



# Rhizosheath formation is positively correlated with retention of water in the rhizosphere and water deficit stress tolerance in pearl millet



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## ABSTRACT

Plants have evolved adaptive strategies to enhance hydromineral uptake, with rhizosheath formation emerging as a key mechanism improving water deficit tolerance. In pearl millet (*Cenchrus americanus* syn *Pennisetum glaucum*), intraspecific genetic diversity exists in rhizosheath formation and root traits, differences in rhizosheath size correlating with shifts in rhizosphere bacterial composition and diversity. However, the role of rhizosheath size in pearl millet response to abiotic stresses remains unclear. Determining whether rhizosheath size improves drought tolerance would help identifying root-soil interactions characteristics that enhance pearl millet resilience. To investigate this, we conducted a study using four pairs of closely pearl millet lines contrasting in rhizosheath size. Plants were grown in an arenosol under greenhouse conditions. After three weeks of irrigation, they were subjected to two water stress regimes (partial and severe deficit) for two weeks, alongside a well-watered control. Under water stress, rhizosheath mass was positively correlated with soil moisture, plant biomass, leaf water potential ( $\Psi_l$ ), and several root traits (length, diameter, area, volume;  $p < 0.001$ ). Under moderate water stress, high-rhizosheath lines (HRL) maintained higher  $\Psi_l$  (-2.07 MPa vs. -2.63 MPa) and exhibited less shoot biomass reduction (14 % vs. 25 %) than low-rhizosheath lines (LRL). Under severe stress, HRL showed greater resilience, with a smaller root biomass reduction (54 % vs. 71 %). These results suggest that lines with more developed rhizosheath tend to maintain root system development and sustain plant growth under moderate water deficit, likely by improving rhizosphere water retention. Our work supports incorporating rhizosheath traits in pearl millet breeding programs to enhance drought tolerance.

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## Introduction

The rhizosphere is defined as the localized volume of soil under the direct or indirect influence of plant roots and their associated microbiota [1]. Some authors define the rhizosphere more precisely by subdividing it into three distinct zones: the rhizosphere in the strict sense, which is the soil near the roots, the rhizoplane corresponding to the surface of the root with the soil particles strongly adhering to the root, and the root itself, which also contains endophytic microorganisms [2]. In a recent study, Mo et al. [3] subdivided the rhizosphere in two parts: the rhizospheric soil that is removed from the root surface after gentle shaking referred as external rhizospheric soil, and the soil that is still firmly attached to the root surface referred as internal rhizospheric soil, also named rhizospheric sheath or rhizosheath. Quantitatively, the rhizosheath is defined as the weight of soil particles that adhere strongly to the root surface after excavation, shaking and removal of soil that does not adhere to the root [4].

Many functions have been attributed to the rhizosheath, such as its role as the main source of organic matter in the soil [5], in protecting roots against drought [6], in helping plants in gaining better access to phosphorus [7,8], zinc and nitrogen under stressful conditions, helping plants to better tolerate severe soil acidity [9] and mechanical stress [10]. Thus, the rhizosheath plays an important role in the uptake of nutrients and the maintenance of soil moisture around the roots in dry soils, and therefore in plant tolerance to abiotic stresses [11,12]. The rhizosheath's porosity fundamentally influences soil quality, fertility and sustainability [13]. The rhizosheath plays a critical role in enhancing crop growth and nutrient acquisition by improving soil-root interactions and facilitating water and mineral uptake [14].

Pearl millet is a major subsistence crop in the Sahelian region of West Africa [15]. Pearl millet is widely grown in semi-arid regions in Africa and Asia where climatic conditions do not allow normal growth of other cereals such as sorghum, maize or rice, and plays an important role for the subsistence of local populations [16,17]. It is mostly grown in rainfed low input agrosystems in areas characterized by low soil fertility and limited rainfall [16,18]. Before and after the 1970s, many countries in the Sahel suffered from dramatic drought episodes, unprecedented in recent history. This drought period is considered as the strongest signal of the 20th century announcing climate change, due to its duration of two decades and its magnitude [17]. It should also be noted that uncertainty in the onset of rains represents a major climatic risk for many farmers with significant negative impacts on pearl millet yields [19]. There is an urgent need to find ways to increase the resilience of pearl millet which plays a major role in food security in arid and semi-arid regions.

Intraspecific genetic variability in rhizosheath size in pearl millet has been evidenced and was positively correlated with the composition and diversity of rhizosphere bacterial communities [20]. In addition, correlation was reported between the level of soil aggregation and the intensity of some enzymatic activities (chitinase, acid phosphomonoesterase, FDA-hydrolysis and  $\beta$ -glucosidase) in the rhizosphere of pearl millet plants grown in the field [21]. Recently, it was reported that rhizosheath size in pearl millet is genetically controlled and regions of the genomes regulating this trait were identified [1]. However, the potential role of rhizosheath size in pearl millet response to abiotic stresses has yet to be demonstrated. Understanding whether this root zone is linked with plant performance under water deficit conditions may point out characteristics of root-soil interactions that promote pearl millet resilience in a context of increasing water scarcity, particularly in a context of climate change, and thus provide breeding targets to develop more resilient plants.

In this study, we investigated the role of the rhizosheath in enhancing water deficit tolerance in pearl millet. We hypothesized that (1) intraspecific genetic variability exists for rhizosheath formation among recombinant pearl millet lines, and (2) greater rhizosheath formation may improve drought resilience by enhancing soil water retention and root system development under water-limiting conditions. To test this, we screened recombinant pearl millet lines, selected genotypes with contrasted rhizosheath sizes, and cultivated them under three different water regimes: well-watered, moderate water deficit, and severe water deficit. Physiological parameters were then analyzed to assess functional link between rhizosheath formation and drought adaptation.

## Material and methods

### Pearl millet genetic material

191 recombinant pearl millet lines developed at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, Niamey, Niger) were used. These lines resulted from crosses between a high-rhizosheath line (ICML IS 11,084 or L132) and a low-rhizosheath line (ICML IS 11,139 or L220). The parents L132 and L220 were selected from 181 inbred pearl millet lines phenotyped for their rhizosheath size [22]. The recombinant lines were generated by 2 back crosses in the ICML IS 11,084 background and 4 cycles of selfing of the resulting plants (BC2F4).

### Growth conditions

Interlocked bottomless "WM" shaped plastic pots (Thermoflan, Molières-Cavaillac, France) with a size of 20 cm in height and 12.5 cm in width were used. Fiberglass mosquito net was placed in the bottom of pots to prevent soil loss. Each pot contained 1.5 kg of an arenosol (8 % water retention) with 94.5 % sand, 3 % silt and 2.7 % clay (Supplementary Table 1). The soil was sampled in an experimental field of the CNRA (Centre National de Recherches Agronomiques, Bambe, Senegal; 14.42°N, 16.28°W) and sieved at 4 mm. Before sowing, soil was moistened with 100 mL of water per pot corresponding to 80 % of field capacity and three seeds were sown at a depth of 1 cm. Plants were watered daily with 20 mL of water. Plants were thinned out after 7 days to leave one plant per pot. Eight plants were analyzed for each pearl millet line. The plants were grown for 4 weeks under natural light in a greenhouse in ISRA/

IRD Bel Air Campus in Dakar (Lat. 14.701778, Long. -17.426229, altitude 9 m). Phenotyping was performed between April and August 2021. The monthly average midday temperature fluctuated between 23 and 28 °C.

#### Root-adhering soil recovery

Plant watering was stopped 48 h before harvesting to facilitate the separation of root-adhering soil from bulk soil. Plants were harvested by detaching the interlocking parts of "WM" pots and gently removing the root system out of the soil. Then the plants with their roots were clamped at the crown level and shaken at a constant speed (1100 rpm) for 1 min with a CAT S50 electric shaker (Cat Ingenieurbuero™) to separate the bulk soil from the soil that had adhered strongly to root defined here as the root-adhering soil (RAS). Roots were carefully washed afterwards in a cup with demineralized water to collect RAS. The water in the cup was then removed by oven drying at 105 °C for three days and weighted. The ratio between mass of RAS and dry weight of root tissue (RT); RAS/RT was used to estimate the rhizosphere aggregation intensity (rhizosheath size) as previously described in Ndour et al. [20].

#### Genotyping and genetic relatedness analysis of recombinant pearl millet lines

tGBS® Genotyping by Sequencing was performed by Freedom Markers (USA) to identify Single Nucleotide Polymorphism (SNPs). 175 recombinant inbred lines were sequenced using an Illumina HiSeq X platform, and the sequences were aligned to the reference genome of *Cenchrus americanus* ASM217483v2, obtained from the NCBI ([https://www.ncbi.nlm.nih.gov/assembly/GCA\\_00217483.2](https://www.ncbi.nlm.nih.gov/assembly/GCA_00217483.2)). A set of 50,003 SNPs (MCR50 SNPs), representing SNPs found in 50 % of the samples, were generated. The occurrences of each genotype (0: missing data, 1: homozygous reference allele, 2: homozygous alternate allele, 3: heterozygous) were calculated for each SNP in the genotyping data. SNPs with at least one individual homozygous for the alternate allele were then selected. The allele frequency for a BC2F4 population is expected at 7/8 for major alleles and 1/8 for minor alleles. SNPs with an allele frequency falling in the 95 % interval estimated by 1000 binomial sampling around this expected frequency were kept. In total, 1280 SNPs were kept after the different filtering steps. The genetic distance between recombinant inbred lines was then calculated based on the frequency of shared alleles. We first built a genotype matrix of the frequency of alternate alleles, varying by individual from 0, 0.5 or 1. The genetic distance between lines was calculated using the mean of the absolute differences between the values of each pair of individuals:

$$d_{ij} = \frac{1}{N} \sum_{k=1}^N |q_{ik} - q_{jk}|$$

Where:  $d_{ij}$  is the genetic distance between lines  $i$  and  $j$   $q_{ik}$  is the genotype frequency of alternate allele of SNP  $k$  for line  $i$   $q_{jk}$  is the genotype frequency of alternate allele of SNP  $k$  for line  $j$

$N$  is the total number of markers for which genotype data is available (non-missing) for both lines  $i$  and  $j$ .

The genetic analysis of the BC2F4 did not perfectly fit expectation from this type of cross (imperfect inbreeding or potential cross-pollination). We consequently also calculated the distances without assuming BC2F4 notably the ideal allele frequencies of  $1/8$  and  $7/8$ . Both analyses led to similar results in terms of distance between lines (see results).

Recombinant lines were selected by sorting the distance values in ascending order and choosing those with distances between the minimum (excluding 0, i.e., identical) and the mean distance. From the top 500 pairs of recombinant lines based on this ordered list, the 15 pairs with the most contrasting rhizosheath sizes (high-rhizosheath and low-rhizosheath) were identified. Out of these, 3 pairs were chosen for the experiment. We estimated the probability of having two individuals with a lower genetic distance than the chosen pair. This probability was obtained by positioning the chosen pair distance in the distribution of genetic distance of all comparisons. A low value indicates that a smaller number of pairs of individuals have this distance or a lower one.

#### Water stress experiment

The study was carried out, after screening and selection, on 4 pairs of pearl millet lines contrasted for rhizosheath size (3 lines with high rhizosheath size, 3 lines with low rhizosheath size and the 2 parent lines). Each treatment was repeated three times for each pearl millet line. Plants were grown for 5 weeks under natural light in a greenhouse in the same conditions as in the large-scale phenotyping trial. Two weeks before harvest, plants were subjected to three irrigation treatments: (1) well-watered (WW) plants were watered every day with 20 mL corresponding to the average daily evaporation loss, (2) partial water deficit stress (PWS) plants were watered every day with 10 mL corresponding to half of average daily evaporation loss, (3) total water deficit stress (TWS) plants were not watered during this period. The average daily evaporation loss was determined by weighing the pots with soil in a preliminary experiment and tracking variations in soil weight [22]. Three pots, containing soil moistened with 100 mL water, were weighed daily at the same time using a precision balance. The difference in weight between two consecutive days corresponds to the amount of water lost through evaporation. The amount of water lost was then added to maintain the moisture content, so that the procedure could be repeated over 3 days and give a daily watering rate to be used as the well-watered treatment.

#### Midday leaf water potential

Leaf water potential was measured on flag leaves the day before harvest (2 weeks after water deficit stress was applied). Measurements were made with a pressure chamber in full sunlight between 1PM and 2PM. The device used a modified 1.5 ton commercial

hydraulic car jack to apply pressure from beneath a rubber flexible membrane to a leaf sample positioned on a filter paper between the membrane and a plexiglass plate placed on top of the leaf [23]. Since this is a destructive method, for each line and at the same distance on the flag leaves, a sample was taken and lines were grouped according to the size of the rhizosheath, with four high rhizosheath (HRL) genotypes and four low rhizosheath (LRL) genotypes and 3 repeats per genotype. This gives a total of 12 samples for HRL and 12 samples for LRL.

#### *Chlorophyll content*

The leaf chlorophyll content was measured at 3 weeks after sowing (before the water deficit stresses were applied) and 4 weeks after sowing (1 week after water deficit stresses were applied) using a SPAD 502 PLUS meter. For each line, measurements were performed on three different leaves per plant and the average chlorophyll content directly made by SPAD was reported. Lines were also grouped according to rhizosheath size, with four high rhizosheath (HRL) genotypes and four low rhizosheath (LRL) genotypes.

#### *Shoot and root biomass*

After recovering RAS, plants were cut at the crown to separate the aboveground and root parts. Each aboveground part was placed in a labeled envelope and the roots were kept at 4 °C in labeled Falcon tubes containing 70 % alcohol. The roots were dried and weighed after measuring the root architecture traits and root hairs length. Roots and shoots were dried at 65 °C for three days before biomass measurement.

Shoot reduction and root reduction were computed using the following formula:

$$\text{Shoot / Root reduction} = \frac{\text{Stressed biomass} - \text{Unstressed biomass}}{\text{Unstressed biomass}} \times 100$$

#### *Root architecture traits*

Root architecture traits (length, average diameter, root area, root volume) were measured using WinRHIZO software version 2012b after scanning the roots using an Epson Perfection V700 scanner. Roots were separated into two groups based on their diameter as primary and crown roots (0.25 mm < diameter < 1 mm) and lateral roots (diameter < 0.25 mm; [25]).

#### *Root hair length*

Root hair length was measured from images of the root hair zone of three intact lateral roots (with apex) per plant. Images were taken using an optical microscope (BX50F, Olympus) equipped with a digital camera (Micro Publisher 3.3 RTV). For each lateral root, the length of 10 randomly selected root hairs was measured using the free Mesurim software (<http://acces.ens-lyon.fr/acces/logiciels/applications/mesurim>).

#### *Soil moisture measurement*

At harvest, a sample of the bulk soil was also taken from each pot. These samples were weighed and dried afterwards at 105 °C to determine the moisture content of the soil in the pot at the time of harvest using the following formula:

$$H\% = \frac{\text{Bulk soil mass (at harvest)} - \text{Dry Bulk soil mass}}{\text{Dry Bulk soil mass}} \times 100$$

#### *Statistical analyses*

All statistical analyses were performed with R version 4.1.1 (2021-08-10). Normal distribution of data was confirmed by Shapiro-Wilk's test at *p*-value < 0.05. ANOVA model and Tukey's pairwise comparison test (HSD) with a 95 % confidence interval were used to compare the mean parameters of the different lines. For shoot and root reduction, a two-way ANOVA was performed to assess the effect of the interaction between rhizosheath group and the irrigation treatment. In order to see the relationships between the different parameters measured, a correlation matrix with Spearman's test (*p*-value < 0.05) was performed on means. For data that did not follow a normal distribution, the Kruskall-Wallis test followed by Dunn's post-hoc pairwise comparison test (*p*-value < 0.05) was used to compare differences.

## **Results**

#### *Variability in rhizosheath size within the recombinant lines*

Rhizosheath size (expressed as a ratio between root adhering soil and total root biomass, RAS/RT) was measured on 191 recombinant pearl millet lines as well as their two parents (L132, L220; Fig. 1A). For each line, eight plants were phenotyped by sowing

the whole panel with one pot per line, in eight successive blocks. As expected, the parent lines (indicated by red arrows on Fig. 1A) showed significant differences in rhizosheath formation, with the low rhizosheath parent L220 being significantly different from the high rhizosheath parent L132 (RAS/RT values respectively 41.3 and 95.2). A large diversity of rhizosheath size was observed in the recombinant lines. The mean RAS/RT for each line ranged from 23.0 for BIL107 to 103.5 for BIL112. Since both soil moisture and temperature are known to influence the rhizosphere aggregation, the humidity effect and the block effect (potential temperature effect) on the RAS/RT ratio were examined. No significant correlation was found ( $p$ -value  $< 0.05$ ) indicating that these two factors did not interfere with the screening process.

Based on the rhizosheath size of the parents, 3 groups of recombinant lines were defined based on an ANOVA (Tukey test  $p$ -value  $< 0.05$ ) (**Supplementary data**):

- a group of lines with high rhizosheath size (high-rhizosheath lines, HRL) which are significantly different from the parent lines with low rhizosheath size. Their rhizosheath size is greater than 73.8.
- a group of low rhizosheath size lines (low-rhizosheath lines, LRL) for which rhizosheath size is  $< 62.8$ , and which are significantly different from the high rhizosheath size parent line.
- a group of intermediate lines for which average rhizosheath size is between 63.3 and 73.4, which are neither different from the low rhizosheath parent line nor from the high rhizosheath parent line.

#### Selection of recombinant lines

Following phenotyping of the entire biparental population, we selected six recombinant pearl millet inbred lines, three LRL (BIL1, BIL107 and BIL108) and three HRL (BIL54, BIL126 and BIL217) together with as a control parent lines (L220/LRL and L132/HRL) for subsequent experiments (Fig. 1B). The selection of these pearl millet lines was made by pairs (one LRL with one HRL) on the basis of their contrasted rhizosheath size and on the genetic proximity within each pair (Table 1).

The genetic distances between the selected line pairs ranged from 0.077 to 0.110 (Table 1), indicating that they share 89.0–92.3 % of their alleles for the analyzed SNPs and are thus genetically closely related. The probability to have lower distance between inbred lines than the pair chosen was low ( $p$ -value  $< 0.005$ , Table 1). The heterozygosity observed among inbreds was 22 % compared to an expected 3 %. Consequently, we recalculated the genetic distance between recombinant lines without presuming them to be perfect BC2F4, leading to similar results ( $p$ -value  $< 0.008$ , Table 1).

#### The extent of water deficit stress as function of treatments applied

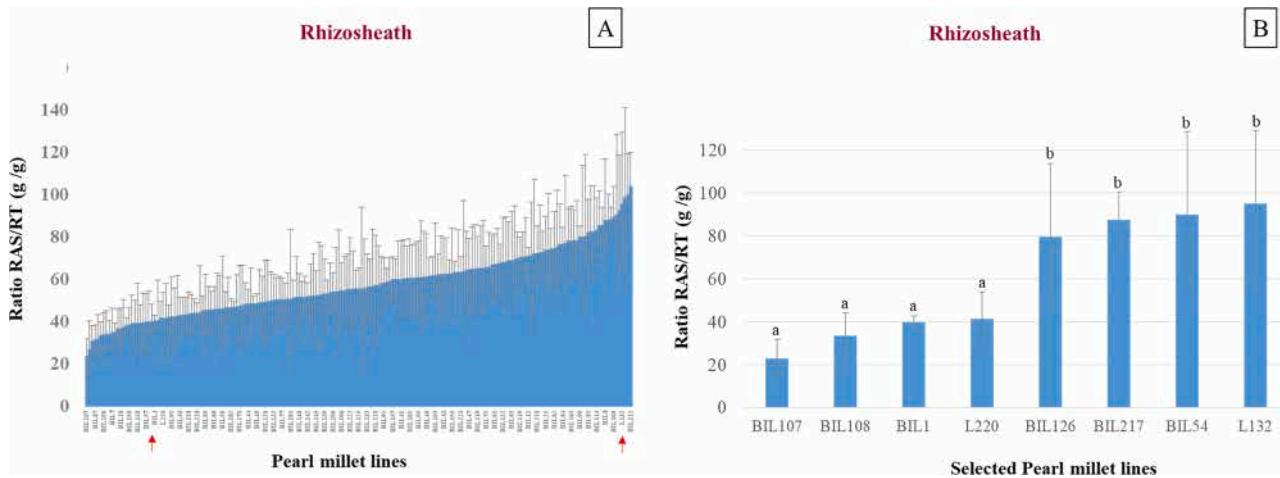
In order to characterize the intensity of the stress applied to the plants in the different treatments, we measured midday leaf water potential after two weeks of water deficit and soil moisture at harvest. We found that the midday water potential was positively correlated with the soil moisture [ $p$ -value  $< 0.0001$ ,  $r^2 = 0.7$ ] (Fig 2A). This relationship shows that the more water available in the soil, the less stressed the plants were, and vice versa. Likewise, we observed that with TWS treatment (green), considered in our work as extreme water deficit stress, we had the lowest leaf water potentials (below  $-5$  MPa, Fig. 2B). In contrast, with WW treatment (in blue), representing the optimum water conditions, measured leaf water potential values were highest ( $\approx -0.94$  MPa). Concerning the PWS treatment, i.e. application of moderate water deficit stress, values were intermediate between WW (well-watered) and TWS (total water deficit stress) with  $\approx -2.35$  MPa. The variation in leaf water potential between the WW, PWS and TWS treatments was significant (Tukey test,  $p$ -value  $< 0.05$ ). This shows that the impact of irrigation treatments on the plants was substantial, with different intensities in terms of impact on plant water status.

#### Correlation between plant physiological parameters and rhizosheath size

To assess drought response variability among the selected lines, plants were grown under irrigated conditions for three weeks prior to stress imposition. Two different water deficit regimes were then applied for two weeks: (i) partial water deficit stress (PWS) implemented by reducing daily watering quantity by 50 %, (ii) total water stress (TWS) achieved through complete water withdrawal. Well-watered (WW) plants maintained under optimal irrigation served as controls. We measured different physiological parameters across all treatments and performed correlation analyses to identify relationships between observed traits under both well-watered and water-stressed conditions.

In well-watered conditions (without applied water deficit stress), RAS/RT was positively correlated with total root length (RL), total root surface area (RS), average root diameter (RD), total root volume (RV), thick root length (TRL), thick root surface (TRS) and average root hairs length (RHL; Fig. 3A and **Supplementary Table 2**). It was negatively correlated with the Root/Shoot ratio (R/S). The RAS/RT was not correlated with root biomass (RT), above-ground biomass (S), leaf water potential at harvest ( $\Psi_l$ ) and chlorophyll content (CC), fine root length (FRL) and fine root surface area (FRS). We also observed that soil humidity (H %) was not significantly correlated with any of the parameters measured in these conditions.

In water deficit stress conditions (Fig. 3B and **Supplementary Table 3**), rhizosheath size was strongly and positively correlated with soil moisture, shoot biomass (S), midday leaf water potential at two weeks after water deficit stress ( $\Psi_l 2sw$ ) and with root traits: root biomass (RT), total root length (RL), total surface area (RS), average diameter (RD), total volume (RV), fine root length (FRL), thick root length (TRL), fine root surface area (FRS) and thick root area surface (TRS). The rhizosheath size was also weakly and positively correlated to average root hairs length (RHL). It was not correlated with chlorophyll content before water deficit stress (CC),



**Fig. 1.** Rhizosheath size of different pearl millet lines. (A) RAS/RT ratio of 193 pearl millet lines. Red arrows indicate parent lines. (B) RAS/RT ratio of 8 pearl millet lines selected. Each histogram represents the mean of 8 replicates with their standard deviations. Different letters indicate significant differences between lines according to Tukey's pairwise comparison test (HSD) with a 95 % confidence interval.

**Table 1**

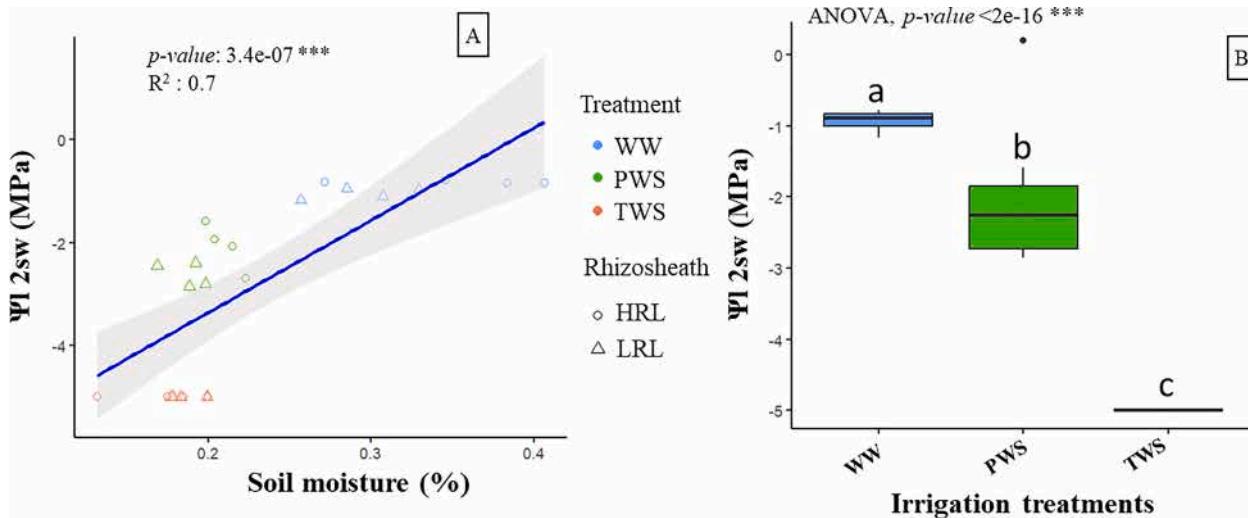
Genetic distance of selected recombinant pearl millet lines. Position: The relative position of the pairs of individual lines.

Pairs of lines		BC2F4 hypothesis		All markers	
HRL	LRL	Distance	Position	Distance	Position
BIL54	BIL1	0.088	0.0005	0.077	0.0015
BIL126	BIL107	0.109	0.0042	0.087	0.0035
BIL217	BIL108	0.110	0.0046	0.096	0.0076

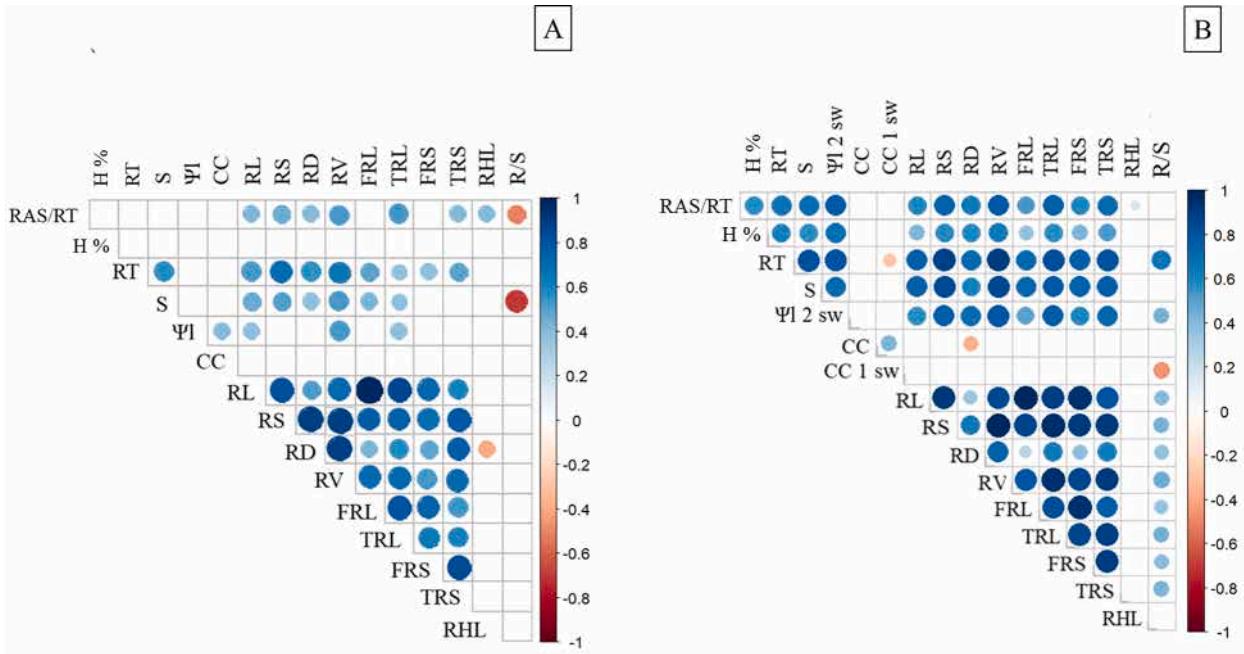
chlorophyll content one week after water deficit stress (CC 1sw) and Root/Shoot ratio.

Note that a detailed picture of the regressions between RAS/RT and the other parameters is given in **supplementary Figs. 1, 2 and 3**.*High-rhizosheath lines produce more biomass in water deficit stress conditions*We analyzed relationships between: (i) shoot and root biomass and leaf water potential at two weeks after water deficit stress ( $\Psi$ 1 2sw) and (ii) shoot and root biomass and rhizosheath size (RAS/RT).Without water stress deficit, no significant differences were observed between HRL and LRL in terms of root and above-ground biomass (**Supplementary Fig. 4**). However, we observed a positive correlation between shoot biomass reduction and midday leaf water potential at two weeks after water deficit stress ( $\Psi$ 1 2sw) [ $p$ -value <0.001,  $r^2 = 0.91$ ] (**Fig. 4A**). The same positive correlation was observed for root reduction and midday leaf water potential at two weeks after water deficit stress ( $\Psi$ 1 2sw) [ $p$ -value <0.001,  $r^2 = 0.8$ ] (**Fig. 4D**). Hence increasing water deficit stress led to a decrease in  $\Psi$ 1 2sw and, consequently, a decrease in biomass production. Interestingly, we found that the RAS/RT ratio was negatively correlated with shoot biomass reduction [ $p$ -value <0.001,  $r^2 = 0.77$ ] (**Fig. 4B**) and root biomass reduction [ $p$ -value <0.001,  $r^2 = 0.64$ ] (**Fig. 4E**), suggesting that shoot and root biomass were less affected by water deficit stress in plants with a large rhizosheath.Two-way ANOVA tests, with treatment and rhizosheath as factors, have evidenced only a significant impact of treatment on shoot reduction ( $p$ -value <0.0001) and root reduction ( $p$ -value = 0.00442), indicating that in this analysis the effect of treatment (PWS vs TWS) is much larger than potential variations due to genotypic effects. In order to more specifically if there was a genotypic effect under each given water shortage condition, we opted for a simplified approach by performing a one-way statistical test between HRL and LRL for PWS and TWS separately. Under partial water deficit stress, the reduction of shoot biomass for HRL was lower than that of LRL (14 % vs 25 % respectively, significant difference; **Fig. 4C**). This suggests that under partial water deficit stress, the response of root biomass to water deficit stress is the same for HRL and LRL, but that shoot biomass was more impacted for LRL than for HRL. Conversely, under total water deficit stress, the reduction in root biomass was less for HRL than for LRL (54 % vs 71 %, significant difference; **Fig. 4F**), while reduction in shoot biomass was not significantly different between HRL and LRL.We further analyzed the impact of water deficit stress and rhizosheath size on leaf physiology. In absence of water deficit stress, leaf water potential averaged  $-1.05$  MPa for LRL and  $-0.82$  MPa for HRL with no significant difference (**Fig. 5**). Under partial water deficit stress, leaf water potential was significantly different between LRL and HRL ( $-2.63$  MPa vs  $-2.07$  MPa; **Fig. 5**). Leaf water potential was therefore less affected by stress for HRL than for LRL.**Discussion**

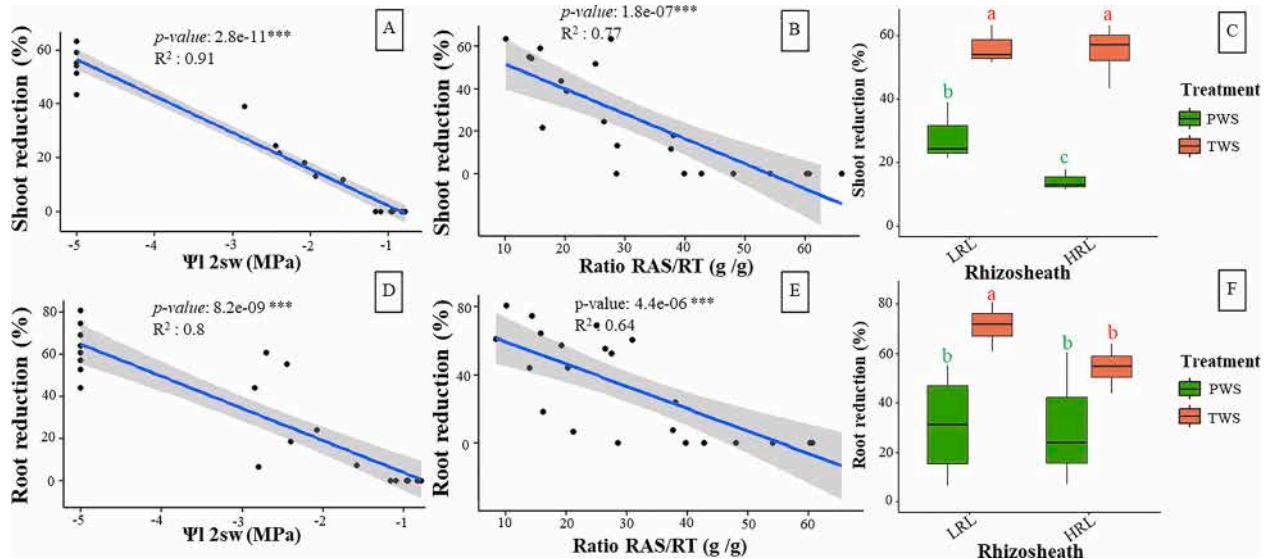
In this study we analyzed the interaction between rhizosheath formation and water stress in pearl millet. We tested the hypothesis that intraspecific genetic variability of the rhizosheath trait may exist within recombinant lines pearl millet and that an increase in rhizosheath formation could improve water retention and promote root development under drought conditions, thus contributing to better plant tolerance to water deficit. Analyses assessed the effects of water availability on several plant physiological parameters. While the millet lines originally identified by Ndour et al. [20] could be considered to perform this assessment, it is likely that these inbred lines being highly diverse in terms of genetic material may differ from each other by many characters besides those responsible for rhizosheath formation. Consequently, when comparing the physiological or agronomic performance of these lines, confusion of effects and epistatic interactions of multiple QTLs [24] could arise and mask potential roles of our target phenotype. This is why we decided to undertake a strategy of backcrossing/selfing from a cross between selected HAL and LAL formerly identified by Ndour et al. [20] to generate lines with a largely common genetic stock, also known as near-isogenic lines (NILs) [25]. For the most part, these lines share a common HAL genetic background and vary in introgressed chromosome fragments from the LAL. This strategy has a double advantage: further confirm and support the genetic inheritance of the aggregation character, and give a material with more homogenous properties for physiological and agronomic studies. To our knowledge, this is the first time that this strategy is employed in a Sahelian crop to assess variability in rhizosheath formation. This would allow us to lower the occurrence of confounding effects and more specifically test the impact of the rhizosphere aggregation phenotype. However, due to the high level of heterozygosity at the population level, we were unable to conduct the planned QTL (Quantitative Trait Loci) analysis as initially intended. We were nevertheless able to identify pairs of lines genetically related (89.0–92.3 % of common alleles for the analyzed SNPs) for our physiological analyses.



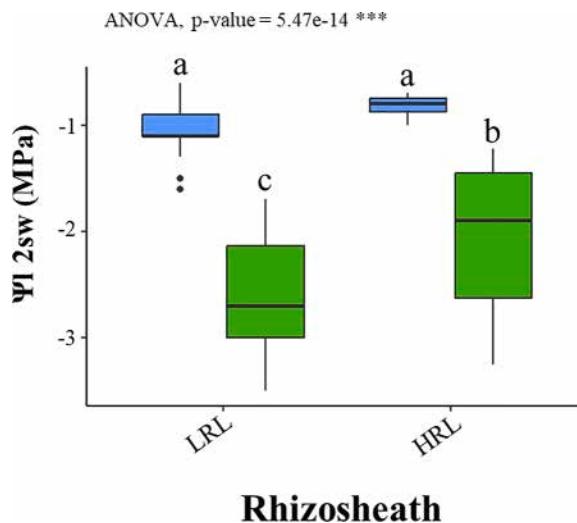
**Fig. 2.** Impact of applied irrigation treatments. (A) Linear regression between soil moisture and midday leaf water potential at two weeks after water deficit stress ( $\Psi_l$  2sw). Points represent the mean  $\Psi_l$  2sw value of each pearl millet line with colors representing irrigation treatments and shape representing the rhizosheath size potential (High: HRL and Low: LRL). (B) Boxplot showing midday leaf water potential at two weeks after water deficit stress ( $\Psi_l$  2sw) of different types of irrigation treatments. Each box represents the average  $\Psi_l$  2sw according to irrigation treatments: WW (well-watered), PWS (partial water deficit stress), TWS (total water deficit stress). Different letters indicate significant differences between irrigation treatments according to Tukey's pairwise comparison test (HSD) with a 95% confidence interval. Spearman's test was used to determine r-squared and p-value.



**Fig. 3.** Correlation between plant physiological parameters and rhizosheath size. Correlogram of physiological parameters of 8 contrasting rhizosheath pearl millet lines under normal irrigation conditions (A) and under water deficit stress (B). Root adhering soil per unit of root biomass tissue (RAS/RT), soil humidity (H %), root biomass tissue (RT), above-ground biomass (S), leaf water potential unstressed ( $\Psi_1$ ), leaf water potential at two weeks after water deficit stress ( $\Psi_1$  2sw), chlorophyll content before water deficit stress (CC), chlorophyll content at one week after water deficit stress (CC 1sw), total root length (RL), total root surface area (RS), average root diameter (RD), total root volume (RV), fine root length (FRL), thick root length (TRL), fine root surface area (FRS), thick root surface area (TRS), average root hair length (RHL), Root/Shoot ratio (R/S). Spearman's test was used for correlation and empty cage indicates no-significant correlation between parameters ( $p < 0.05$ ).



**Fig. 4.** Relations between rhizosheath, midday leaf water potential at two weeks after water deficit stress ( $\Psi_l$  2sw), shoot and root biomass reduction. (A) Linear regression between shoot biomass reduction and midday leaf water potential at two weeks after water deficit stress ( $\Psi_l$  2sw). (B) Linear regression between shoot biomass reduction and RAS/RT. (D) Linear regression between root biomass reduction and leaf water potential at two weeks after water deficit stress ( $\Psi_l$  2sw). (E) Linear regression between shoot biomass reduction and RAS/RT. Points represent the mean value of each pearl millet line with colors representing irrigation treatments and shape, the rhizosheath size potential. Boxplot showing the response of shoot (C) and root (F) biomass to different types of water deficit stress after 2 stressed weeks of 8 different pearl millet lines grouped according to their rhizosheath size potential (High and Low). Each box represents the average of 4 high-rhizosheath lines and 4 low-rhizosheath lines according to treatments. Different letters indicate significant differences between pearl millet lines groups according to Kruskal-Wallis test followed by Dunn's pairwise comparison test ( $p < 0.05$ ). Spearman's test was used to determine  $R^2$  and  $p$ -value.



## Rhizosheath

**Fig. 5.** Boxplot showing the leaf water potential under well-watered conditions (WW - blue) and after 2 weeks of partial water deficit stress (PWS - green), for 8 different pearl millet lines grouped according to their rhizosheath size potential (High and Low). Each box therefore represents the average of 4 high-rhizosheath lines and 4 low-rhizosheath lines according to treatments. Different letters show significant differences between pearl millet lines groups according to Tukey's pairwise comparison test (HSD) with a 95 % confidence interval.

### The extent of water stress

We showed that in the well-watered treatment, with average midday leaf water potential value equal to  $-0.94$  MPa, the plants were not under water deficit stress. In contrast, the PWS and TWS treatments, with average potential equal to  $-2.35$  MPa and below  $-5$  MPa respectively, showed that plants underwent moderate to severe water stress with these treatments. These results are consistent with the observations of Matsuura & An [26], who measured mid-day leaf water potential using a pressure chamber in foxtail millet. They reported water potentials ranging from  $-0.8$  to  $-1.12$  MPa under normal conditions and from  $-1.72$  to  $-2.02$  MPa under water deficit. Similarly, Subramanian & Maheswari [27] showed that unstressed pearl millet had a leaf water potential of  $-1.52$  MPa, while this decreased to  $-2.09$  MPa under water-stressed conditions. These results therefore confirm that leaf water potential can be used as a reliable indicator to categorize deficit water stress, i.e. moderate water stress and severe water stress, in plants.

### Reduction of rhizosheath, root and shoot with water deficit stress

In water stress conditions, we observed a reduction of rhizosheath size proportional to the intensity of water deficit stress, which confirms that soil moisture plays a very important role in rhizosheath formation or stability, particularly in sandy soils. Our results corroborate those of Steiner et al. [28] that demonstrated, in maize, an overall reduction in the total mass of the rhizosheath and the mass of the rhizosheath normalized by root length or root biomass in drought conditions. According to Rahim et al. [29], rhizosheath formation under dry conditions is closely linked to mucilage concentration. In these conditions, the mucilage is too concentrated to diffuse far into the soil, which consequently leads to a reduction of rhizosheath size. This might explain the strong negative correlation that we observed between intensity of water stress and rhizosheath size.

On the other hand, the application of water stress induced a reduction of above-ground and root biomass, as well as all the root traits we measured. Schweiger et al. [30] emphasized that under water deficit stress conditions, photosynthesis is limited by closure of stomata to avoid water loss, leading to a decrease of  $\text{CO}_2$  input and/or by damage to photosynthetic machinery. Also, Yang et al. [31] observed on wheat, in addition to reducing the photosynthesis rate by over 62 %, water deficit stress decreased root exudation by up to 89 %. This reduction of rhizodeposition in the rhizosphere was also observed by Steiner et al. [28] in maize under water deficit stress. All these factors might also explain these reductions.

### Correlation between soil moisture, LWP and rhizosheath

We observed that rhizosheath size was positively correlated with soil moisture at harvest. Since, the roots after 5 weeks of cultivation occupied almost the entire pot at harvest, soil moisture at harvest was likely equivalent to water content in the rhizosphere. This result underlines the role of rhizosheath in maintaining soil moisture around the roots, particularly under conditions of abiotic stress. This is confirmed by the fact that we observed that despite reduced watering, lines with high rhizosheath size, under moderated deficit water stress condition, had higher leaf water potentials compared to lines with a low rhizosheath size (21.3 % difference). This result aligns with the work of Hosseini et al. [32] who observed that in moderate moisture, maize with rhizosheath improved soil-plant-water relationships. Similarly, under water stress conditions, when the soil water content dropped to 50 % of field capacity, Rabbi et al.

[33] found that the tolerant cultivar of chickpea had significantly greater rhizosheath mass, higher average stomatal conductance to water vapor and higher mid-day whole plant water potential than the sensitive cultivar with lower rhizosheath mass. Similarly, rhizosheath mass was shown to have a positive relationship with transpiration rate in wheat [34,35]. These results could be linked to the water holding capacity of mucilage and other polymeric substances exuded by roots and microorganisms in the rhizosphere. In their work, Ahmad et al. [34] showed that the metabolic profiles of different pearl millet lines reflect their levels of rhizosheath formation. Their results also highlighted the fact that interconnection between root exudates and microbiota jointly shapes the structure of rhizosheath. We therefore hypothesize that, in our context of water stress, plants with higher rhizosheath size have more root exudation or trigger more microorganism-led exopolymer production, and consequently deposit more mucilage into the rhizosphere. This should in return favor water retention. Then, these mechanisms require further experimental validation, such as direct measurements of exudates, soil porosity and microbial activity in stress conditions.

#### *Positive correlation between root traits and rhizosheath in water stress deficit*

The positive correlation between rhizosheath size and parameters linked to root system development may suggest that root growth also favors the aggregation of soil particles. But the correlation could also be understood in the reverse way: for instance Liu et al. [35] proposed that the rhizosheath has a significant impact on root architecture by creating macropores and which promote steeper and deeper root growth. In our work, we also found that rhizosheath size negatively correlated with shoot biomass reduction and root biomass reduction under water deficit stress. Also, we observed that under moderated water deficit stress, the reduction of shoot biomass for high-rhizosheath lines was 11 % lower for low-rhizosheath lines. Under severe water deficit stress, the reduction in root biomass was 17 % less for high-rhizosheath lines than for low-rhizosheath lines. Whereas, without water stress, RAS/RT is not correlated with either root or above-ground biomass. Moreover, high-rhizosheath lines did not show significant differences in either root or above-ground biomass compared with low-rhizosheath lines in the absence of stress. This means that high-rhizosheath lines are less responsive and therefore less sensitive to water deficit stress than low-rhizosheath lines in terms of root or shoot biomass impairment. This hypothesis is further reinforced by the fact that, in water deficit stress conditions, we observed a higher positive correlation, compared with non-stressed conditions, between rhizosheath size and both above-ground and root biomass, leaf water potential at two weeks after water deficit stress and all root traits. These results corroborate the work of Liu et al. [36] who observed that rhizosheath size was correlated with more numerous and deeper roots under drought stress in foxtail millet. It suggested that plants that can produce longer, denser root hairs, thereby forming large rhizosheaths, are able to sustain root growth in water scarce conditions. All these results show that rhizosheath could potentially contribute to protecting plants against water loss during a dry period, as sheath structure creates a buffer barrier between plant roots and environment [3,37]. According to Mo et al. [3] and Cheraghi et al. [38], the rhizosheath can serve as a protective layer shielding plant root, particularly young roots, from heat stress and desiccation. This thesis is in line with our observations that under water deficit stress conditions, rhizosheath size was weakly and positively correlated with root hair length ( $r = 0.32$ , explains 10.24 % of variability) and also fine roots (length ( $r = 0.51$  explains 26.01 % of variability) and surface area ( $r = 0.56$  explains 31.36 % of variability)), which suggests that these fragile parts are protected by larger rhizosheaths.

However, it is important to note that with the application of stress, high rhizosheath (HRL) lines tend to also better maintain root architectural characteristics than low rhizosheath (LRL) lines. Therefore, in addition to the significant relationship between rhizosheath size and resilience to water stress that likely involves root protection and improvement in distribution and retention of water in the soil, these results indicate that the impact of root architecture is not to be dismissed in getting the full picture of the observed responses to water stress.

When the soil is drying, air gaps can form around plant roots as the soil and roots shrink. These gaps can considerably hinder water absorption by the roots and amplify the stress. North & Nobel [39] observed that the rhizosheath delayed the appearance of these air cavities, helping to maintain optimum soil moisture levels and improving mechanical contact between root and soil, which may give a mechanical explanation of beneficial effect of rhizosheath under drought. The positive influence of rhizosheath on water uptake in dry conditions, combined with the development of the root system, should be advantageous for nutrients uptake as well [6], because nutrient acquisition is highly dependent on the mass water flow to roots and mineral nutrients generally dissolve in water before absorption by roots [3]. Accordingly, James et al. [7] have shown that it is possible to develop wheat lines with improved phosphorus efficiency acquisition based on phenotypic selection for rhizosheath size. However, the trade-offs between rhizosheath size and energy costs, in particular the carbon costs associated with producing larger rhizosheaths, remain an interesting area of investigation.

#### **Conclusion**

This study demonstrates the critical role of rhizosheath in enhancing drought resilience among closely genetically related pearl millet lines. Our results reveal significant positive correlations between rhizosheath size and key drought tolerance indicators, including soil moisture, plant biomass, leaf water potential, and root system architecture. Under partial water deficit stress, high-rhizosheath lines (HRL) maintained superior leaf water potential ( $-2.07$  vs  $-2.63$  MPa in LRL) and showed reduced shoot biomass loss (14 % vs 25 %), compared to the low-rhizosheath lines (LRL). Under severe water deficit, HRL exhibited significantly greater root system preservation in root biomass than (54 % vs 71 % biomass reduction in LRL). These findings suggest a dual protective mechanism: (1) under moderate water stress, rhizosheath-mediated water retention in the rhizosphere promotes root development and sustains plant growth, while (2) under severe stress, it may physically protect root system from desiccation damage. Our work provides evidence which would advocate for incorporating rhizosheath traits phenotyping in pearl millet breeding programs to enhance its

drought tolerance, particularly in changing climates in Africa. But a limitation of our work is that the quantification of rhizosheath (rinsing roots) was not compatible with specific determination of rhizospheric moisture, which would have been an interesting parameter to better understand the observed protective effects. Future research directions should focus on direct measurements, such as imaging or the use of sensors, which will enable a more accurate assessment of the impact of rhizosheath on water retention in the rhizosphere. It would also be interesting to study root exudate profiles and their role in rhizosheath formation, as well as its genetic determinants. The characterization of rhizosheath-associated microbiota could provide critical insights into their functional contributions to the observed drought resilience phenotypes, particularly regarding water retention, nutrient cycling, and root system protection. An exploration of the trade-offs between root growth, rhizosheath production and water stress management would be very interesting for a better understanding of these dynamics. Also, field validation across diverse environments and developmental stages and on different varieties would be needed to better understand how these processes can be leveraged for sustainable agriculture facing climate change.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.sciaf.2025.e03103](https://doi.org/10.1016/j.sciaf.2025.e03103).

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