNA gene library preparation guide (Illumina 2013). Sequencing was performed utilizing the Illumina MiSeq Sequencer (Illumina, San Diego, CA) with a MiSeq Reagent Kit v3 to generate  $2 \times 300$  paired-end reads at the ARC-BTP.

### Bioinformatics analyses

Bacterial and fungal community patterns were analysed using the Quantitative Insights Into Microbial Ecology package (QIIME 2 v.2010.10) (Caporaso et al. 2010). The deblur plugin was used to remove chimeras and sequence variant calling of the Illumina amplicon sequences (Amir et al. 2017). The resultant sequences were used to determine differences in bacterial and fungal communities between asymptomatic and symptomatic samples. Sequences were clustered to operational taxonomic units (OTUs) using a cut-off of 97% similarity. The resulting OTUs were compared against the trained, full-length SILVA 138 database for bacterial taxonomic classification (Yilmaz et al. 2014). Fungal OTUs were compared against the UNITE database for

taxonomic analysis of ITS sequences (Abarenkov et al. 2010).

Exploratory analyses were performed in R v.3.5.1 and Bioconductor v.3.0 (Gentleman et al. 2004). Briefly, two indices were computed (Simpson and Shannon) in phyloseq (McMurdie and Holmes 2013) to measure diversity by accounting for “evenness” and richness. These indices were obtained using the plot\_anova\_diversity function of the microbiomeSeq package (Heruth et al. 2016).  $\beta$ -Diversity was visualized using PCoA ordinations generated with the Bray–Curtis distance metric. Ordinations were created with the phyloseq and ggplot2 (v2.1.0) packages.  $\beta$ -Diversity was measured using PERMANOVA with the betadisper function from the microbiomeSeq package (Heruth et al. 2016; Oksanen et al. 2007). Differential abundance analysis between the groups was obtained through the use of DESeq2 R package (Love et al. 2014). The taxonomical counts were normalized, and significant differentially abundant taxa with ( $p$ -adjusted value  $< 0.05$ ) were visualized using ggplot2 package. Taxonomic classification data was visualised using phyloseq and microbiomeSeq package (Suppl. Text. 1).

## Results

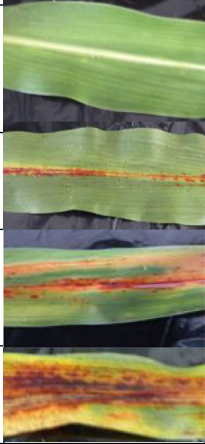
Foliar assessments reveal discrete pathogen groups which were evenly distributed

The results from foliar assessments delineated the grouping of RILs into the following disease groups: resistant (R) ( $n = 11$ ), moderately resistant (MR) ( $n = 10$ ), susceptible (S) ( $n = 12$ ) and highly susceptible (HS) ( $n = 12$ ) (Table 1). Comparison of the distribution among disease groups were done using a Chi-square test. This analysis showed that the number of samples per disease group was evenly distributed.

Fungal and bacterial alpha diversity of sorghum leaves

Estimates of indices measuring richness (Shannon) and evenness (1-Simpson index), showed that fungal alpha-diversity differences between disease groups were statistically significant. The HS disease group had a significantly higher Shannon–Weaver index compared to the R group (ANOVA,  $p$  value = 0.034;  $df = 19$ ) as shown in Fig. 1a. Fungal population diversity measure 1-Simpson index indicated a significant difference

**Table 1** The samples from individual RILs of which manual foliar disease rating was done based on visual symptoms of the leaves collected according to a rating scale described by TeBeest et al. (2004)

Severity scale	Symptoms and lesions	Disease reaction	No of samples	Visual symptoms
2 3	1-5% leaf area 6-10 % leaf area	Resistant (R)	11	
4 5	11-20 % leaf area 21-30 % leaf area	Moderately resistant (MR)	10	
6 7	31-40 % leaf area 41-50 % leaf area	Susceptible (S)	12	
8 9	51-75% leaf area > 75% leaf area	Highly susceptible (HS)	12	

between HS and R (ANOVA,  $p$  value = 0.05;  $df$  = 19). Indices measuring evenness (1-Simpson index) suggest that the fungal populations on HS plants were less diverse compared to those on R plants. A significant difference in the Shannon–Weaver index between S and R (ANOVA,  $p$  value = 0.0136;  $df$  = 18) was observed, with S disease group indicating higher species richness. The MR group did not show any significant difference in 1-Simpson index diversity. However, in terms of the Shannon index, the MR group was associated with high species richness (see Table S2 for detailed statistics).

Similarly, the bacterial alpha-diversity differences resulted in HS disease group significantly harbouring high bacterial species richness (Shannon), compared to R group (ANOVA,  $p$  value = 0.034;  $df$  = 19) (Fig. 1b). However, diversity measures which account for evenness (1-Simpson index) suggest that HS samples were dominated by fewer species (ANOVA,  $p$  value = 0.05;  $df$  = 19). The R group was significantly more diverse (ANOVA,  $p$  value = 0.05;  $df$  = 19) consistent with low 1-Simpson index values compared to the other disease groups, based on species evenness (Fig. 3b). The Shannon index of the S disease group was associated with high species richness compared with the R group (ANOVA,  $p$  value = 0.026;  $df$  = 18). The diversity measures for the MR group did not show any significant difference with the other disease groups (see Table S3 for detailed statistics).

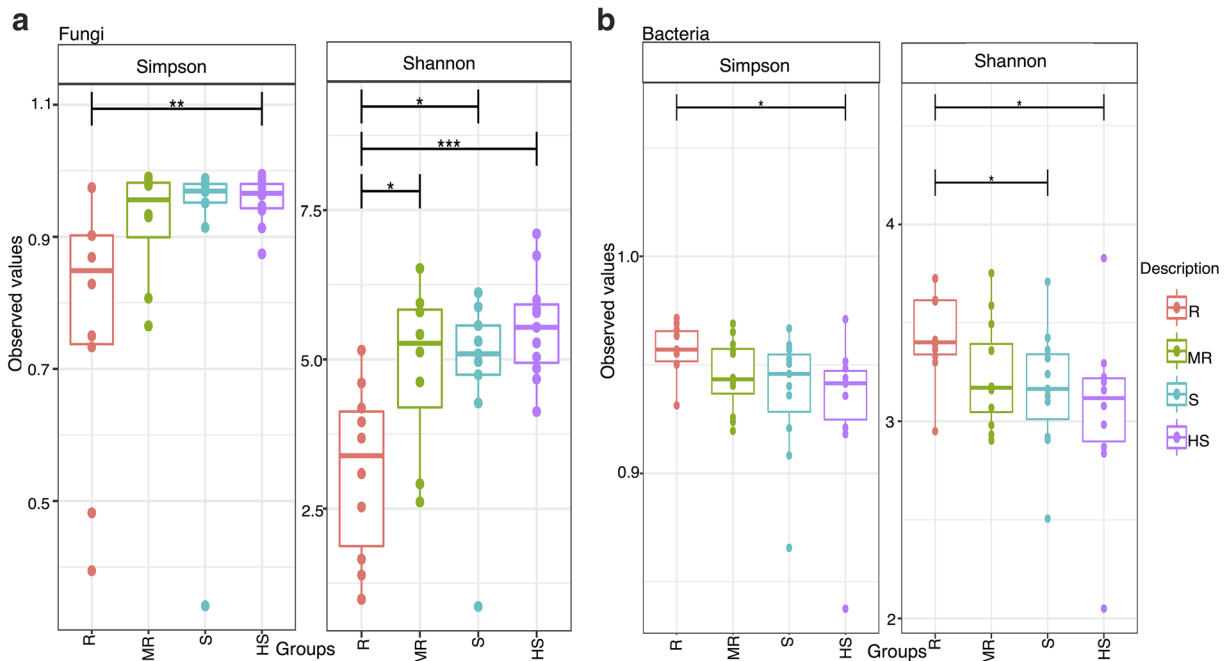
#### Beta-diversity and disease severity

PCoA showed a cluster for each disease group and the differences between the clusters were tested for significance using PERMANOVA (Fig. 2a). Beta-dispersion, used to measure variances in fungal abundance, revealed the significant differences in the microbial community dispersion (within-group variation in beta-diversity) between MR and HS ( $P_{\text{PERMDISP}} = 0.002$ ;  $df$  = 19) and R and HS ( $P_{\text{PERMDISP}} = 0.005$ ;  $df$  = 19). For bacterial abundance variation, beta-dispersion revealed a significant difference between the R and S group ( $P_{\text{PERMDISP}} = 0.04$ ;  $df$  = 19) (Fig. 2b). Permutation analysis of variance (PERMANOVA) and corresponding R-squared ( $R^2$ ) revealed that microbial communities were significantly differentiated across all the disease groups, with ( $R^2 = 0.216$ ,  $P_{\text{PERMANOVA}} = 0.001$ ;  $df$  = 41) for fungi and ( $R^2 = 0.16$ ,  $P_{\text{PERMANOVA}} = 0.001$ ;  $df$  = 41) for bacteria (see Table S2 and 3 for detailed statistics).

#### Fungal and bacterial composition of sorghum leaves

The impact of microbial communities on sorghum RILs after exposure to natural infection by pathogens was assessed in this study. The bacterial and fungal composition observed consisted of both reported disease-causing and beneficial taxa. The most dominant fungal pathogenic species, for the plants designated as HS, were the well-known phytopathogen members of the



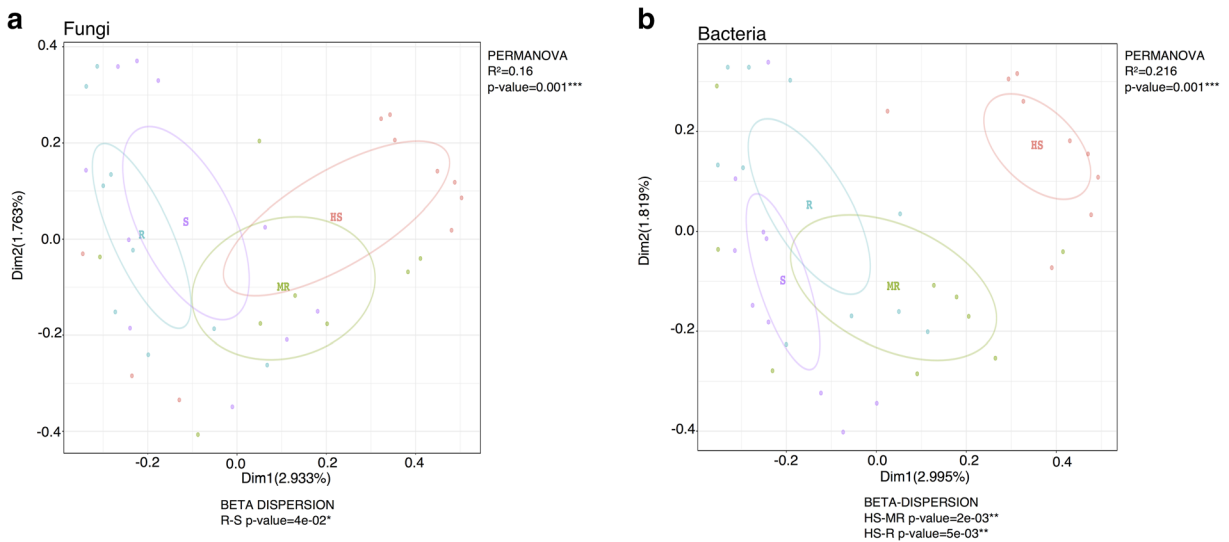


**Fig. 1** **a** Fungal alpha diversity metrics based on Shannon's and 1-Simpson's diversity indices on disease groups R, MR, S and HS. **b** The bacterial alpha diversity metrics based on Shannon's and 1-Simpson's diversity indices on disease groups R, MR, S and HS. The box plots represent the observed bacterial and fungal values based on richness, and evenness of different disease groups. The lines represent the interquartile range and asterisks above the boxplots represents statistically significant differences at ( $p$  value

$<0.05$ ) plotted using the `plot_anova_diversity` function within MicrobiomeSeq R package. For 1-Simpson index box (\*\* $p$  value = 0.05;  $df = 19$  between HS and R), for Shannon index box plot (\*\* $p$  value = 0.034,  $df = 19$  between HS and R), (\*  $p$  value = 0.013;  $df = 18$  between R and S) in Fungi. For bacterial alpha diversity 1-Simpson index box (\*  $p$  value = 0.05;  $df = 19$  between HS and R), for Shannon index box plot (\*  $p$  value = 0.034;  $df = 19$  between HS and R), (\*  $p$  value = 0.026;  $df = 18$  between R and S)

family *Nectriaceae* (40%) and the genus *Gibberella* (40%). Members of the genus *Epicoccum* (family *Didymellaceae*), were exclusively found in the R and S disease groups and accounted for over 5% relative abundance. In addition to these genera, members of families *Didymellaceae* (*Ascochyta* genus) and *Ustilaginaceae* (*Ustilago* genus) were found in the susceptible disease groups albeit in lower abundance ( $<5\%$ ). Furthermore, members of the families *Didymellaceae* (*Didymella* genus) and *Massarinaceae* (*Sclerostagonospora* genus), were also amongst the potential pathogenic taxa found across all disease groups, although in low percentages (Fig. 3a and b; Table 2). Table 2 further highlights the known potential sorghum pathogenic taxa and the pathogenic taxa not commonly found in sorghum. The dominant phyla were Ascomycota and Basidiomycota respectively, which in total comprised over 55% of the OTUs. Chytridiomycota, Kickxellomycota, Glomeromycota, Mucoromycota and Rozellamycota were detected at much lower relative abundances, cumulatively 9% of fungal

OTUs. Unassigned fungi encompassed less than 13% of OTUs. The most substantial differences were observed among the different disease groups, with a higher relative abundance of OTUs found in the HS samples. Members of the family, *Pleosporaceae*, *Bulleribasidiaceae*, *Tremellaceae* with taxa in the genera *Phoma* (30%), *Didymella* (30%) and *Papiliotrema* (20%) dominated the samples designated as the R group (Fig. 3a and b). The S and HS disease groups had a high proportion of fungal OTUs, which agrees with results from Shannon diversity analyses (Fig. 1a). The R and MR groups had a similar fungal composition, however, the relative abundance of genera identified as *Papiliotrema*, *Phoma* and *Cladosporium* was high in the R group. Similarly, the S and HS disease groups had the same microbial composition with a high relative abundance (more than 20%) of *Gibberella* genus in the HS disease group, while *Epicoccum* was more abundant in the R and S disease group. Surprisingly, our analyses showed that the sorghum fungal community had more OTUs (478) compared



**Fig. 2** **a** Beta diversity of fungal communities represented via Principal coordinates analysis (PCoA) based on Bray-Curtis distance from the PERMANOVA analysis (betadisper function) across disease groupings (R, MR, S and HS). **b** Beta diversity of bacterial communities represented via Principal coordinates

analysis (PCoA). Statistically significant groups are represented by asterisks with  $p$ -values in PERMANOVA (beta dispersion). Groupings is based on the categorical factors depicted with ellipses representing the standard error around the centroid

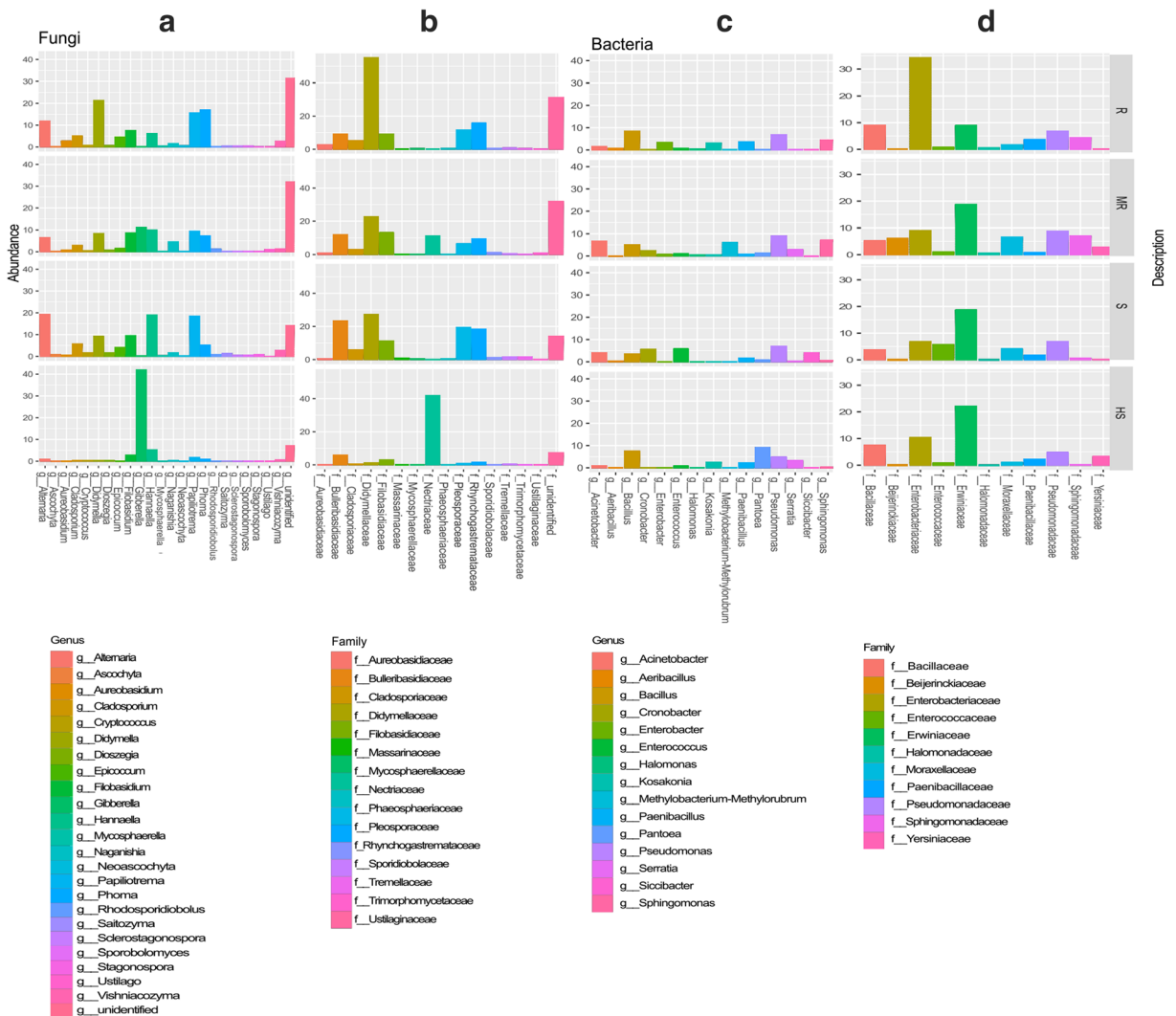
to the bacterial OTUs (246) Table S4 and 5. The majority of these fungal OTUs (301) were shared and corresponded to 63% of OTUs distributed across all the disease groups. The HS disease group had the highest proportion of 11 unique fungal OTUs which corresponded to 2.3% of the total. The distribution of fungal OTUs in HS relative to R group is shown in Fig. 4a.

Analysis of bacterial abundance, revealing the composition of commensal and pathogenic bacteria of sorghum RILs across all disease groups at the family and genus levels is shown in Fig. 3c and d and Table 3. The known pathogen *Pantoea* (~ 10%) were exclusively associated with the HS disease group. The S disease group had a higher relative abundance of the genera *Siccibacter* and the pathogenic genus *Cronobacter* (~ 5% for both genera). It was found that Proteobacteria and Firmicutes had the highest relative abundances among all the disease groups. The dominant families across all disease groups were *Erwiniaceae*, *Bacillaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* with members of the genera, *Bacillus* and *Pseudomonas* highly dominant across all the disease groups. Among all disease groups, sequences assigned to members of the genera *Bacillus* and *Kosakonia* were found at high relative abundances in both R and HS disease group. The

relative abundance of *Bacillus* sequences in both R and HS was evenly distributed (~ 10%). Similarly, an even distribution of relative abundance of *Kosakonia* sequences was observed in the R and HS disease group. These taxa were followed by members of the genus *Sphingomonas* which were highly abundant and evenly distributed (~ 10%) in the R and MR group. Bacterial sequences were assigned to 246 OTUs, with 81 of the OTUs, corresponding to 32.9% of the total, shared amongst all disease groups. The HS disease group harboured the highest proportion of unique OTUs (21), corresponding to 8.5%, while MR and S had the lowest number of unique OTUs (14), corresponding to 5.7%. Disease groupings S and HS shared 5 OTUs that corresponded to 5.3% of bacterial OTUs with only 5 OTUs corresponding to 2% shared between the R and MR groups (Fig. 4b).

#### Differential taxa abundance

Differential abundance analysis was conducted to determine the taxa that showed significant abundance between the R and HS disease group (differential abundance was performed between these groups as they showed significant differential abundance at  $p$  value  $\leq 0.05$ ). The relative difference in abundance was



**Fig. 3** **a** The relative abundance of fungal families across disease groups (R, MR, S and HS), **b** The relative abundance of fungal genera across different disease groups **c** Bacterial family relative

abundance across disease groups, **d** The relative abundance of bacterial genera across different disease groups. Each bar represents a disease group, the colour distinguishes the taxon in each group

expressed as log<sub>2</sub> fold change, with more represented differentially abundant taxa expressed at a log<sub>2</sub> fold change of >0 and the less represented differentially abundant taxa expressed at a log<sub>2</sub> fold change <0 ( $p < 0.05$ ). Eleven (11) fungal genera, *Gibberella*, *Epicoccum*, *Alternaria*, *Papillotiroma*, *Phoma*, *Aerobasidium*, *Cladosporium*, *Filobasidium*, *Ascochyta* and *Didymella* showed significant differential abundance in the HS disease group, while *Hannaella* genus was significantly enriched in the R group.

The bacterial analyses showed 15 differentially abundant genera between the two disease groups and included *Methylorubrum*, *Aeribacillus*, *Pantoea*, *Serratia*,

*Halomonas*, *Enterobacter*, *Kosakonia*, *Sphingomonas*, *Acinetobacter*, *Paenibacillus*, *Enterococcus*, *Siccibacter*, *Pseudomonas*, *Bacillus* and *Cronobacter*. Members of the genera *Pantoea* and *Serratia* were significantly enriched in the HS disease group while *Methylorubrum* and *Aeribacillus* were highly enriched in the R group.

## Discussion

The community dynamics of microbial assemblages linked to the rhizosphere of plants are increasingly well

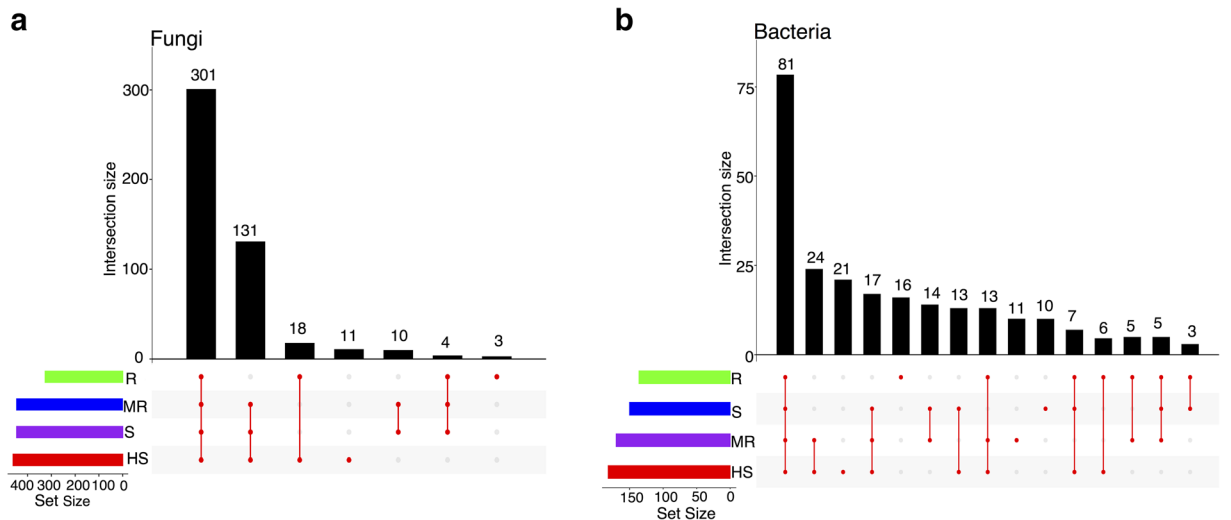


**Table 2** The sorghum reported & non-reported plant pathogenic fungi found in various disease groups (R, MR, S and HS) in this study

Potential pathogenic taxa	Disease group	Percentage (%) of samples	Recorded symptoms	Reference
<i>Epicoccum</i>	S	<10	Grain mold of sorghum	(Oliveira et al. 2017a)
<i>Mycosphaerella</i>	R, MR, S, HS	<5	Charcoal rot in sorghum	(Bandara et al. 2017; Bandara et al. 2018; Das and Padmaja 2016; Hassan 2018)
<i>Sclerostagonospora</i> Anamorph <i>Leptosphaeria</i> <i>Ascochyta</i>	MR, S, HS	<5	Blackleg and leaf blotch in sorghum, wheat and maize	(Fitt et al. 2006; Ma et al. 2019; Quaadvlieg et al. 2013)
<i>Didymella</i>	R	>20	Leaf spot and leaf blight	(Moral et al. 2018)
	MR	10		
	S	10		
<i>Ustilago</i>	S	<10	Leaf smut disease	(Kruse et al. 2018; Omayio et al. 2018)
<i>Gibberella</i>	HS	>40	Stalk rot and Sorghum complex disease	(Gilbert and Fernando 2004; Kelly et al. 2017; Nida et al. 2019)
	MR	>10		
<i>Phoma</i>	R	>10	Mycotoxins in sorghum	(Bennett et al. 2018; Oliveira et al. 2017b)
	MR	<10		
	S	5		
	HS	<5		
<i>Alternaria</i>	R	>10	Leafspot	(Astoreca et al. 2019; Wei et al. 2020)
	MR	<10		
	S	20		
	HS	<5		

documented. Unfortunately, less is currently known regarding the composition and community dynamics of the foliar microbiome (Peñuelas and Terradas 2014). This study provides novel insights into the leaf

microbial community structure and diversity of sorghum RILs. Our results suggest that previous studies may have underestimated the effects of natural infection in selecting the microbial communities in sorghum



**Fig. 4 a** Upset plot showing shared and unique fungal OTUs across disease groups **b** Upset plot showing shared and unique bacterial OTUs across disease groups. The total size of each

disease group is represented on the left barplot. The overlapping red lines indicates the number of OTUs across all disease groups and connecting bar indicates multiple disease groups

**Table 3** The sorghum reported & non-reported plant pathogenic bacteria found in various disease groups (R, MR, S and HS) in this study

Potential pathogenic taxa	Disease group	Percentage (%) of samples	Recorded symptoms	Reference
<i>Pantoea</i>	HS	10	Bacterial leaf spot of sorghum	(Cota et al. 2010; Lana et al. 2012)
<i>Serratia</i>	S	<10	Cucurbit Yellow Vine Disease	(Besler and Little 2017)
<i>Pseudomonas</i>	R, MR, S, HS	<10	Bacterial leaf stripe of sorghum	(Kaplin et al. 2017)

plants, as there are studies on naturally coexisting soil and rhizosphere microbial consortia (Nemergut et al. 2013; Zegeye et al. 2019). We found a strong correlation between the diseased groups and the sorghum microbiota after natural infection. This finding suggests that naturally occurring pathogens may considerably shape the structure of microbiota, favouring some taxa. The results of alpha diversity analysis (Fig. 1a and b), displayed clear contrasts between disease groups, which supports this hypothesis. The beta diversity analysis (Fig. 2a and b) indicated a distinction between the RILs displaying disease symptoms and those that did not show disease symptoms (S; HS and R; MR samples).

Among the most abundant taxa, we identified a considerable portion of fungal and bacterial genera which have reported beneficial and pathogenic attributes known to inhabit the cereal phyllosphere, together with a range of non-pathogenic yeasts, and filamentous fungi (Fig. 3a and c). The filamentous fungal genera such as *Cladosporium*, *Alternaria* and *Sporobolomyces* found in this study is in agreement with previous observations since these genera frequently colonize the phyllosphere as epiphytes and endophytes (Arnold et al. 2000; Glushakova and Chernov 2004; Inácio et al. 2002; Kinge et al. 2019; Rana et al. 2019). Consistent with our findings, members of the genera *Aerobasidium*, *Fusarium*, *Alternaria*, *Cladosporium* and *Phoma* were previously found in the wheat and maize phyllosphere (Fig. 3a) (Ripa et al. 2019; Szilagyi-Zecchin et al. 2016). Blixt et al. (2010) detected that *Udeniomyces*, *Dioszegia* and *Cryptococcus* fungal genera were found on the leaves of cereal crops (wheat) which were absent in our sorghum phyllosphere disease groups. The bacterial composition analysis, revealed high relative abundances of Proteobacteria and Firmicutes among all the disease groups. Previous studies showed that phyllosphere bacterial communities were dominated by the phyla Proteobacteria followed by Actinobacteria, Bacteroidetes and Firmicutes (Knief et al. 2010;

Müller et al. 2016). Many genera such as *Bacillus*, *Methylobacterium*, *Pantoea*, *Sphingomonas* and *Pseudomonas*, which were also identified in this study, have been reported from the phyllosphere environment of different crop plants (Fig. 3c) (Aquino et al. 2019; Delmotte et al. 2009; Dobrovol'skaya et al. 2017; Kumar et al. 2019; Luo et al. 2012; Meena et al. 2012; Mukhtar et al. 2010). Furthermore, compared to other studies on cereal host plants, we did not detect sequences for *Janthinobacterium*, *Pedobacter* and *Erwinia* bacterial genera which largely dominate the wheat and barley phyllosphere (Kuzniar et al. 2020; Newton et al. 2010). This disparity may result from differences in the geographic characteristics and climatic parameters (Finkel et al. 2011).

Fungal and bacterial genera associated with the R, MR, S and HS disease group

The reported fungal pathogens were present at considerably higher proportions compared to bacteria in this study. While sorghum serves as a host to over 100 pathogens, previous studies suggest that fungal pathogens are more likely to colonize plants in comparison to bacterial pathogens (Akinrinlola et al. 2018; Zheng et al. 2016). In the present study, fungal genera *Gibberella*, *Epicoccum* and *Ascochyta*, were differentially abundant in the HS disease groups (Fig. 5a). These genera are pathogenic taxa and have been previously reported to be a major causative agent of grain mould disease, leaf stripe and leaf smut (de Oliveira et al. 2018; Jayashree and Wesely 2018; Kelly et al. 2017; Oliveira et al. 2017a; Sharma et al. 2011; Tivoli and Banniza 2007; Xu et al. 2019). Interestingly, members of the genus *Epicoccum* were also associated with the resistant group, albeit in low levels (Fig. 3a). Fungi belonging to the genus *Epicoccum* are ubiquitous ascomycetes frequently isolated from both healthy and diseased grapevines (Bruez et al. 2014; Del Frari et al. 2019;

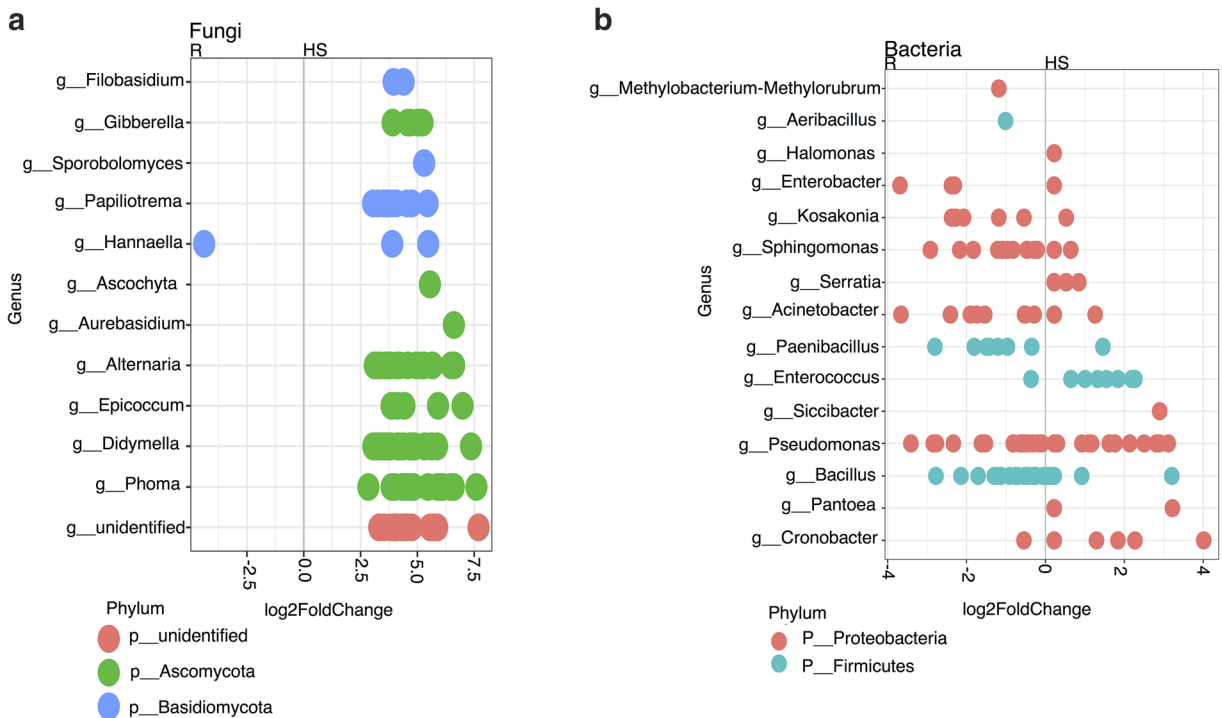
Hofstetter et al. 2012; Pancher et al. 2012). *Pantoea*, a sorghum bacterial pathogen causing leaf spot, was differentially abundant and linked to the HS disease group (Lana et al. 2012) (Fig. 3c and 5b). Members of the *Serratia* genus, known to colonize several dicotyledonous plants (cucurbits, sunflower and alfalfa) causing yellow vine disease was associated with the HS disease group, but there are currently no known reports of *Serratia* on sorghum leaves (Besler and Little 2017; Bolton et al. 2006) (Fig. 3c and 5b). However, not much is known regarding the host specificity of pathogens infecting agriculturally important plants like sorghum (Prospero and Cleary 2017; Rodriguez-Moreno et al. 2018).

It is important to highlight that the R and MR group had many of the fungal and bacterial OTUs that were taxonomically classified, and these taxa have previously been associated with plant growth-promoting and biocontrol activity. These included members of the fungal genera *Papiliotrema*, *Alternaria*, *Didymella* and *Phoma*; and bacterial genera *Enterobacter* and *Sphingomonas* (Knief et al. 2010; Saldajeno et al. 2012; Schisler et al. 2019; Schlemper et al. 2018; Poudel et al. 2016; Turbat et al. 2020; Zhou et al. 2018) (Fig. 3a and c). *Hannaella* yeasts and the *Methylobacterium-Methylorubrum* bacterial genera, which are frequently observed in the phyllosphere of various plant species (Caporaso et al. 2012; Edwards et al. 2015; Nasanit et al. 2015; Nutaratat et al. 2014), were also associated with the R and MR groups. Some members of the *Hannaella* genera are known to produce indole acetic acid (IAA) (Kaewwichian et al. 2015; Mehmood et al. 2018). However, the precise mechanisms of *Hannaella* yeasts in influencing plant performance and host-genotype specifically are unknown (Sun et al. 2014). Little is also known regarding the phylogenetic taxa and functional attributes of the recently classified *Methylorubrum* bacterial genus which was also associated with the R group (Fig. 3c and 5b, Green and Ardley 2018; Grossi et al. 2020). Nevertheless, members of this genus have plant-growth promoting abilities and usually colonize plants that display disease resistance (Ardanov et al. 2012; Bulgari et al. 2011; Koskimaki et al. 2015; Sagaram et al. 2009; Schisler et al. 2019; Schreiner et al. 2010; Rakotoarisoa et al. 2015; Trivedi et al. 2010; Wallace et al. 2018; Zhang et al. 2018). Interestingly, the susceptible disease group had a higher relative abundance of members of the family *Bacillaceae*, which are usually associated with healthy plants (Das 2019; Klein 2008) (Fig. 3d).

Nonetheless, members of this family also contain a plant pathogenic *Bacillus* genus that causes ginger rhizome rot and can survive harsh conditions (such as invasion by pathogens; Yuan and Gao 2015; Zheng et al. 2016). This may explain the high relative abundances of the *Bacillaceae* family in the susceptible disease groups. *Pseudomonas*, a known bacterial pathogen causing sorghum leaf blight, was associated with all the disease groups including R, MR, S and HS (Fig. 3c). The *Pseudomonas* genus has been previously reported to be an attractive biocontrol agent and this could explain its association with the R group in the present study (Gómez-Lama Cabanás et al. 2018; Panpatte et al. 2016; Praveen Kumar et al. 2012). *Alternaria* and *Phoma*, which are known to cause sorghum diseases (Astoreca et al. 2019; Fitt et al. 2006; Wei et al. 2020) were significantly associated with HS (Fig. 3a and 5a).

#### Fungal and bacterial $\alpha$ -diversity and $\beta$ -diversity patterns associated with different disease groups

The alpha and beta-diversity metrics (Fig. 1a and b) showed that the RIL microbiome assemblage was associated with the severity of the disease symptoms. The R group had more fungal and bacterial diversity (evenness index) relative to the HS disease group. There were significant fungal and bacterial variation (beta diversity) between the RILs that showed disease symptoms (S and HS disease groups) and those that did not show disease symptoms (R and MR groups) (Fig. 2a and b). Similar to our study, vines with moderate disease symptoms displayed higher microbial diversity (evenness) than severely symptomatic vines (Deyett and Rolshausen 2019). Our data suggest that the role of plant-driven microbial assemblages may be pivotal for coping with the effects of biotic stresses and may result in increased environmental fitness (Berendsen et al. 2012; Deyett and Rolshausen 2019; Turner et al. 2013). In contrast with severely symptomatic vines, the toxic environment (e.g., occlusion of xylem vessel with tyloses and decrease of hydraulic conductivity; Deyett et al. 2019) is not conducive to microbial survival. A similar result was also observed in a soil microbiome study where soil surrounding healthy tobacco plants harboured more diverse microbial communities, contrasted to soil samples collected around bacterial wilt affected plants (Yang et al. 2017). Other studies have also shown that plants with a diverse microbial community were less prone to pathogen invasion than those with less diverse



**Fig. 5** **a** Fungal genera and phyla; and **b** bacterial genera and phyla showing significant differential abundance between disease groups (R and HS) as detected by DESeq2. The log<sub>2</sub> fold change is plotted at the x-axis, and the y-axis is represented by the fungal and

bacterial genera significantly associated with R and HS disease groups at  $p$  values  $<0.05$ . Coloured circles represent the phylum of the presented genera

microbial communities (Berg et al. 2017; Shade 2017; van Elsas et al. 2012; Yang et al. 2017). This is likely due to increased competition for available resources among potential pathogens and other microorganisms in the less diverse community (Shade 2017). Our results indicate that the bacterial and fungal diversity (as measured by the Simpson diversity index) was significantly different between the R and HS disease groups. This finding suggests that highly diverse plant microbiomes may reduce the possibility of detrimental disease outbreaks as pathogens are likely to be outcompeted (Berg et al. 2017; Shade 2017; Yang et al. 2017).

#### Fungal and bacterial OTUs distribution between the disease groups

The largest number of unique OTUs were associated with the susceptible disease group in both bacterial and fungal datasets (Fig. 4a and b). This is consistent with previous studies which have shown that diseased hosts tend to harbour more unique OTUs when compared to healthy hosts (Rosenzweig et al.

2012). The fungal communities associated with the highly susceptible disease group was the most complex (richness). Zhang et al. (2018) recently reported an increase in the fungal community richness (Shannon index) and linked this to increased disease pressure. Another report revealed that soils with *Fusarium* wilt were colonized by more complex bacterial communities (richness) and also harboured significantly different community structure compared to healthy soils (Zhang et al. 2011; Zhou et al. 2019). Our results on the leaf microbiome demonstrate similar patterns and possibly suggest that plant diseases may affect shifts in the phyllosphere of fungal and bacterial communities on sorghum leaves.

#### Conclusion

To the best of our knowledge, this is the first study to assess both the fungal and bacterial composition in the leaves of sorghum RILs. We show that natural pathogen infection results in distinct foliar microbial communities in sorghum RILs. Together, our results

suggest that different ‘resident’ consortia found in naturally infected and uninfected sorghum plants may be viable biocontrol and plant-growth promoting targets. These results have consequences for crop breeding, and the analysis of microbial diversity and community composition can be useful biomarkers for assessing disease status in plants. Future cultivation-based studies may clarify the influence of potentially beneficial taxa and their precise biological functions *in vitro* and *in planta*.

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**Availability of data** The nucleotide sequence data reported are available in the NCBI GenBank databases under the BioSample accessions number (SAMN13439454 – SAMN13439498) for bacteria and NCBI Biosample accessions (SAMN13439386 – SAMN13439430) for fungi, SRA accession number PRJNA614545.

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