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ABSTRACT

Aims: The interactions between plant roots and the associated microbiota impact soil aggregation, water retention and plant nutrient availability. Thus, selection of plant genotypes that promote microbial species involved in root-adhering soil aggregation and rhizosheath formation could help improve yield sustainably. Here, we tested pearl millet genotypic variation in both root-adhering soil aggregation, microbiological and biochemical characteristic.

Methods: A collection of 181 pearl millet inbred lines was phenotyped for their rhizosheath size, and thirteen contrasting genotypes were selected and grown under field conditions, and their root-adhering soil (RAS) was sampled. Microbial biomass, pH, mineral N content and six enzymatic activities involved in main nutrients cycles were analyzed, and metabarcoding of 16S rDNA and ITS were performed for bacterial and fungal diversity.

Results: Enzymatic activities (chitinase, acid phosphomonoesterase, FDA-hydrolysis and β-glucosidase) were higher in RAS of larger rhizosheath lines than that of smaller rhizosheath one. Bacterial β-diversity showed a separation of the most contrasting lines in the principal coordinate analysis performed with the Bray-Curtis distance. Some bacteria from the *Gaiellaceae* and *Sphingomonadaceae* families and the *Bradyrhizobium* genus were associated with the large rhizosheath phenotype. Concerning the fungal community, we noticed a negative correlation between the specific richness and the rhizosheath size and *Trichoderma* genus was positively associated to the rhizosheath size.

Conclusions: This study demonstrates that in pearl millet, rhizosheath size is related to soil nutrient dynamics and microbiota diversity. However, it also shows that other factors shape this trait and their relative importance must be determined.

KEYWORDS:

Pearl millet; Rhizosphere microbial diversity; Rhizosphere enzymatic activities; Rhizosheath; Metabarcoding

DECLARATIONS:

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ABBREVIATIONS:

CCA: Canonical correspondence analysis

CNRA: Centre national de recherche agronomique

FDA: 3'6'-diacetyl-fluorescein

GLM: Generalized linear model

ICRISAT: international crops research institute for the semi-arid tropics

ITS: Internal transcribed spacer

OTU: operational taxonomic unit

PCA: Principal component analysis

PCoA: Principal coordinate analysis

PERMANOVA: Permutational analysis of variance

p-NP: *p*-nitrophenol

p-NPG: para-nitrophenyl β-D-glucopyranoside

p-NP-NAG: para-nitrophenyl-N-acetyl-glucosaminide

p-NPP: para-nitrophenyl phosphate

QIIME: Quantitative insight into microbial ecology

RAS: Root-adhering soil mass

rDNA: Ribosomal deoxyribonucleic acid

RDP: Ribosomal database project

RT: Root mass

THAM: tris-hydroxymethyl aminomethane

INTRODUCTION

Pearl millet is the sixth most cultivated cereal around the World. It is mainly cultivated in the semi-arid regions of the Indian subcontinent and sub-Saharan Africa (Haussmann et al. 2012). In the latter, subsistence farmers recently privileged short cycle varieties that give optimal yield to cope with the reduction of the rainy season (Vigouroux et al. 2011). Despite this adaptation, pearl millet yields remain very low (less than 1 ton/ha) compared to those of other cereals. Besides water limitation, the factors explaining pearl millet low productivity in the Sahel region are soil acidity and low nutrient content (Brück et al. 2003). The latter is one of the main factors as a result of exportation of harvest residue and low input of fertilizer and manure due to the low financial capacity of farmers. Therefore, new strategies need to be developed to improve pearl millet productivity in low fertility soils together with soil resilience. This can be elaborated through the combination of plant breeding and rhizosphere soil microbial ecology management, as the interactions between root, soil and microbiota can impact both soil aggregation (water availability) and nutrient cycling (mineral nutrition). These interactions in the rhizosphere are in large part driven by root carbon deposition.

The profile of root exudates may vary depending on the plant genotype (Aulakh et al. 2001; Czarnota et al. 2003; Micallef et al. 2009; Mönchgesang et al. 2016). In addition, a significant influence of plant genotype on rhizosphere soil microbial diversity has been reported in different species including *Arabidopsis thaliana* , maize (*Zea mays*), barley (*Hordeum vulgare*) and pear millet (*Pennisetum glaucum*) (Lundberg et al. 2012; Peiffer et al. 2013; Bulgarelli et al. 2015; Ndour et al. 2017). Indeed, the impact of plant genetic traits on microbial communities was attributed to quantitative or qualitative variation in root exudation which constitute an important source of carbon for rhizosphere microbiota (Micallef et al. 2009; Neumann et al. 2014; Schreiter et al. 2014; Carvalhais et al. 2015). This effect on microbial diversity could also have an impact on soil enzymatic activities and then on nutrient cycling if the microorganisms involved in such processes are impacted.

Plant rhizosphere is known to be one of the most dynamic microbial and enzymatic hotspots (Kuzyakov and Blagodatskaya 2015; Kuzyakov and Razavi 2019). This is related to rhizodeposition and root exudation of easily degradable organic substances that stimulate the microbial extracellular enzyme activities (Ge et al. 2017; Ma et al. 2018), but also to the high density of active microorganisms that have an important role in biogeochemical processes such as organic matter mineralization, pH setup and nutrient availability (Allison and Vitousek 2005; Blagodatskaya et al. 2014). For pearl millet, microbial activity and some enzyme activities (acid phosphomonoesterase and arylsulfatase activities) have been shown to be greater in the rhizosphere than in bulk soil (Diakhaté et al. 2016). Moreover, plants can modify directly the availability of nutrients such as phosphorus (Lambers et al. 2006), zinc (Hoffland et al. 2006) and iron (Ishimaru et al. 2007) through root exudation. These plant driven nutrient dynamics depend on enzymes production and distribution in the rhizosphere, which are related to root morphology, root exudation and rhizodeposition (Kuzyakov 2002; Ma et al. 2018). Thus, plant genetic control of carbon deposition could impact diversity and activity of the microbiota and therefore could modify nutrients cycling and availability in soil. For instance, two maize lines with contrasting nutrient use efficiency showed significant difference in their

rhizosphere microbial biomass, beta-glucosidase and urease activities in parallel with significant difference in the composition of their rhizosphere microbiota (Pathan et al. 2015).

Moreover, several studies have evidenced a positive impact of a higher rhizosheath (synomym of root-adhering soil aggregation; Ndour et al. 2020) on plant tolerance to abiotic stresses including drought (Shane et al. 2009; Benard et al. 2016; Liu et al. 2018, 2019; Basirat et al. 2019), phosphorus (George et al. 2014) or nitrogen deficiency (Wullstein 1991; Othman et al. 2004), and soil acidity (Haling et al. 2010; Delhaize et al. 2012). Importantly, numerous studies reported a plant genotypic control on this rhizosheath size in many cereals crop including barley, wheat, maize and pearl millet (George et al. 2014; Delhaize et al. 2015; Ndour et al. 2017; Brown et al. 2017). Notably, George et al (2014) have demonstrated that the specific rhizosheath varied reproducibly between barley lines in both greenhouse and field conditions, and that this variation had an impact on plant growth under water and phosphate deficiency conditions. Therefore, the interactions between plant root and soil microbiota is of great importance for soil carbon management, nutrient mobilization and soil water retention and could be considered in plant breeding programs for sustainable crop production. In a recent study performed under greenhouse conditions, we found that rhizosphere soil bacterial communities changed significantly from one pearl millet line to the other, and this variation was related to intensity of root-adhering soil aggregation (Ndour et al. 2017). So this rhizosphere aggregation, genetically driven in pearl millet, is reported in other plants to be particularly important for plant nutrition and improving soil water retention (Amellal et al. 1998; Alami et al. 2000). However, the impact of this pearl millet intraspecific variability on microbial diversity, soil activity and nutrient availability, has not yet been demonstrated under field conditions. This is particularly important as root exudation which is reported as potential driver, is reported to change with plant growth stage (Aulakh et al. 2001; Jones et al. 2004; Vives-Peris et al. 2020), and genetic variations could influence microbial communities in the rhizosphere under field conditions. These questions should be answered before the consideration of this trait as valuable criterion for plant breeding programs.

The aim of this study was to determine in a field experiment whether different pearl millet lines selected for their contrasting rhizosheath size, present differences in their rhizosphere microbial activity and diversity and to check if such differences would be related with the rhizosheath size. For that, we measured and compared the microbial biomass and different enzymatic activities involved in major nutrients cycling in the root-adhering soil sampled from 13 pearl millet lines. The bacterial and fungal diversity was analyzed using metabarcoding of respectively 16S rDNA genes and ITS region. We hypothesized that (i) the different pearl millet genotypes would present significant differences in their microbial diversity and activities, and chemical properties in the root-adhering soil fraction when cultivated in field condition and (ii) the differences in these microbial and chemical characteristics could be correlated to those of the rhizosheath size.

MATERIALS AND MEHTODS

Root-adhering soil mass phenotyping and selection of pearl millet lines under greenhouse conditions

We used a panel of 181 pearl millet lines developed at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) from landrace and improved open-pollinated cultivars of West African origin previously described (Debieu et al. 2018). The soil used was sampled in an experimental field of the CNRA (Centre National de Recherche Agronomique), Bambey, Senegal (14.42°N, 16.28°W) and sieved at 4 mm. These pearl millet lines were evaluated for their rhizosheath size using the ratio between the root-adhering soil mass and the root tissue mass (RAS/RT) according to Ndour et al. (2017). Briefly, 1.5 kg of soil was filled in "WM" shaped pots installed in plastic crates. Pearl millet lines were sown according to a complete random block design with 7 repetitions was performed (with the sowing of successive block separated by 15 days for the harvest purpose). Pearl millet lines were cultivated in a greenhouse for 28 days under natural light and temperatures ranging from 23 to 35 °C (April to September 2015) with measurement of the soil mass closely adhering to the roots (RAS) and the root tissue mass (RT), and calculation of the rhizosheath size. From this screening, 13 contrasting lines (from very small to large rhizosheath size) were selected, taking into account germplasm availability, rhizosheath size stability and plant development. These 13 lines were sown in a second experiment to confirm again their phenotypes by using the same experimental procedure. We investigated the genetic relationship between these selected lines using 268,321 SNPs identified through Genotyping-by-Sequencing (GBS) (Debieu et al. 2018). SNPRelate (Zheng et al. 2012) was used to calculate the Identity-by-State (IBS) pairwise distances between genotypes.

Analyses of root-adhering soil chemical and biochemical properties

The analyses of the microbial diversity and activities of the root-adhering soil were performed on the 13 pearl millet lines we selected in the first part of this study. Pearl millet lines were sown during the dry season of 2016 (planting: March 10th) in a field experiment with the permission of the ISRA (Institut Sénégalais de Recherches Agricoles). The field experiment was located at the CNRA (Centre National de Recherche Agronomique), Bambey, Senegal (14.42°N, 16.28°W). The soil was sandy and had a low organic matter content (0.4 %) and low level of silt and clay (12%). As the experiment was conducted in the dry season, the plants were irrigated twice a week with 30 mm water. The root-adhering soil was sampled in a field trial comprising the 181 inbred lines of pearl millet set up for the phenotyping agromorphological traits (Debieu et al. 2018). This trial was set up using an incomplete randomized blocks design and we sampled the root-adhering soil fraction of the 13 lines we selected.

For each of these 13 lines, root-adhering soil samples were taken from four different plants 77 days after sowing (after flowering stage of millet lines). The root-adhering soils were sampled in the horizon 0-20 cm at 10 cm distance from the plant stem base. In addition, four replicates of bulk soil were sampled for control in the same horizon (0-20 cm) of an adjacent unplanted plot that had the same physico-chemical characteristics and similarly watered. All soil samples were divided into two fractions: the first one was stored in freezer (-20 °C) and was used for the microbial biomass and microbial diversity analyses; the second one was stored in refrigerator (4 °C) and was used for microbial activity and soil chemical analyses.

- *pH and inorganic N*

Soil pH was measured in 1:2.5 soil-to-water suspensions using a pH meter (Delta 320, Mettler Toledo, Switzerland). Exchangeable ammonium and nitrate concentrations were determined by colorimetry (Technicon, Auto Analyzer III) after extraction in 1 M KCl solution (Bremner 1965). Inorganic N was obtained by summing the exchangeable ammonium and nitrate content of each soil sample.

- *Soil respiration*

Soil respiration was evaluated by measuring the amount of CO_2 released after humidification over a period of seven days. Ten grams of air dried soil of each soil sample were placed in plasma bottle, moistened to 80% of their water holding capacity and incubated at 28 °C for seven days. The amount of CO_2 released was measured daily using gas chromatography with an Agilent 490 Micro GC (Agilent Technologies) by sampling and analysis of the ambient air of the bottle. The average amount of CO_2 was calculated over the seven days and the results were expressed in µg C-CO_2. g^{-1} soil. day^{-1}.

- *Microbial biomass*

Microbial biomass estimation was carried out using the fumigation-extraction method (Amato and Ladd 1988). Ten grams of control soil and root-adhering soil samples were fumigated (incubated for 10 days in a chloroform saturated atmosphere) for microbial cells lysis. Then, the α-amino N was extracted from fumigated soil and from non-fumigated samples using 2 M KCl. A reagent containing ninhydrin was then added to the solution and a colorimetric determination of this nitrogen was carried out at 570 nm. The α-amino N originating from the microbial lysis was determined by coloration difference between fumigated and non-fumigated soil sample using spectrophotometry. Microbial-C biomass was estimated by multiplying the α-amino N concentration from microbial cells by the factor 21 (Amato and Ladd 1988), and expressed as µg C. g^{-1} dry soil.

- *Enzyme activities*

The β-glucosidase activity was measured according to Hayano (1973) with some modifications. Three replicates and one control tube containing 100 mg were prepared for each fresh soil sample. In the three replicate tubes, 0.1 mL of phosphate citrate buffer at pH 5.8 and 100 µL of 5 mM para-nitrophenyl β-D-glucopyranoside (*p*-NPG, Sigma) were added. In control tubes, the substrate (*p*-NPG) was replaced with distilled water. A blank was also carried out in another tube by addition of all reagents without the soil. After incubation at 37 °C for 2 h, the reaction was stopped by adding 3 mL of 0.2 M Na_2CO_3. Then the optical density of the supernatants was measured at 400 nm after a centrifugation at 10,000 rpm for 5 min. The coloration due to the reaction was corrected for blank and control tube coloration. The β-glucosidase activity was calculated using a calibration solution and expressed in µg *p*-NP.g^{-1} fresh soil. h^{-1}.

The acid phosphomonoesterase activity was measured according to Tabatabai and Bremner (1969) with minor changes. For each fresh soil sample, three replicates of 100 mg were placed in tubes. In each tube we added 400 µL of phosphate citrate buffer (Mac Ilvain) at pH 5.8 and 100 µL of para-nitrophenyl phosphate (*p*-NPP). After

incubation at 37 °C during 1 h, the reaction was stopped by alkalinization with 400 µL of NaOH 0.5 M and 100 µL of $CaCl_2$ 0.5 M. The mixture was then centrifuged for 5 min at 10,000 rpm and the optical density of the supernatant was measured using a spectrophotometer at 400 nm. The acid phosphomonoesterase activity was expressed in µg *p*-NP.g^{-1} fresh soil. h^{-1} after calibration of the spectrometer using a known *p*-NPP concentration solution.

The hydrolysis of FDA (3'6'-diacetyl-fluorescein) gives an estimation of microbial activity as it is performed by both fungal and bacterial enzymes and was determined by the method described by Adam and Duncan (2001). Briefly, 1 g of soil, 15 mL of 60 mM phosphate buffer at pH 7, and 200 µL of 4.8 mM FDA were mixed in a tube. After incubation at 30 °C for 1 h, the reaction is stopped by addition of acetone (10 mL). The mixture was then centrifuged at 10,000 rpm for 5 min and the optical density of the supernatant measured at 490 nm. The results are expressed in µg fluorescein. g^{-1} fresh soil. h^{-1}, after calibration with a standard solution.

The urease activity was assayed according to Kandeler and Gerber (1988). One g soil, 1.4 mL of phosphate buffer at pH 7, and 100 µL of 1.2 M urea were mixed. After incubation at 37 °C for 2 h, the reaction was stopped by addition of 3 mL of 2 M KCl. The solution was then agitated for 30 min at room temperature before being centrifuged at 10,000 rpm for 5 min. To reveal the ammonium production, 1 mL of the supernatant was mixed with 9 mL of deionized water, 5 mL of a mixture of sodium salicylate and sodium nitroprusside 1 M and 2 mL of 39 M sodium dichloro-iso-cyanurate, then agitated during 30 min and the optical density measured at 660 nm. The results are expressed in µg N-$NH_4^{+\cdot}$ g^{-1} fresh soil. h^{-1} after calibration of the spectrophotometer with a standard N-NH_4^+solution.

The chitinase activity was assayed according to Parham and Deng (2000). The reaction consists of the hydrolysis of para-nitrophenyl-N-acetyl-glucosaminide (*p*-NP-NAG) that produces *p*-nitrophenol (*p*-NP). The *p*-NP concentration was measured by colorimetry at 400 nm. To do this, 0.25 g of fresh soil, 1 mL of sodium acetate trihydrate 0.1 M (CH_3CO_2Na-$3H_2O$) at pH 5.5 and 250 µL of the *p*-NP-NAG substrate were mixed. After incubation at 37 °C for 1 h, the reaction was stopped by adding 250 µL of 0.5 M $CaCl_2$ and 1 mL of 0.5 M NaOH. The mixture was centrifuged at 1000 rpm for 5 min. The optical density was measured at 400 nm and the quantity of *p*-NP was calculated after calibration with a *p*-NP standard solution. The results were expressed in µg *p*-NP.g^{-1} fresh soil.h^{-1}.

The dehydrogenase activity was assayed using the method of Mersi and Schinner (1991) as modified by Prosser et al (2011). Five mL of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) and 3.5 mL of 1 M tris-hydroxymethyl aminomethane (THAM) at pH 8.5 were added to 1 g of soil sample. After homogenization and incubation of the mixture at 37 °C for 2 h, 10 mL of a mixture of ethanol and N, N-dimethylformamide were added. Then, the optical density of the solution was measured at 464 nm. The results were expressed in µg INTF.g^{-1} fresh soil. h^{-1}.

Analyses of root-adhering soil microbial diversity

DNA was extracted from 0.25 g of the root-adhering soil and of the control soil frozen samples using *FastDNA spin kit for soil* (MP biomedical, USA) according to the supplier's recommendations. The extracted DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The composition of bacterial and fungal communities was studied by sequencing the16S r-RNA gene and the ITS region from the DNA extracts. Sequencing was performed by MR DNA (503 Clovis Rd., Shallowater, TX, USA) using the Ion Torrent PGM sequencer (Life Technology Corp) according to the supplier's instructions. For bacteria, the V4 region of 16S ribosomal DNA was targeted using primers 515F/806R (Caporaso et al. 2011). The diversity of fungal communities was studied by sequencing the ITS regions using the primers: ITS1F 5'-CTTGGTCATTTAGAGGAAGTAA-3' / ITS2R: 5'-GCTGCGTTCTTCATCGATGC-3'(Orgiazzi et al. 2012). For both 16S and ITS sequencing, PCR was performed using the HotStar Taq plus Master Mix reaction kit (Qiagen, Germantown, MD, USA) under the following conditions: 94 °C for 3 min followed by 28 cycles consisting of a 94 °C step for 30 s, a step of 53 °C for 40 s and 72 °C for 1 min. A final elongation step of 72 °C for 5 min was added at the end of these 28 cycles. We processed data using Quantitative Insight Into Microbial Ecology (QIIME) software version 1.9 (Caporaso et al. 2010).

16S rRNA amplicon sequence processing: raw data were checked for quality, mismatches and homopolymers. Sequences with a quality score average lower than 25, sequences with homopolymers, and sequences shorter than 200 pb were removed from the analysis. Using an inhouse PHP script (LEMiRE/BIAM, Cadarache, France), we conducted mitochondria and chloroplast sequences filtering after the denoising and chimera check. The sequences were then demultiplexed according to their barcode. Sequences representing less than 0.1 % of total sequence count were filtered. Thereafter the open reference operational taxonomic unit (OTU) picking method from QIIME was used. It consists of the clustering of sequences against the Greengenes database at 97% threshold. Sequences that did not match any entries in these references were then clustered using the *de novo* OTU picking at 97% similarity using Usearch. Taxonomic assignment of OTUs was performed using the RDP classifier and the Greengenes database (DeSantis et al. 2006). The phylogenetic position of bacteria in the different treatment was then determined using the QIIME pipeline script. To compare bacterial α-diversity of RAS fraction of the 13 pearl millet lines, we used Shannon diversity index, Phylogenetic Diversity index (PD-Whole), Chao1 richness estimator and the number of OTUs. To do this, we performed rarefaction of data by picking randomly 24839 sequences in which the different α-diversity parameters were calculated.

ITS amplicon sequence processing: sequences with a quality score average lower than 25, sequences with homopolymers were removed. As we have done for bacteria, mitochondria and chloroplasts sequences were filtered after the denoising and chimera check. Therefore, a demultiplexing step was performed using barcodes to attribute valid sequences to their corresponding treatments. The sequences representing less than 0.1 % of total count were filtered from the dataset and the remaining sequences were then clustered into OTUs by the Usearch method at 97% pairwise identity using the QIIME *de novo* picking strategy (Edgar 2010). The OTUs representative sequences were classified using RDP and the non-redundant Unite sequence database at 97% identity. Using QIIME, fungal α-diversity metrics was calculated using 19362 ITS sequences per sample to compare the different treatments and the taxonomical composition was determined for each sample up to species level if possible. Sequence data from both 16S rDNA and ITS sequencing are available in NCBI under the SRA accession number PRJNA475215.

Statistical analysis

Statistical analysis was performed using R statistical environment (version 3.3.3). The normality of different variables was tested using the Shapiro test ($p < 0.05$). To test pearl-millet line effect on the different parameters (RAS/RT ratio, microbial biomass, pH, mineral N, soil respiration, enzyme activities), we constructed general linear models (GLM) using the "glm" function in R. Each model was fitted by considering data distribution and using the corresponding link function *i.e*. Gaussian (link="identity") for normally distributed variables and Poisson (link="log") for not normally distributed variables. Then, ANOVAs were fitted to these models using the "Chisq" test, and Tukey HSD post-hoc tests (library multicomp) were performed ($p < 0.05$) to identify the significant differences between treatments (13 lines + control soil). To compute bacterial and fungal beta-diversity of the soil samples, Bray-Curtis distance matrices were generated from the OTU tables and Principal Coordinate Analyses (PCoA) were performed. The significance of samples separation in the PCoA plot was tested using PERMANOVA (Adonis, 999 permutations) of the Bray-Curtis distance matrixes. Correlation analyses were performed using the Spearman method to test possible relation between the Bray-Curtis distance of the rhizobacterial communities of the selected pearl millet lines and their phylogenetic distances. Moreover, a principal component analysis (PCA) was performed on the enzyme activities to evaluate the relationship between root-adhering soil mass phenotype and enzyme activities in the rhizosphere. To test the correlation of enzymatic activities, N content, and pH (constrained variables) with microbial communities, canonical correspondence analyses (CCA) were performed using bacterial and fungal genus matrices and the matrix of these chemical and biochemical data (*vegan package*). Then, the *anova.cca* function was applied to assess the significance of the correlations ($p < 0.05$, 999 permutations). To detect bacterial and fungal OTUs that were enriched or depleted in the rhizosphere of the most contrasting lines compared to the control soil, the "group significance script" (available in QIIME) was performed with the *g*-test option. After this, mean abundances of the discriminant taxa (genus or family) corresponding to these OTUs were compared in the different lines libraries by carrying out a GLM and ANOVA.

RESULTS

Plant phenotyping and lines selection for microbiological analyses

The phenotyping of the 181 pearl millet lines revealed a large variation on the rhizosheath size (RAS/RT ratio), ranging from 7.4 (L220) to 26.3 (L132) **(Fig. 1)**. The results of the second phenotyping experiment confirmed significant differences of the rhizosheath size, ranging from 7.8 (L220) to 25.6 (L24) **(Table 1)**. The four lines (L24, L64, L132 and L253) with significantly higher RAS/RT ratios were named "*large rhizosheath lines*", and L220 with significantly lower RAS/RT ratio was named "*small rhizosheath line*". Other lines with RAS/RT ratio not significantly different from the mean value (L3, L55, L62, L71, L137, L126, L177 and L216) were considered as "*intermediary rhizosheath lines*".

pH and inorganic N

The pH values of RAS fraction from the different millet lines ranged from 6.6 (L132) to 8.4 (L177). Compared to control soil, there was a significant decrease of pH in the RAS fraction of 3 lines, two large rhizosheath lines (L132, L253) and one intermediary rhizosheath line L71. There was no significant change in root-adhering soil pH for other lines compared to the control **(Fig.2A).**

Mineral N (including the exchangeable ammonium and nitrate content) varied significantly ($p < 0.0001$) between 2.5 (L177) and 16.3 µg $N.g^{-1}$ dry soil (L253) in the RAS **(Fig.2B)**. Even if the line L253 showed a mineral N content in root-adhering soil not significantly different from those of the control soil, it showed much more mineral N compared to most of the intermediary lines and the small rhizosheath line L220 (more than two-fold increase). This increased mineral N content was due to nitrate content in RAS of L253 pearl millet line (significantly higher than the control soil and most of the other pearl millet lines, **Supplementary file 1: Fig. S1**) but not to the ammonium content **(Supplementary file 1: Fig. S2**).

Microbial biomass

The microbial biomass was 18.5 µg C.g dry $soil^{-1}$ in the control soil and varied from 5.5 (L220) to 35 µg $C.g^{-1}$ dry soil (L71) in the RAS fraction of the 13 pearl millet lines **(Fig. 2C)**. None of the microbial biomass C values of these RAS fractions were significantly different compared to the control soil. The main significant differences were observed between the small rhizosheath line (L220) and one intermediary rhizosheath line (L3) and on the other side the large rhizosheath line (L64) and two intermediary rhizosheath lines (L71 and L62) (4-fold average increase).

Soil respiration

Respiratory activity of RAS samples ranged from 2.6 (L253, L132) to 3.8 µg $C\text{-}CO_2$ g^{-1} dry soil day^{-1} (L3), always higher than that of the control soil (2.1 µg $C\text{-}CO_2$ g^{-1} dry soil. day^{-1}) (**Fig. 2D**). However, only the RAS of the large rhizosheath line L64, and two intermediary rhizosheath lines (L3, L126) showed a significant increase of respiratory activity compared to the control soil ($p < 0.05$).

Enzymatic activities

Six enzymatic activities (β-glucosidase, acid phosphomonoesterase, chitinase, FDA hydrolysis, dehydrogenase and urease) were determined on the RAS fraction of the 13 pearl millet lines and on the control soil (**Table 2**). The Principal Component Analysis (PCA) performed on these enzymatic activities showed a total inertia percentage of 62.8 % by combining the components F1 and F2. The biplot of the samples from pearl millet lines and the different variables in the F1 * F2 plane is shown in **Fig. 3.** The F1 component (dim1: 48 % of the variability) was mainly explained by four enzymatic activities: chitinase, acid phosphomonoesterase, FDA hydrolysis and β-glucosidase. The F2 component (dim 2: 14 % of the variability) was explained by dehydrogenase and urease **(Supplementary file 1: Fig. S3 and S4).** We found that the enzymatic activities of RAS samples from three of the four large rhizosheath lines (L64, L132, L253) were clearly separated from those of the small rhizosheath line (L220) and of the control soil. The fourth large rhizosheath line (L24) was grouped with the small rhizosheath line L220 and the control soil **(Fig. 3).** Due to high variability of the enzymatic activities data, very few significant differences were evidenced (**Table 2**). However, some enzymatic activities measured in the RAS of large rhizosheath lines were significantly higher ($p < 0.05$) than the ones of the control soil and of the small rhizosheath line (L220). Acid phosphomonoesterase and chitinase were, respectively, 8-fold and 3-fold higher in the RAS fraction of L253 than in control soil. Compared to the small rhizosheath line (L220), these two activities were, respectively, 6-fold and 5-fold higher in RAS fraction of this large rhizosheath line (L253). Chitinase activity was 3-fold and 4-fold higher in the RAS of L64 than in, respectively, control soil and RAS of the small rhizosheath line (L220).

Bacterial diversity

The sequencing of 16S rRNA gene in root-adhering soil samples produced 2.06 x 10^6 sequences (min=26449, max= 53890 sequences per sample). The results showed that the Shannon diversity index calculated from the abundances of the defined OTUs did not show any significant variability between the different treatments (including control soil) (**Table 3)**. However, the general trend is that the highest value for these estimators of α-diversity was observed in the control soil. The only significant decrease of α-diversity in RAS of pearl millet lines was observed with the intermediary rhizosheath line L71 (PD-Whole and OTU richness). We used Bray-Curtis distance matrix and a PCoA to assess the ß-diversity of bacterial community in RAS fractions of pearl millet lines and in the control soil (**Fig. 4A**). The projection of the individuals showed that the soil sampled from three large rhizosheath lines (L64, L132, L253) were distantly separated from the one of small rhizosheath line (L220) and of the control soil ($p < 0.001$; F.Model = 2.93; PERMANOVA, 999 permutations). Again, the fourth large rhizosheath line (L24) is closer to the control soil and the small rhizosheath line (L220) **(Fig. 4A).** Moreover, the CCA performed revealed significant relations between bacterial community and N content, pH and enzymes activities we measured in the soil samples ($p < 0.05$). These variables used as constraints explained 21 % of the community distribution **(Supplementary file 2)**.

The projection of soil samples and these variables in the two first axes confirmed the separation between the control soil and the small rhizosheath line (L220) and the 3 large rhizosheath lines (L132, L64 and L253), as we observed in the PCA of enzyme activities and the PCoA of bacterial communities **(Supplementary file 1 Fig S5).** However, we did not find a significant correlation between the Bray-Curtis distances based on rhizobacterial communities of the RAS of pearl millet lines and the phylogenetic distances of these lines ($p > 0.05$, **Supplementary file 1: Fig S6**) based on genotyping-by-sequencing data.

At the phylum level, the two dominant phyla in this soil and in the RAS of the 13 pearl millet lines tested were *Proteobacteria* and *Firmicutes*, which constituted together more than 50% of the total bacterial phyla. These two dominant phyla were followed by *Actinobacteria* (from 12 to 20%) and *Acidobacteria* (4 and 8%) according to the treatments **(Supplementary file 1: Fig. S7).** At this level of taxonomic analysis of the microbiota composition in the RAS fraction, there was no difference between large and small rhizosheath lines, and between each of them compared to the control soil **(Supplementary file 1: Fig. S7).**

At the OTU level, using the QIIME *g-test* script we detected the dominant OTUs differentially abundant in the RAS fraction of the large rhizosheath lines (L24, L64, L132, L253) compared to the control soil. The most discriminant OTUs showing significant increase compared to control soil ($p <$ e-12) were affiliated to the following taxa: *Bradyrhizobium*, *Mesorhizobium* and *Kaistobacter* (*Alphaproteobacteria*), and *Gaiellaceae* (*Actinobacteria*). The most discriminant OTUs showing significant decrease compared to control soil were affiliated to *Ammoniphilus***.** To compare the abundance of these taxa in the RAS samples of the different pearl millet lines, we used a GLM to fit an ANOVA and used the Tukey HSD post-hoc test. Their differential distribution on the bacterial sequence libraries can be observed in **Table 4.** The abundance of *Bradyrhizobium*, *Mesorhizobium*, *Kaistobacter* and *Gaiellaceae* was increased in the RAS of the four large rhizosheath lines (L24, L64, L132, L253) when compared to that of the small rhizosheath line (L220), with only one exception (abundance of *Kaistobacter* decreased in RAS of L24). Concomitantly the abundance of *Ammoniphilus* was decreased in the rhizosphere of the large rhizosheath lines (L24, L64, L132, L253).

Fungal diversity

After the sequencing of ITS gene in root-adhering soil of the 13 pearl millet lines, we obtained 2.71 x 10^6 sequences (min =19816, max= 105293 sequences per samples). Using the three diversity indices (Shannon index, Chao1 and OTU number), there was no significant difference of fungal α-diversity between RAS fractions of three large rhizosheath pearl millet lines (L24, L64, L253) and that of the control soil (**Table 3**). However, for these three lines, the fungal specific richness (OTUs number and Chao1 estimator) of the RAS fraction was significantly lower than that of the small rhizosheath line (L220). This tendency of a reduced fungal α-diversity in the RAS of large rhizosheath lines was confirmed by the negative correlations between the RAS/RT ratio and the different α-diversity metrics **(Fig. 5)**. The fungal ß-diversity was evaluated by a PCoA performed on the Bray-Curtis distance matrix**.** The projection of the individuals (soil samples) in the two first components PC1 and PC2 explained only 21 % of the distribution. In this projection, there was no separation between the RAS sampled from the different pearl millet lines in relation to their rhizosheath size **(Fig. 4B)**. The CCA performed did not reveal any significant relation between

fungal community and N content, pH and enzymes activities we measured in RAS sample ($p > 0.05$, **Supplementary file 2**).

The taxonomical composition of the soil samples from the different pearl millet lines was determined at the phylum level (**Supplementary file 1: Fig. S8)**. Overall, there was no relationship between the RAS/RT ratio and the abundance of fungal groups in the RAS fraction of the different pearl millet lines. We noticed that 35 to 57% of the fungal sequences (depending on the treatments) could not be affiliated. The *Ascomycota* and a non-identified phylum constituted the most represented phyla with abundances ranging, respectively, from 38 to 62% and from 29 to 58% **(Supplementary file 1: Fig. S8)**, followed by *Basidiomycota* and *Glomeromycota* phyla for which relative abundances ranged respectively from 1.6% to 19% and from 0.1% (for L64) to 2.8% (L177).

The group significance test performed using the QIIME *g-test* script gives fungal OTUs that have differential abundances between the rhizosphere of pearl millet lines and the control soil. However, due to so many non-identified OTUs, only the genus *Trichoderma* has been clearly identified to be differentially distributed in the RAS of large rhizosheath lines compared to control soil and the small rhizosheath line. Indeed, *Trichoderma* sequences were significantly more abundant ($p < 0.05$) in the rhizosphere of two large rhizosheath lines (L132: n=77 sequences [0.11% of sequence count]; L253: n=87 sequences [0.30 % of sequence count]) compared to one of the small rhizosheath line (L220: n=10 sequences [0.02% of sequence count]) and the control soil (n=25 sequences [0.02% of sequence count]).

DISCUSSION

In a previous study, Ndour et al. (2017) found a 2.9-fold variation in the rhizosheath size using a subset of 9 pearl millet lines selected for bacterial diversity study. The rhizosheath size we measured in the present work are consistent with these previous results, as we found a 2.85-fold variation in the rhizosheath size in 13 different pearl millet lines that we selected for microbial diversity and activities analyses. Moreover, in both studies (pot and field experiment), the same dominant bacterial phyla were found in the rhizosphere of pearl millet lines (*Proteobacteria*, *Firmicutes and Actinobacteria*). This is not surprising since the soil used in the pot experiment of this previous work was sampled in the same area that the field experiment of this present work. However, the relative importance of such dominant phyla varied according to the millet lines in the two studies. Since the pearl millet lines used in these two studies are different, it was not possible to make further comparisons of the composition of microbial taxa in RAS between these two studies.

pH and microbial biomass in the RAS of the contrasting pearl millet lines

We noticed for three of the large rhizosheath lines (L64 L132, L253,) a global reduction of the pH compared to the control soil (significant differences for L253 and L132, pH 6.6-6.7) in contrast with the small rhizosheath line (L220) and the control soil value (respectively pH= 8.1 and 7.9). This reduction of pH in the RAS of the large rhizosheath lines could be related to a higher amount of root exudates deposed in the rhizosphere of these lines compared to the small rhizosheath one. Indeed, root exudates include many organics compounds such as amino acids, organic acids that could modify the pH and then impact the microbial diversity (Li et al., 2016; Rousk et al., 2010; Zhalnina et al., 2015) and activities in the rhizosphere. Importantly, it has been shown a positive correlation between root exudates amount and the stability of soil aggregates (Traoré et al. 2000) or the rhizosheath size (Liu et al. 2019).

The microbial biomass of the large rhizosheath lines (L64, L132, L253) was globally higher than the one of the small rhizosheath line (L220) even if the differences were significant only with the L64 line. In a previous study, using quantitative PCR of 16S rRNA gene, we found that different pearl millet lines with contrasting RAS/RT ratios did not show any significant difference in bacterial community density when grown in greenhouse conditions (Ndour et al. 2017). An element of explanation may reside in the difference in plant age that can influence the rhizodeposition and then impact soil microbiota as sampling of RAS was performed 28 days after sowing in the first study (Ndour et al. 2017) and 77 days after sowing in the present work. Accordingly, Pausch et al. (2016) reported significant variations of microbial biomass in barley rhizosphere between two different sampling times (tillering *vs* head emergence). On the other hand, Pausch et al. (2016) reported that different barley genotypes did not show any significant difference in the microbial biomasses measured in their rhizosphere. This could be related to the difference in genetic distances between barley genotypes and between pearl millet genotypes used in these studies. Even if significant only for the line L64, the microbial biomass was more important in the RAS of large rhizosheath lines (L24, L64, L253, L132) compared to those of the small rhizosheath line (L220; two to five-fold variation). This suggests that the size of the microbial community may contribute to the determination of the RAS/RT ratio in pearl millet. It is important to note that our microbial biomass values are low compared to those founded by Diakhaté et al.

(2016). This could be related to the lower organic carbon content of the soil (0.4 % vs 0.52 %) and the different protocol of the estimation as we used the fumigation-extraction method whereas, in this comparative study, the authors used the respiratory rate of glucose to estimate soil microbial biomass.

Microbial activity in the RAS of the contrasting pearl millet lines

Several enzymatic activities have been reported to be elevated in the rhizosphere of plants compared to unplanted control soil (Pathan et al. 2015; Giagnoni et al. 2016). Particularly, acid phosphomonoesterase activity was significantly higher in soybean rhizosphere than in control soil (Fraser et al. 2017). In our study, the different enzymatic activities measured in the RAS of pearl millet lines and in control soil showed very different patterns. Chitinase, acid phosphomonoesterase and to a lesser extent FDA activities were generally stimulated in the RAS of pearl millet lines compared to control soil. On the other hand, beta-glucosidase, urease and dehydrogenase activities were not significantly different between the RAS of pearl millet lines and the control soil. The differential responses between FDA and dehydrogenase activity, which both indicate general microbial activity, could be explained by the differences in pH in which they were tested. Indeed, the former was measured at pH 7, which is closer to the pH measured in the RAS that the latter measured at pH 8.5. In addition, dehydrogenase is an indicator of viable microbial community activity (active microbiota) whereas the FDA gives an estimation of both viable and dead microbial community activity (intracellular and extracellular enzymes). These variations of enzymatic activities could be explained by a potential variation in root exudation patterns of the different pearl millet lines as reported earlier on different pearl millet varieties (Venkateswarlu and Rao 1985). Indeed, root exudates shape rhizosphere microbial diversity (Paterson et al. 2007; Haichar et al. 2008; Micallef et al. 2009; Turner et al. 2013) and then could select or deplete some microbial populations that could be differentially competent for different soil functions. For instance, Pathan et al. (2015) and Giagnoni et al. (2016) reported concomitantly variations in beta-glucosidase and urease activities, and variations in the bacterial and fungal communities structure in the rhizosphere of two maize lines. Moreover, it is important to note that this influence of root exudates on enzymatic activities could also be direct as it was reported that plants can also release phosphatases directly into soil to hydrolyze soil phytates in phosphorus deficient conditions (Hammond et al. 2004; Rengel and Marschner 2005).

The principal component analysis carried out on these enzymatic activities showed differences between pearl millet lines according to their root-adhering soil mass (rhizosheath size) phenotype. Chitinase, acid phosphomonoesterase, beta-glucosidase and FDA enzymatic activities appear globally higher in the rhizosphere of lines with high RAS/RT ratio (L64, L132, L253). In barley, it was shown that the RAS/RT ratio varied significantly between different lines (George et al., 2014) and the increase of this ratio was associated with an improvement of plant growth in phosphorus and water deficit conditions (Brown et al., 2012; George et al., 2014). Thus, this growth improvement could be explained by a greater phosphatase activity in the rhizosphere of lines with high RAS/RT ratio (large rhizosheath line). We therefore propose two hypotheses to explain this convergence between some enzymatic activities and the rhizosheath size. The first one is that pearl millet root exudation could be variable between the different lines, as reported in numerous other cereal species (Aulakh et al., 2001; Czarnota et al., 2003; Micallef et al., 2009; Mönchgesang et al., 2016). Thus, a more important root exudation in the rhizosphere of the large

rhizosheath lines could lead to a decrease of the pH (that potentially stimulated some enzymatic activities: chitinase, beta-glucosidase, and acid phosphomonoesterase, that have their optimal potential under acid pH conditions), as well as an increase of aggregates stability and then a larger rhizosheath as reported (Traoré et al. 2000; Liu et al. 2019). Moreover, a variation in root exudate amount could also affect rhizosphere microbial populations involved in root-adhering soil aggregation and rhizosheath formation such as EPS-producing bacteria as reported in several studies (Alami et al. 2000; Bezzate et al. 2000; Kaci et al. 2005; Berge et al. 2009). The second hypothesis is a genotypic variation of root hair characteristics (density and length) between the different millet lines as reported in others cereal species (Delhaize et al. 2015; James et al. 2016; Adu et al. 2017). It has been shown that root hair density and length had an impact on several root traits such as exudation (Yan et al., 2004), microbial diversity (Robertson-Albertyn et al., 2017), rhizosheath formation (Delhaize et al., 2012; George et al., 2014) as well as leaf phosphorus accumulation, plant biomass and phosphorus deficiency tolerance (Brown et al., 2012). Importantly, these different processes could have an impact in nutrient recycling in the rhizosphere.

Bacterial diversity in the RAS of the contrasting pearl millet lines

The analysis of the bacterial α-diversity that refers to the diversity within a sample (Jurasinski et al. 2009) showed very few significant differences between samples from the RAS of different pearl millet lines and control soil: only one exception for L71 line which was different from L3, L55, L137 and the control soil for PD-whole and OTU richness **(Table 4).** This result is quite different from that obtained by Peiffer et al. (2013), which showed high variability on specific richness (OTU richness) between the rhizosphere samples from different maize lines. They found that plant genetic effect explained 19 % of the variability of OTUs number. This discrepancy may be explained by many factors such as the intraspecific genetic distance, which may be greater in their panel than in ours but also by difference in soil physicochemical characteristics that could have a stronger influence on bacterial diversity. The analysis of beta-diversity or "differential diversity" (Jurasinski et al. 2009) between the rhizosphere samples of the pearl millet lines, using the Principal Coordinate Analyses of Bray-Curtis distance matrix, evidenced a separation between millet lines. As reported by Peiffer et al. (2013), we could not relate the kinship matrix for the pearl millet lines to the rhizobacteria beta-diversity distances matrix. This may suggest a control of the microbial community by a few major localized alleles of the genome or an indirect control of host plant on rhizosphere microbiota. Previous studies also showed that the plant genotype explains a small but significant part of the beta-diversity variation in the rhizosphere (Lundberg et al. 2012; Peiffer et al. 2013; Ndour et al. 2017; Robertson-Albertyn et al. 2017). Interestingly, we found that the three large rhizosheath lines (L64, L132 and L253) are clearly different from the small rhizosheath line (L220) and the control soil **(Fig. 4A)** and that this separation of pearl millet lines within the PCoA plot was similar to that we observed in the PCA plot performed with the enzymatic activities. This suggests that some bacterial taxa that were differentially distributed in RAS of contrasting rhizosheath lines (species belonging to *Gaiellaceae and Sphingomonadaceae* families and *Bradyrhizobium* genus) could play specific role in both soil chitinase, acid phosphomonoesterase and beta-glucosidase activities, and in RAS aggregation (rhizosheath formation).

However, it remains very challenging to highlight bacterial species responsible for rhizosheath formation by plant using metabarcoding approach because (i) most of the time attribution cannot be made at the species level and, (ii) the rhizosheath size is a very integrative phenotype which responds to various biotic and abiotic drivers (Brown et al. 2017; Pang et al. 2017; Ndour et al. 2020). Concerning the bacteria component, several studies have reported (i) an increase of the rhizosheath size after inoculation of plant with an exopolysaccharides (EPS)-producing bacterial strains (Alami et al. 2000; Kaci et al. 2005; Sandhya et al. 2009), (ii) a correlation of some rhizobacterial taxa abundances with the rhizosheath size (Ndour et al. 2017; Zhang et al. 2020). However these bacteria belong to different phylogenetic groups: *Rhizobiaceae/Alphaproteobacteria* (Alami et al. 2000; Berge et al. 2009), *Enterobacteriaceae/Gammaproteobacteria* (Amellal et al. 1998; Zhang et al. 2020), *Paenibacillaceae and Bacillaceae/Firmicutes* (Bezzate et al. 2000; Fernández Bidondo et al. 2012) as well as their mechanisms used to shape the rhizosphere are very diversified: production of EPS (for instance levane; Bezzate et al. 2000) , ethylene production (1-aminocyclopropane-1-carboxylic acid [ACC] deaminase activity) and plant hormonal balance resulting in root hairs formation (Zhang et al. 2020).

Fungal diversity in the RAS of the contrasting pearl millet lines

The impact of plant genotype on the diversity of rhizosphere fungal populations was already demonstrated (Yao and Wu 2010; Yu et al. 2016). Our study confirms these results and further shows a significant decrease in the specific richness of the fungal community in the RAS of two large rhizosheath lines (L24 and L64) compared to small rhizosheath line (L220) and one intermediary rhizosheath line (L137). Interestingly, negative correlation was found between RAS/RT ratio (rhizosheath size) and fungal specific richness. This could mean that the large rhizosheath pearl millet lines could be associated to the selection of specific fungi leading to a decrease in global fungal diversity. It has been demonstrated a positive impact of arbuscular mycorrhiza on soil aggregation in several studies (Wu et al. 2014, 2015; Rillig et al. 2015). However, the g-test we have performed did not highlight any OTU matching for arbuscular mycorrhiza to differentiate RAS from large and small rhizosheath line. We evidenced an enrichment of the *Trichoderma* genus in the RAS of two large rhizosheath lines among the four. Interestingly, some recent studies reported an increase of polysaccharides amount in the rhizosphere and a PGPR effect after inoculation of plants (including chickpea, wheat and cotton) with *Trichoderma* species (Velmourougane et al. 2017, 2019). So we can hypothesize that some species belonging to this genus could contribute to root-adhering soil aggregation depending on pearl millet line.

CONCLUSION

The data presented show that, in pearl millet, the RAS/RT ratio phenotype (rhizosheath size) could be correlated with different rhizosphere microbial parameters (bacterial and fungal diversity, enzymatic activities) which may act concomitantly or independently, depending on the genotype. We identified some bacterial and fungal taxa associated with a large rhizosheath phenotype and noticed a decrease of the pH in the rhizosphere of some large rhizosheath lines indicating a role of root exudation as a potential driver. Moreover, our study highlighted that, in pearl millet, the root-adhering soil aggregation process (rhizosheath formation) is very complex and could potentially involve other mechanisms which were identified as drivers such as root architecture (Adu et al. 2017; Burak et al. 2018), root hair characteristics (length and density; Delhaize et al. 2012, 2015; George et al. 2014), and the composition of root exudates (Traoré et al. 2000; Liu et al. 2019). The relative importance of these different drivers will be investigated in future studies. Nevertheless, rhizosheath size is a promising trait to cope with abiotic stress and identification of plant genes controlling this trait could contribute to sustainable agriculture particularly in arid and semi-arid regions where this cereal is cultivated.

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