Iron-induced Transcriptional and Microbiome Changes in Host Roots Associated with Drought Tolerance in Sorghum

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Abstract

Iron (Fe) metabolism is linked to drought tolerance, and to microbial community composition, by unknown mechanisms. In this growth incubator study, exogenous Fe caused a significant recovery from growth retardation in sorghum exposed to drought, including improved photosynthetic attributes. The elevation of Fe in roots of Fe-treated plants was correlated with increased *SbFER1* (*Ferritin 1*) expression, elevated siderophore levels, and decreased H_2O_2, suggesting that ferritin minimizes oxidative stress under drought. RNA-sequencing analysis in roots demonstrated the Fe-associated induction of genes associated with auxin transport, plus increased indole-3-acetic acid and abscisic acid. Amplicon sequencing indicated alterations of both bacterial and fungal communities in roots by both drought and added Fe. Among fungi, drought reduced *Curvularia* and *Pseudallescheria* regardless of Fe levels, while *Taralomyces* increased with drought but declined when Fe was augmented during drought. *Trichoderma* levels remained similar under control and drought conditions but dramatically decreased with elevated Fe. In 16S analysis, drought caused a massive increase in *Trinickia*, which is further amplified by Fe treatment, while the significant increase in *Burkholderia* by drought was minimized by Fe treatment. The substantial decrease in *Herbaspirillum* with drought stress was not reversed by Fe supplementation. The abundance of *Streptomyces*, a known modulator of plant drought tolerance, did not change dramatically with water stress, but split-root assays suggested that elevated Fe is required for *Streptomyces* to assist sorghum in recovery from drought. These findings indicate that Fe-mediated drought tolerance in sorghum is linked to ferritin-driven redox regulation and shifts in fungal and bacterial communities.

Keywords: Auxin, bioenergy crop, ferritin, microbial alternations, photosynthetic efficiency.

Running title: Fe-mediated drought tolerance in sorghum

Introduction

Drought stress, leading to adverse impacts on growth, development, and productivity (Lamaoui et al. 2018), is ubiquitous across crops and natural plant populations but varies dramatically in intensity and frequency. Primary drought stress symptoms, especially wilting and leaf curling, are reflective of reduced turgor pressure and water loss reduction strategies (Ruehr et al. 2019; Zoghi et al. 2019). Additional drought stress manifestations include leaf yellowing, stunted growth, and premature leaf senescence (Khan et al. 2018). Water deficiency disrupts essential physiological processes like photosynthesis, nutrient uptake, and hormone regulation, ultimately compromising the plant's ability to thrive (Hussain et al. 2018). Additionally, crops subjected to drought stress often show increased susceptibility to pests and diseases (Duveiller et al. 2007). This major agricultural problem is expected to be more prevalent under current climate change scenarios. As global temperatures rise and weather patterns shift, many regions are experiencing unusually long periods of low precipitation and resultant water shortages. Sorghum (*Sorghum bicolor*) is known for its exceptional drought tolerance, but its yields are affected by prolonged periods of water scarcity (Rajarajan et al. 2021). Studies predict that drought, associated with temperature increases, could cause up to a 44% yield loss in US sorghum production in the near future, emphasizing the need for additional efforts to improve resilience, especially during the pre-flowering phase (Tack et al. 2017).

Plants employ diverse strategies to regulate water loss, including stomatal and osmotic adjustments through the accumulation of compatible solutes and the enhanced production of antioxidant enzymes and metabolites to counteract reactive oxygen species (ROS) generated under drought conditions (Seleiman et al. 2021; Takahashi et al. 2020). Hormones, particularly abscisic acid (ABA) and auxin play a pivotal role in orchestrating these responses by modulating stomatal closure and activating stress-responsive genes, ultimately enhancing a plant's ability to withstand periods of water shortage (Iqbal et al. 2022; Sadok and Schoppach, 2019). Iron (Fe) and ferritin play a crucial role in the drought tolerance of plants because Fe is an essential component of antioxidant enzymes that help neutralize reactive oxygen species generated during drought stress (Zhang et al. 2023; Ndou et al. 2023). Ferritin, a Fe-storage protein, acts as a central player in the plant's antioxidant defense system by regulating Fe availability and preventing the excessive accumulation of free Fe that could lead to ROS production (Briat et al. 2010).

Microbes can improve plant stress resistance, including drought tolerance, by instigating a range of processes that alter plant biochemistry and physiology (Poudel et al. 2021; Timmusk et al. 2013). Fungal communities, including AMF (arbuscular mycorrhizal fungi), are sometimes associated with drought stress tolerance in some crops (Chen et al. 2021; Ferrol et al. 2019). Studies suggest that AMF can directly influence plant performance by modifying the plant–water relationship, leading to increased plant productivity during periods of drought stress (Ma et al. 2020; Rapparini and Peñuelas, 2014). *Trichoderma*, a fungal genus, has gained attention for its potential role in enhancing drought tolerance in plants. Two ways in which *Trichoderma* are believed to contribute to drought tolerance are by inducing a systemic drought stress response in plants and by participating in the production of specific metabolites that positively impact plant responses to drought (Scudeletti et al. 2021; Mona et al. 2017). Monoderm bacterial lineages,

such as Actinobacteria, Chloroflexi, and Firmicutes, induce increased desiccation tolerance in plants, associated with thicker plant cell walls, and these microbes' increased abundances are driven by drought-responsive plant metabolism (Xu and Coleman-Derr 2019). Inoculation of *Bacillus amyloliquefaciens* S-134 on wheat plants increased the production of auxins, stimulated root growth, and assisted plants in adapting to water stress conditions. Particularly, *Streptomyces* are reported to produce auxin and siderophores that can promote mineral availability, stress tolerance, and growth in plants (Anwar et al. 2016; Palaniyandi et al. 2014). Studies have shown that drought tolerance in sorghum is linked to Fe metabolism and that the loss of a Fe transporter has implications for the composition of the bacterial community (Xu et al. 2021).

Previous experiments have shown that drought tolerance in sorghum is linked to Fe metabolism and that the loss of a Fe transporter has implications for the composition of the bacterial community (Xu et al. 2021). A recent study proposed that optimizing Fe homeostasis through vacuolar Fe transporters and ferritin enhances Fe accumulation in sorghum seeds when subjected to drought stress (Araki et al. 2022). However, the interplay between Fe and drought tolerance, as well as the mechanism of action and its interactions with both prokaryotic and eukaryotic microbes in sorghum, remain unclear. Our study aims to investigate the impact of exogenous Fe on host responses and endophytic microbes in sorghum subjected to drought stress. By investigating the physiological and molecular mechanisms underlying Fe-mediated drought tolerance, we provide valuable insights into the potential of enhancing crop resilience in water-limited environments through targeted management of Fe availability and associated microbial communities.

Materials and methods

Plant cultivation and growth conditions

Seeds of sorghum (USDA-GRIN accession PI 591038) were surface sterilized using a 1% sodium hypochlorite bleach solution for 5 min. Following sterilization, the seeds were rinsed three times with sterile water and subsequently placed in a tray for germination at 25°C in an incubator for a duration of 2 d. Plants were cultivated in pots in a mixture of field soil and SunGro potting mix (1:3) in three different treatments: control, drought, and drought + Fe-EDTA (1g/1000 g soil) supplementation. Drought conditions were initiated by reducing water availability to 75% compared to the controls (well-watered) two weeks after transplanting the seedlings into the soil pots. Daily monitoring of soil moisture content was conducted to maintain 75% and 25% soil moisture levels for control and drought-stressed plants, respectively. Plants were cultivated following a randomized complete block design (RCBD), with 9 replications (3 plants/pot) per treatment in a growth chamber (10 h light/14 h darkness, 250 μmol m^{-2} s^{-1}) at 25°C for 6 weeks before measuring physiological properties and harvesting samples for other physiological and nucleic acid analyses, for all experiments.

Determination of morphological and gas-exchange attributes

The height of each plant was measured using a measuring tape. The aboveground portions of the plant were harvested and dried for 3 days at 75°C in an incubator before obtaining the dry weight. Furthermore, the relative water content (%) in the leaf was measured as previously described (Soltys-Kalina et al. 2016). Relative water content (RWC) of leaves was measured on the fully expanded trifoliate leaves at the tip of the main stem as previously described (Schonfeld et al. 1988). Briefly, the fresh weight (FW) of leaves from each treatment was recorded

immediately after removal. The turgid weight (TW) was measured after soaking the leaves in distilled water in beakers for 24 h at room temperature. Following the soaking period, the leaves were quickly and carefully blotted dry with tissue paper to prepare for turgid weight determination. The dry weight (DW) of the leaves was obtained by oven-drying the leaf samples for 72 h at 70ºC. RWC was calculated using the formula: RWC (%) = (FW – DW) / (TW – DW) x 100.

Gas exchange and photosynthetic parameters of the leaves were measured using the LI-6800 portable Photosynthesis System (LI-COR Biosciences, USA). The photosynthetic rate, transpiration rate, stomatal conductance, and intrinsic water-use-efficiency (iWUE) were assessed on the central portion of the fourth fully expanded leaf under the following conditions: 2 cm^2 leaf area, flow rate of 600 μmol s^{-1}, relative humidity of 65%, CO_2 reference of 400 μmol mol^{-1}, leaf vapor pressure deficit of 1.5 kPa, leaf temperature of 25°C, and leaf-level light intensity of 250 μmol photons m^{-2} s^{-1}. Measurements were conducted three times, averaged for statistical analyses, and calculated as previously described (de Oliveira et al. 2022).

Elemental analysis of plant tissues

After harvesting, the roots of 6-week-old sorghum plants were excised and rinsed with running tap water, followed by immersion in 0.1 mM $CaSO_4$ for 10 min, and a final wash with deionized water. Subsequently, the roots and leaves were dried in an incubator for 3 days at 75°C. Elemental concentrations were analyzed using ICP-MS (inductively coupled plasma mass spectrometry) at the Agricultural & Environmental Services Laboratories at the University of Georgia. The concentration of elements in the samples was determined by comparing the intensity of ion peaks in the spectrum to calibration standards.

Fe localization in roots using Perls-DAB staining

Perls-DAB staining assays were used to evaluate the accumulation of Fe in roots, following a modified procedure (Roschzttardtz et al. 2010). In brief, roots were immersed in 4 mL of a solution containing 2% (v/v) HCl and 2% (w/v) potassium ferrocyanide for 30 min. Subsequently, the samples were rinsed with H_2O and treated with 4 ml of 10 mM NaN_3 and 0.3% H_2O_2 in methanol for 45 min. After washing with 100 mM Na-phosphate buffer (pH 7.4), the samples underwent a 30 min incubation in the same buffer containing 0.025% (w/v) DAB and 0.005% (v/v) H_2O_2, followed by two washes with H_2O. Finally, the samples were cleared using 15% glycerol and examined under an optical microscope.

Determination of H_2O_2 in roots

For H_2O_2 determination, root samples were mixed in a solution of 0.1% trichloroacetic acid, as previously described (Alexieva et al. 2001). Briefly, the samples were finely ground into a powder using a mortar and pestle. The extracted fluid underwent centrifugation for 15 min at 10,000 rpm to separate cellular residues. The resulting upper aqueous phase was supplemented with potassium iodide (1 M) and phosphate buffer (10 mM, pH adjusted to 7.0), and then left in darkness for 60 min. Finally, the optical density of the solution was measured at 390 nm using a spectrophotometer (ThermoFisher, Waltham, USA), and the concentration of H_2O_2 was calculated based on the standard curve.

RNA-seq analysis

Before RNA extraction, roots were meticulously cleansed twice in sterile phosphate-buffered saline (PBS) through vortexing for 10 sec, followed by two rinses with sterile water to minimize surface contaminants. Subsequently, samples were pulverized into a powder using a pre-cooled pestle and mortar with liquid nitrogen. The resulting powdered material was then utilized for RNA extraction. Total RNA extraction was performed using the SV Total RNA Isolation System (Promega, USA), following the manufacturer's instructions. A total of 1 µg of RNA per sample, possessing a RIN value >8, was employed as input material for RNA-seq sample preparations. Sequencing libraries were constructed using the KAPA HiFi HotStart Library Amplification Kit (Kapa Biosystems, USA), per the manufacturer's instructions. The resulting libraries were sequenced on the Illumina NextSeq SE 100bp platform. Overall, >80% of reads passed filters with Phred score values exceeding 33% per sample.

Adaptor sequences and low-quality sequences were respectively removed and filtered from raw reads using Trimmomatic v0.38 (Bolger et al. 2014). The sorghum reference genome (Sbicolor_454_v3.0.1) was obtained from Phytozome (McCormick et al. 2017) and the filtered reads were aligned to the reference genome using HISAT2 (v2.0.5) software. HT-Seq was utilized to count the read numbers mapped to each gene (Anders et al. 2014). Differentially expressed genes (DEGs) were identified using the Limma R package v3.19 (Law et al. 2016) and read counts were normalized by using the FPKM (fragments per kilobase of exon per million mapped fragments) method. Genes were identified as up-regulated or down-regulated using a cut-off threshold of $\log_2$ fold change (Log_2FC) ≥ 1 and ≤ -1, respectively, with a false discovery rate (FDR) ≤ 0.05. Venn diagrams and heatmaps were generated using the VennDetail R package v1.20 (Hinder et al. 2017) and the ClustVis (https://biit.cs.ut.ee/clustvis/) tool (Metsalu et al. 2015), respectively. Gene ontology for sorghum was retrieved using Phytozome (Goodstein et al. 2012) resources. Gene annotation for molecular function (MF), biological process (BP), and cellular component (CC) was performed through the g:Profiler web tool (Kolberg et al. 2023).

Phytohormone analysis in roots

Phytohormone concentrations in sorghum roots were determined using LC-MS (Liquid Chromatography-Mass Spectrometry) at the Michigan State University Mass Spectrometry and Metabolomics Core. In brief, approximately 100 mg of fresh root material was homogenized in liquid nitrogen and extracted using a solution of 80:20 (v/v) methanol: water, 0.1% formic acid, and 0.1 g/L butylated hydroxytoluene. The samples were then placed in pre-frozen bead beater adaptors and beaten (30/s for 30 s) in a Tissue Lyser II (Qiagen, USA). Following a brief centrifugation (20 s) at 4°C to settle ground tissue at the tube bottom, the samples were further incubated on a shaker at 5,000 rpm at 4°C for 16 h. Subsequently, the samples were vortexed and centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was transferred to autosampler vials. Standards for each phytohormone (Sigma-Aldrich, USA) were prepared in 80% methanol and 0.1% formic acid.

Amplicon sequencing targeting fungi and bacteria

Harvested roots were cleaned by vortexing in sterile phosphate-buffered saline (PBS) for 10 seconds, followed by two rinses with sterile water to remove surface contaminants. Approximately 0.2 g aliquots of cleaned root samples were used for DNA extraction following the procedure outlined by Clarke (2009), along with RNase and Proteinase K treatments. Four biological replicates were used for microbiome analysis. Amplicon libraries were prepared for

sequencing using a two-step PCR approach (Kozich *et al.* 2013). Two primer sets targeting regions of the ribosomal DNA gene were used to generate the initial libraries. The internal transcribed spacer (ITS) was used to target fungal communities (fITS7: GTGARTCATCGAATCTTTG and ITS4: TCCTCCGCTTATTGATATGC), whereas the small sub-unit encoding region (16S) was used to target bacterial communities (515F: TGTGYCAGCMGCCGCGGTAA and 806R: GGACTACNVGGGTWTCTAAT). Primers were adapted to include a 12N UDI sequence immediately prior to the target gene sequence to aid in cluster delineation when sequencing and Illumina P5/P7 complementary overhangs to facilitate barcode index addition in Step 2 PCR. Step 1 PCRs were carried out using Hot Start GoTaq master mixes (Promega, Madison, WI USA) in 12.5 ul volumes containing 2 ul of DNA, 2 mM dNTPs, 10 pmol of each primer. PCR conditions were the same for both the 16S and ITS amplicon generation: 95C for 5 min, 35 cycles at 94C for 30 s, 55C for 30s, 72C 30s, and 72C for 5 min. PCR steps were carried out using a BioRad T100 thermal cycler (Bio-Rad, Hercules, CA USA). Amplicons were dual barcoded with Illumina i7/i5 style barcodes in step 2 PCR using Phusion Hot Start II master mix (Thermo Fisher, Waltham, MA USA) to ensure high-fidelity barcode additions. Amplicons were sequenced using the NextSeq2000 platform on a P2 flow cell run in paired-end 300 bp mode (Illumina, San Diego, USA) by the Georgia Genomics and Bioinformatics Core (Athens, GA USA).

Adapter and primer sequences were trimmed from the raw sequencing reads using *Cutadapt v. 4.5* (Martin 2011). Next, low-quality reads were removed using the filterAndTrim function of *dada2* (*v. 1.28.0*) implemented in R (*v. 4.3.0*, Callahan et al. 2016), followed by error model estimation, dereplication, sample inference, and nonchimeric removal to generate a table of amplicon sequence variants (ASVs). 16S and ITS ASVs were aggregated to 97% OTUs through open-reference clustering against the Greengenes2 16S database (McDonald et al. 2024) and the UNITE ITS database (dynamic_s_all_25.07.2023, Abarenkov et al. 2024) with *vSearch* implemented in QIIME2 *v.2023.7).* 16S taxonomy was assigned using phylogenetic tree decoration, as previously described (McDonald et al. 2024). ITS taxonomy was assigned using CONSTAX2 (Liber *et al.* 2021), which carries out consensus taxonomy assignment from multiple approaches, again using the UNITE database. ITS taxonomy assignments were additionally curated to amend species descriptions to match those assigned during closed-reference OTU clustering, and to fill in missed family-level assignments where lower-order assignments were made. Further taxonomic curation was performed using *phyloseq* implemented in R (Callahan et al. 2016). 16S OTUs were filtered to include only OTUs assigned to the Bacteria kingdom, while those assigned to the 'Cyanobacteria' phylum were removed. This was done to ensure that chloroplast amplicons were not included in the analysis. ITS OTUs were filtered to remove any OTUs where the 'higher level taxonomy' assignment through CONSTAX2 was not to the Fungi kingdom, and where taxonomy was not assignable at the Class level. Alpha diversity metrics (observed species richness and Shannon index) were calculated using *phyloseq*. Alpha diversity metrics were compared between treatment groups using ANOVA, followed by post-hoc testing where appropriate with 'hsd' multiple testing corrections employed. Beta diversity was assessed through Bray-Curtis dissimilarities calculated between samples and statistically compared between treatments with PERMANOVA analysis using vegan (*v. 2.6.4*). Community dissimilarity between samples and treatments was visualized through non-metric multidimensional scaling (NMDS), for which the optimum number of dimensions was determined through examining ordination stress across multiple values and choosing the lowest

set of dimensions where stress was less 0.05. Two methods were employed to determine which taxa were more associated with different treatments at the genus level. Indicator species analysis was conducted using the R package indicspecies (*v. 1.7.14*) on a core set of genera (>0.001 RA in > 10% of samples) consisting of 80 out of 306 genera. The resulting p-values were adjusted for multiple testing using "fdr" correction. The relative abundances of the top ten most abundant genera of bacteria and fungi were also compared between treatments using ANOVA, again followed by post-hoc testing where appropriate.

Measurement of root siderophore levels

We conducted a Chrome Azurol S (CAS) assay to determine siderophore amounts in rhizosphere extracts homogenized with 80% methanol (Himpsl and Mobley 2019). Briefly, the rhizosphere soil was collected by washing off the soil tightly attached to the root surface. The soil extracts underwent centrifugation for 15 min at 10,000 rpm. Subsequently, 500 µl of the supernatant was combined with an equal volume of CAS solution and allowed to incubate at room temperature for 5 min. The absorbance was measured at 630 nm relative to a reference consisting of 1 mL of CAS reagent. Siderophore levels were determined per gram of rhizosphere sample using the formula: % siderophore unit = [(Ar - As)] / Ar × 100, where Ar represents the absorbance at 630 nm of the reference (solely CAS assay solution), and As denotes the absorbance at 630 nm of the sample (CAS assay solution + soil extracts).

Split-root assay to investigate local/systemic signaling

The plants were grown from sterilized seeds for 1 week in soil pots without exogenous Fe or *Streptomyces griseus*, before splitting the root system into two different compartments. Briefly, the main taproot in the middle was excised and then the rest of the lateral root system was evenly split into two separate plastic pots, each isolated by a plastic barrier. The treatment compartments were supplemented with or without exogenous Fe or *S. griseus*, while the control received no Fe or *S. griseus*. The split-root cultivation was performed in four different conditions (control/control, drought/drought, drought with Fe-EDTA/drought with *S. griseus*, and drought with *S. griseus*/drought with *S. griseus*) in the same environmental conditions as previously described under plant cultivation. Drought conditions were initiated when the roots were split, and the data were collected five weeks later, from 7-week-old plants.

Statistical analysis of plant characteristics

All measurements were conducted with at least three independent biological replicates for each treatment. To identify significant differences between treatment groups ($P < 0.05$), analysis of variance (ANOVA) was carried out with treatment as an independent variable, followed by Duncan's Multiple Range Test (DMRT) for both roots and shoots individually using SPSS Statistics 20.0 (IBM). Graphical representation was performed using GraphPad Prism 6.0.

Results

Iron-derived improvement in morphological and nutritional traits under drought stress

Drought stress caused a substantial decline in aboveground shoot and belowground root systems compared to well-watered controls in these growth chamber experiments with soils containing natural field microbiomes. The addition of Fe to the soil led to a visible improvement in both above- and belowground traits under drought conditions (Fig. 1A and 1B). The root length showed a significant decrease under drought regardless of Fe supplementation when compared to

the control Fig. 1C). The results also indicated that root dry weight, shoot height, shoot dry weight and leaf relative water content (RWC) significantly decreased in response to drought stress. However, the addition of exogenous Fe in the soil lessened the relative negative effect of the drought in comparison to the control and shoot height change with drought stress and Fe added was reduced to a statistically insignificant decrease (Fig. 1D, 1E, 1F, and 1G). The Soil Plant Analysis Development (SPAD) scores- a measure of overall plant health and especially nitrogen status (Zhang et al. 2022) were also significantly different between treatments and decreased dramatically under drought stress, but this effect was more than reversed by Fe addition to drought-stressed sorghum (Fig. 1H).

Changes in nutritional elements and Fe storage

ICP-MS was employed to investigate changes in elemental concentrations in the roots and shoots of sorghum plants cultivated under drought conditions with or without Fe supplementation. Fe concentration was significantly different between treatments, exhibiting a significant decline in the roots of drought-exposed sorghum (Fig. 2A). The introduction of exogenous Fe to droughted plants, however, resulted in a significant increase in Fe levels in the roots of plants cultivated in the presence of both drought and Fe supplementation, relative to controls and drought-exposed plants without Fe supplementation. Less change in Fe content in the shoots was seen between treatments, although the concentration of Fe significantly decreased due to drought compared to the control plants but this decrease was effectively reversed by Fe addition to the soil (Fig. 2A). Zn concentration significantly increased in the roots of drought-imposed plants, with or without Fe supplementation relative to controls (Fig. 2B), but the shoot Zn concentrations were also changed, but not significantly, by the treatments (Fig. 2B). Similarly, shoot Mn, S and Ca all exhibited no significant differences associated with the different treatments (Fig. 2C, 2D and 2F) though the shoot Ca content trended upwards under drought and Fe supplementation. Root mineral concentration responded significantly to drought for all three of these minerals, with decreases observed for Mn and S, but an increase for Ca. Only the drought effects on Mn were reversed by Fe treatment. In both the root and shoot, drought did not cause any significant alterations in Mg levels, but sorghum plants cultivated with exogenous Fe under drought conditions showed a significant increase in shoot Mg levels (Fig. 2E).

Root staining revealed the accumulation of Fe predominantly in the apoplast. Microscopic visualization demonstrated that sorghum exposed to drought exhibited a lower accumulation of Fe in the roots compared to controls. The addition of Fe in the soil caused a substantial increase in Fe accumulation in the roots compared to plants cultivated in control and drought-exposed conditions (Fig. 2G). We also found that drought stress caused no significant changes in the siderophore level in the roots of sorghum compared to non-treated controls. However, sorghum plants under drought supplemented with Fe had a significantly higher siderophore level in the roots compared to drought-exposed plants that were not Fe-supplemented (Fig. 2H). Furthermore, the concentration of H_2O_2 in roots of sorghum exposed to drought increased significantly, but the addition of Fe to the soil caused a significant decline in root H_2O_2 concentration in these drought-stressed plants (Fig. 2I).

Improvement in gas-exchange parameters in leaves

We measured the gas-exchange parameters in the leaves of sorghum cultivated under different treatments of drought and Fe supplementation. In this study, drought caused a significant

decrease in the photosynthetic rate compared to well-watered controls. However, the presence of exogenous Fe in the soil led to a significant increase in the photosynthetic rate, to a level significantly higher than that of the drought-exposed plants and similar to the control plants (Table 1). The transpiration rate, stomatal conductance, and water use efficiency (intrinsic) showed a significant decline in drought-exposed sorghum compared to controls. However, the plants treated with exogenous Fe caused a significant increase in these features relative to the plants cultivated under drought conditions without Fe supplementation (Table 1).

Changes in phytohormone profile in the root

Drought-exposed plants showed a significant decrease in IAA concentrations in roots relative to controls. However, the addition of Fe in the soil caused a significant increase in IAA levels compared to controls and plants cultivated under drought (Table 2). Sorghum plants exposed to drought showed a significant increase in ABA concentrations in roots in contrast to control plants with added Fe boosting the ABA level even higher (Table 2). Relative to well-watered controls, drought caused a significant decline in JA concentrations in the roots of sorghum, cultivated with or without the addition of exogenous Fe (Table 2). In this study, GA3 levels showed no significant changes in the roots of sorghum cultivated under different treatments of drought and Fe supplementation (Table 2).

RNA-seq analysis of differentially expressed genes in roots of sorghum

Transcript abundances in sorghum roots were seen to be quite different for the three treatments (Fig. 3). Principal component analysis (PCA) based on three biological replicates revealed that the first two principal components, PC1 and PC2, contributed 39.6% and 13.2% of the total variance, respectively (Fig. 3A). Control plants clustered far from the drought plus or minus Fe samples, which were not resolved. We analyzed differentially expressed genes (DEGs) in sorghum roots exposed to the standard three conditions for this project: control, drought, and drought supplemented with Fe. The results highlight the significantly different gene activity in response to these different conditions (Fig. 3B and 3C). There were 1,039 upregulated (3B) and 533 downregulated (3C) genes in 'drought vs. control' comparison, and 653 upregulated and 1,222 downregulated genes in 'drought supplemented with Fe vs. control' comparison. In total, 6,139 DEGs were identified in roots, including 61 upregulated and 6,078 downregulated genes in 'drought supplemented with Fe vs. drought' comparison (Fig. 3B and 3C). A Venn diagram analysis to identify the common and specific DEGs across different comparisons showed 502 upregulated and 351 downregulated DEGs compared to controls and in comparison, of the two treatments (Fig. 3B and 3C).

We further annotated the functions of DEGs between drought with Fe supplementation and drought using the Phytozome (V. 13) database for sorghum. We selected several differentially expressed ($P < 0.05$) genes known to be associated with drought and other abiotic stress responses in earlier plant studies for more detailed investigation (Supplementary Table S1). For instance, the expression of *SOBIC.008G004800* (*Ferritin 1*) showed significant upregulation in 'drought vs control' and 'drought+Fe vs drought' conditions, with the latter being higher than the former. Unexpectedly, from these results (drought>control and drought+Fe>drought), this gene showed decreased expression (C>A) when comparing 'drought+Fe vs control' (Fig. 3D). *SOBIC.008G165800*-encoding threonine-specific protein kinase, *SOBIC.009G016633*-encoding MYB-like DNA-binding protein MYB, *SOBIC.007G182300*-encoding ATP-binding cassette

transporter, *SOBIC.001G510800*-encoding large subunit ribosomal protein L23e and *SOBIC.001G190700*-encoding ABC transporter G family member 30-related exhibited significantly downregulated in ‘drought vs control’ conditions, but significantly upregulated in ‘drought supplemented with Fe compared to drought alone. However, these genes displayed no significant changes between drought under Fe supplementation and control conditions (Fig. 3D). Although the expression of *SOBIC.001G102400* transcript involved in methylerythritol phosphate pathway II and *SOBIC.001G113200*-encoding histidine kinase 4 showed no significant changes in ‘drought vs control’ and Fe supplementation under drought compared to control conditions; however, the addition of Fe under drought compared to drought alone, exhibited a significant upregulation of these two genes in the roots of sorghum (Fig. 3D). Furthermore, *SOBIC.002G138000* transcript-encoding histone H2B and *SOBIC.006G241000*-encoding watched-related homeobox 4 exhibited no significant changes in ‘drought vs control’ conditions. It is worth noting that these two genes demonstrated significant inductions in the roots of sorghum under drought supplemented with Fe compared to drought alone, as well as drought supplemented with Fe compared to control conditions (Fig. 3D).

Furthermore, SOBIC.010G246600, known as xyloglucan endotransglycosylase, showed a significant downregulation when comparing drought stress with Fe supplementation to drought stress alone (Fig. 3E). Conversely, there is a significant upregulation of SOBIC.010G246600 when comparing drought stress alone to control conditions. However, there is no significant change in expression levels of SOBIC.010G246600 between drought stress with Fe supplementation and control conditions (Fig. 3E). SOBIC.007G206500, identified as sulfotransferase, exhibited significant downregulation under drought with Fe compared to drought stress alone. Conversely, there were no changes when comparing drought stress alone to controls, whereas a significant downregulation occurred between drought stress with Fe supplementation and control conditions (Fig. 3E). SOBIC.002G404800 (mitochondrial phosphate transporter) showed significant downregulation in response to drought stress with Fe versus drought stress alone but showed significant upregulation when comparing drought stress alone to control conditions (Fig. 3E). However, SOBIC.002G404800 showed no significant alternation in expression levels in ‘drought+Fe vs control’ conditions (Fig. 3E). Furthermore, SOBIC.001G244500 (sulfate transporter 3.1-related gene) and SOBIC.001G278650 (Glutathione S-transferase) significantly induced under drought stress supplemented with Fe compared to drought alone. Again, these two genes showed a significant upregulation under drought relative to controls but showed no significant changes in 'drought vs control' conditions (Fig. 3E). In this study, SOBIC.001G396700 (xyloglucan 6-xylosyltransferase), SOBIC.003G227300 (WRKY71 transcription factor 71), SOBIC.003G353400 (S-protein acyltransferase) and SOBIC.010G268500 (multi-copper oxidase) showed a significant decline in the expression levels under drought with Fe supplementation compared to drought. However, these genes showed aa significant upregulation when comparing drought to control conditions but did not show any significant changes in "drought+Fe vs control conditions (Fig. 3E). Lastly, SOBIC.005G137100 (a chalcone synthase WHP1) showed a significant downregulation in ‘drought+Fe vs drought’ but showed no changes when comparing drought stress alone to control. However, SOBIC.005G137100 significantly declined in drought-stressed plants supplemented with Fe relative to controls (Fig. 3E).

We have analyzed the biological significance of DEGs in roots of sorghum in drought+Fe vs drought' conditions. Several pathways significantly enriched were associated with the upregulated DEGs, including but not limited to various molecular functions (catalytic activity, tetrapyrrole binding, hydrolase activity, acting on ester bonds, iron ion binding), biological processes (secondary metabolic process, external encapsulating structure organization, cell wall organization or biogenesis) and cellular components (cell periphery, extracellular region) in plants (Table 3).

Changes in diversity and composition of bacterial and fungal communities

Total bacterial and total fungal communities in sorghum roots were analyzed by ribosomal DNA amplicon sequencing, and found to be significantly altered by the imposition of experimental treatments (PERMANOVA Bacteria: F = 13.56, df = 2, p = 5e-4; Fungi: F = 4.65, df = 2, p = 5e-4). Pairwise analysis of bacterial community composition revealed that all three treatments harbored distinct communities (Fig 4A), whereas a similar analysis of the fungal dataset revealed that the drought treatment caused a shift in the fungal community regardless, but this was not altered by Fe-EDTA addition (Fig 4A).

The strong selective pressure of drought is displayed through the analysis of alpha diversity metrics where experimental treatment was found to significantly alter both observed OTU richness (ANOVA Bacteria F = 60.28, df = 2, p = 6.14e-6; Fungi F = 14.03, df = 2, p = 0.002, Fig 4B) and Shannon evenness of communities (ANOVA Bacteria F = 228, df = 2, p = 1.95e-8; Fungi F = 21.21, df = 2, p = 0.0004, Fig 4B). For the bacterial and fungal datasets, relative to the unstressed control, the drought treatment was associated with a 33% and 39% reduction in observed OTU richness. This was exacerbated by the Fe-EDTA treatment in bacteria, which was associated with a 62% reduction relative to the control treatment (Fig 4B). A similar pattern was observed for the Shannon Index, where drought was associated with 44% and 36% reductions relative to the control, respectively, with an 85% reduction association with the Fe-EDTA treatment for the bacterial community.

Differential abundance tests were performed at the genus level to balance between the specificity of taxa interactions, statistical power of the analysis, and redundancy, as the majority of OTUs in both datasets are single representatives of their genera. Seven of the ten most abundant bacterial genera were found to be differentially abundant between treatments (Supplementary Table S3). In particular, *Trinickia* was in very low abundance in the control samples and increased through drought. In Fe-EDTA amended and droughted plants *Trinickia* dominated the bacterial community- representing 91% of all sequences (Supplementary Table S3). Two genera phylogenetically related to *Trinickia*- *Burkholderia* and *Paraburkholeria*- also trended towards increased relative abundance in plants subjected to drought relative to the control. The relative abundance of *Burkholderia* significantly declined when Fe-EDTA was added under drought relative to regular drought conditions (Supplementary Table S3). *Herbaspirillum*, *Azospirillum* and *Rhizobium* were negatively affected by the drought treatment, exhibiting lower relative abundances than control samples. Of them, the *Rhizobium* showed a significant increase in roots of sorghum when Fe-EDTA was added under drought in contrast to the plants solely cultivated under drought. Although drought caused no significant changes in a *Streptomyces_G_399870* abundance under drought, the addition of Fe-EDTA under drought demonstrated a significant decline relative to control and drought conditions (Supplementary Table S3).

None of the top ten genera considered for ANOVA were significantly different between treatments. However, the abundance of *Talaromyces* and Fusarium showed a trend towards increase under drought relative to controls. Of the *Talaromyces* genus, we found three species - *Talaromyces coprophilus*, *Talaromyces domesticus*, and *Talaromyces purpureogenus* - in all samples. The abundance of *Pseudallescheria*, *Curvularia*, *Sarocladium*, *Malassezia*, *Exidia*, and *Chromelosporium* showed a non-significant decrease in mean relative abundance under drought either supplemented with Fe-EDTA or not. *Rhizoctonia* was only present above the detection threshold in the Fe-EDTA-amended samples, whereas *Malassezia* was undetected in Fe-EDTA-amended drought samples (Supplementary Table S3).

ANOVA analyses of the ten most abundant bacterial genera were supplemented by indicator taxa analysis using a core set of genera for each dataset, representing at least relative abundances of 0.001 in at least 10% of samples. The core bacterial and fungal communities were represented by 80 genera and 20 genera, respectively. In this study, 26 indicator genera were identified, including *Azospirillum* and *Trinickia_580244* that are included within the top 10 genera (Supplementary Table S4). Through this analysis, *Trinickia_580244* was found to be an indicator of the control and drought-only treatments. In this study, 12 indicator genera were found to be associated with the control treatment, and 10 were found to be associated with both the control and drought treatment (Supplementary Table S4). No indicator taxa were associated with solely the Fe-EDTA or Fe-EDTA plus drought combination. There were no fungal indicator genera found for any treatment. Considering the low innate richness of fungal communities in this study, to better explain community turnover between samples in this case, sample presence/absence of taxa was considered. We found that 14 OTUs were shared across all samples, whereas 18 OTUs were present in all control treatment replicates and completely absent from either drought treatment.

Crosstalk between *Streptomyces* and Fe

We conducted a split-root assay to investigate the relationship of Fe and *S. griseus* in promoting drought tolerance in sorghum cultivated in soil pots. The results demonstrated that those plants cultivated with drought showed a significant decline in leaf chlorophyll score, shoot height, and shoot dry weight relative to the plants cultivated with well-watered conditions in both compartments (Fig. 5A-5E). Interestingly, drought-exposed sorghum supplemented with Fe in one compartment, while the other one remained without exogenous Fe but with *S. griseus* inoculum, exhibited a significant increase in chlorophyll score, shoot height, and shoot dry weight relative to the plants cultivated under drought without any Fe supplementation in both compartments. However, drought-exposed sorghum plants inoculated with *S. griseus* in both compartments without exogenous Fe showed no significant changes in these parameters compared to the plants solely cultivated under drought in both compartments (Fig. 5A-5E).

Discussion

Fe is a vital micronutrient, and its availability is influenced by and linked to soil conditions and the structure of the rhizosphere microbiome community (Lurthy et al. 2021). However, the role of exogenous Fe in the physiology and induction of drought tolerance in plants, particularly in association with soil microbes, is a largely unexplored topic. Our comprehensive transcriptomics and microbial sequencing studies suggest that higher accumulation of Fe induces ferritin in

sorghum roots, acting as a defense mechanism against oxidative stress during drought exposure. This improvement in drought-exposed sorghum mediated by exogenous Fe is synergistically dependent on the association of certain beneficial microbes that are potentially involved in elevated Fe uptake and phytohormone regulation.

In this study, sorghum plants cultivated under drought conditions exhibited a significant improvement in both root and shoot biomass, as well as in leaf chlorophyll score and relative water content when supplemented with exogenous Fe in the soil. Drought stress causes considerable damage to photosynthetic pigments, leading to chlorophyll degradation, which is considered a symptom of oxidative stress that causes growth retardation in plants (Ma et al. 2020; Rahdari et al. 2012). The reduced chlorophyll content under drought conditions may trigger the inactivation of photosynthesis and decreased transpiration efficiency in plants (Fahad et al. 2017). We also observed a decline in gas exchange parameters in sorghum exposed to drought. However, the addition of Fe in the soil caused a significant improvement in photosynthetic rate, transpiration rate, stomatal conductance, and water use efficiency, implying that exogenous Fe contributes to the restoration of sorghum plants to healthy cellular status. Of the gas-exchange parameters, stomatal conductance is most significantly affected by low water availability, because drought-stressed plants typically close their stomata to conserve water (Marchin et al. 2022; Farooq et al. 2009). While this response helps conserve water, it can also affect other physiological processes, such as photosynthesis and overall plant growth. In addition, drought conditions lead to a reduction in water use efficiency and transpiration rate, ultimately resulting in increased leaf and canopy temperatures (Turner et al. 2001). Considering that water use efficiency is a significant indicator of drought tolerance, the enhanced water use efficiency observed in sorghum plants supplemented with exogenous Fe, even under drought conditions, suggests the beneficial role of Fe in enhancing cellular integrity and physiological performance. The Fe-derived enhancement in water use efficiency observed in drought-exposed sorghum aligns with improved plant biomass, achieved by maintaining a sufficient water supply and improved regulation of stomatal closure and transpiration rates.

The nutrient relations of plants are also adversely affected by drought stress (Ge et al. 2012). Furthermore, the composition and activity of soil microbial colonies are adversely impacted by soil water deficit, ultimately disrupting plant nutrient relations (Schimel et al. 2007). In this study, the decline of several nutrient elements in the roots of drought-exposed plants, particularly Fe and Mn, showed a significant improvement following Fe supplementation. Plants modify the length and surface area of their roots, as well as alter their architectural structure, to enhance the capture of less mobile nutrients (Lynch and Brown 2001). We also observed an enhancement in root dry matter, potentially linked to the restoration of minerals indirectly facilitated by Fe supplementation in drought-exposed sorghum. Particularly, the addition of exogenous Fe promoted Fe uptake even under drought, potentially facilitated by higher siderophore levels in the roots. Microbial siderophores released by certain bacterial and fungal species play a crucial role in plant Fe uptake by mobilizing and solubilizing Fe in the rhizosphere, making it more accessible to plants (Ahmed and Holmström, 2014; Kabir and Bennetzen, 2024). We also found that elevated Fe resulted in a higher accumulation of Fe in the roots compared to the shoot. This was further substantiated by microscopic observations, revealing an increased accumulation of Fe in the root tissues, as evidenced by Perl-staining, when exogenous Fe was added to the drought-stressed plants, suggesting enhanced storage in shoot versus root under these conditions.

The overexpression of the native ferritin gene *MusaFer1* enhances both Fe content and oxidative stress tolerance in transgenic banana plants (Parveen et al. 2016). The integration of Fe homeostasis with the generation of ROS is evident, and the distribution of Fe at the root tip plays a role in controlling root growth (Reyt et al. 2015). Concurrently, H_2O_2, a common indicator of ROS damage under abiotic stress, substantially decreased in roots of drought-exposed sorghum with exogenous application of Fe in soil. Excess Fe increases ferritin abundance, enabling the storage of Fe, which contributes to the protection of plants against oxidative stress (Yadav et al. 2017; Reyt et al. 2015). Several studies indicate that ferritin plays a crucial role in preventing Fe deficiency in plants (Zielińska-Dawidziak, 2015; Grusak, 1994). The common bean exhibits the induction of ferritin synthesis in response to water deficit, implying the potential functional significance of ferritin genes in the acclimatization of common beans under conditions of either Fe excess or water deficit (DeLaat et al. 2014). Also, the study of transgenic plants that overexpress ferritins have elucidated the connections between ferritin and the management of ROS, in addition to its role in shielding against oxidative stress during photoinhibition (Murgia et al. 2001; Deák et al. 1999). Consistent with such observations, the restoration and abundance of Fe storage may also facilitate the supply of adequate Fe in drought-exposed plants when supplemented with Fe. In RNA-seq analysis, the upregulation of the *Ferritin 1* (*SOBIC.008G004800*) gene in sorghum roots under drought supplemented with Fe was observed. In another study, the regulation of Fe homeostasis through vacuolar Fe transporters and ferritin was found to enhance Fe accumulation in sorghum seeds that were from plants subjected to drought stress (Araki et al. 2022). This suggests that the increased Fe accumulation in the roots is associated with the induction of ferritin. This induction may play a role in reducing H_2O_2 levels and ensuring an adequate supply of Fe throughout the plant, which could restore chlorophyll synthesis and photosynthetic activities in drought-exposed sorghum.

While exogenous Fe promoted ferritin-mediated ROS scavenging, there were also a few other genes related to phytohormones (histone H2B, ATP-binding cassette transporter, ABC transporter) and osmotic and drought tolerance (histidine kinase 4, Wuschel-related homeobox 4) that exhibited upregulation in the roots of sorghum plants exposed to drought. It is known that phytohormones play a crucial role in drought response by regulating adaptive mechanisms such as stomatal closure, root development, and stress-related gene expression to enhance plant resilience under drought conditions (Ullah et al. 2018; Waadt et al. 2022). In this study, *histone H2B*, a gene involved in the transcriptional regulation of auxin biosynthesis was upregulated in the roots of sorghum due to exogenous Fe cultivated under drought. *H2Bub1* is reported to be associated with the promotion of faster responses to drought stress in cotton (Chen et al. 2019). In a recent study, *H2Bub1* is reported to exhibit a positive association with the transcription of various auxin synthetic genes, contributing to the maintenance of normal auxin homeostasis (Zhang et al. 2021). Furthermore, auxin is found to be a key determinant in mycorrhizal symbiotic association with plants (Kabir and Bennetzen, 2024; Ludwig-Müller and Güther, 2007). Our RNA-seq data additionally reveals the induction of an ATP-binding cassette transporter in the roots of sorghum after exogenous Fe addition under drought conditions. Earlier studies have demonstrated that an ATP-binding cassette transporter regulates auxin levels in various plant organs, including the root pericycle (Dahuja et al. 2021; Jenness et al. 2019). In *Arabidopsis thaliana*, the ABC transporter *AtABCG25*, characterized by ABA efflux activity, is reported to function as a regulator facilitating the transport of ABA from the cytosol to the

apoplast, thereby inducing tolerance to drought (Kuromori et al. in 2010 and 2014). Consistently, our RNA-seq and LC-MS analyses revealed the upregulation of auxin-responsive genes and the synthesis of IAA and ABA in the roots of sorghum supplemented with Fe under drought conditions. Moreover, the histidine kinase proteins that were found to be upregulated in our study are reported to contribute to phytohormone signaling and stress signaling (Nongpiur et al. 2012). In this study, we found the induction of Histidine Kinase 4 as well as WUSCHEL-related homeobox 4 in roots of sorghum supplemented with Fe under water-limiting conditions. The WUSCHEL-related homeobox genes are a family of plant-specific transcription factors that regulate the differentiation of plant stem cells and play a key role in plant organ development (Xu et al. 2023; Li et al. 2020). In poplar, the WUSCHEL-related homeobox gene *PagWOX11/12a* has been discovered to counteract drought stress by promoting root elongation and enhancing biomass growth (Wang et al. 2020). Taken together, our findings suggest that elevated Fe levels indirectly, or at least in part, promote the phytohormone signaling and regulatory responses of sorghum to combat drought stress.

Drought stress causes a major compositional change in the rhizosphere and endophytic communities (Xu et al. 2021; Ma et al. 2020). In this study, amplicon sequencing data showed a reduction in both the diversity and richness of fungal and bacterial microbial communities in the roots under drought conditions, which was further exacerbated by Fe supplementation for bacteria. When considering the bacterial community drought response, drought conditions appear to favor the proliferation of *Burkholderia* and *Trinickia* relative to other taxa, the latter of which appears to further outperform all other bacteria under Fe-EDTA amendments and dominates communities under this treatment. While the nature of amplicon sequencing limits our understanding of bacterial abundance, differences in the ratio of 16S amplicon reads assigned to cyanobacteria/chloroplast in origin and other bacteria (very loosely a proxy in this case of plant genome and bacterial sequences within a sample) is suggestive of a mild increase in bacterial abundance under drought (79% mean increase in chloroplast-filtered reads assigned to bacteria relative to unstressed plants) and a further increase in Fe supplemented plants (351 % mean increase relative to unstressed plants) from which we posit that the observed community differences between treatments are not driven just by a decrease in growth of sensitive taxa, but also an increase in the growth of well-adapted taxa such as *Trinickia* and *Burkholderia*. Metze et al. (2023) showed that drought inhibited the growth of over 90% of bacterial taxa but favored the growth of specialized taxa including those within the phyla Bacillota (Firmicutes) and Actinomycetota (Actinobacteria), including *Streptomyce*s within this clade.

The relatively minor changes in Actinomycetota (and *Streptomyces*, in particular) abundances in drought stress response in our experiments conflict with previous studies conducted in soil systems (Metze et al. 2023, Xu et al. 2021, Fitzpatrick et al. 2018). When investigating specifically root-endophytic bacteria, Garcia et al. (2024) found that two *Streptomyces* OTUs were enriched in drought only in a drought-tolerant line of sorghum but not in a drought-sensitive line. As our experiment was conducted in greenhouse pots, and not in the field as per the other referenced studies, some differences in outcomes would be expected as our observed community is likely a subset of the field community (Kushwaha et al. 2024). For example, Forero et al. (2019) showed that different plant-soil feedback is observed between greenhouse and field trials even when greenhouse pots are inoculated with field soil. In our study, endophyte communities appeared to be extremely biased towards *Proteobacter* even in the control pots

(representing 88.93% of all 16S sequences), which may inhibit other taxa's potential positive responses to drought in this instance, as already abundant taxa within the *Proteobacter* clade are able to further proliferate under drought at the expense of less abundant taxa, possibly through antimicrobial production (Prasad et al. 2021).

Trinickia and *Burkholderia* are very closely related taxa- *Trinickia* was only recently split from the genus *Burkholderia* following whole genome analysis, reflecting the close ecological and physiological similarities between the two groups (Estrada-de Los Santos et al. 2018). The genera belonging to the Burkholderiaceae family are known for containing a diverse set of taxa capable of plant symbiosis and functions in plant growth, stress resilience, and nitrogen fixation (Pal et al. 2022; Esmaeel et al. 2020). Both genera have also been identified as inorganic phosphate-solubilizing bacteria (Damo *et al.* 2022). Burkholderia species have been well documented to be associated with an increase in the drought stress resilience of host plants in inoculation studies (Naveed et al. 2014; Naveed et al. 2014; Huang et al. 2017; Tallapragada, Dikshit, and Seshagiri 2016). Studies have also reported that switchgrass inoculated with *Burkholderia* were more photosynthetically active, allocated more biomass, and produced more tillers than uninoculated plants (Wang et al. 2015; Kim et al. 2012). *Burkholderia* may alleviate drought stress through siderophore production, which are Fe-chelating compounds that help plants survive in environments with low Fe levels (Pal et al. 2022; Barrera-Galicia et al. 2021). In this study, drought caused a significant decrease in Fe uptake in sorghum. Hence, the higher abundance of *Burkholderia* may be linked to the poor availability of Fe and its ability to withstand these conditions relative to other microbial groups. This was further supported by the drastic disappearance of *Burkholderia* when Fe was supplemented under drought conditions as Fe became more bioavailable. *Trinickia* must have further, yet unknown, adaptations that allow it to outperform *Burkholderia* in this scenario.

In our first greenhouse study, fungal communities were dominated by *Talaromyces* under all experimental treatments. *Talaromyces* are primarily known for their role in the decomposition of organic matter (Heo et al. 2019). Some of the ten most abundant fungal taxa did trend towards reduction in relative abundance under drought (*Pseudallescheria*, *Curvularia*, *Sarocladium*, *Chromelosporium*, *Exidia*) while *Fusarium* and *Talaromyces* showed a relative increase under drought, though these conclusions lack firm statistical support. The greenhouse conditions of this study apparently selected for a narrower range of fungal taxa that had variable abundance responses to drought than seen for bacteria. Fungi are generally agreed to be more drought resistant than bacteria (Sun et al. 2020), and the stochasticity between replicates likely points to their being little selection pressure within this experiment from either the soil environment caused by drought, or from the plant in response to any beneficial associations with this narrow set of fungal taxa. *Fusarium* often exhibits abundance increases through opportunistic colonization of stressed root systems, often functioning as a pathogen (e.g., *Fusarium* root rot), and has previously been shown to increase under drought (Pierre et al. 2023). *Talaromyces* may likewise be opportunistically utilizing a potential increase in decaying root matter as an organic carbon source under drought. Unlike in the bacterial dataset, the ITS amplicon cannot readily be used to generate a loose proxy of fungal abundance (because so many fungal ITS regions do not amplify well), so we are unable to conclude whether *Fusarium* is indeed increasing in abundance in these samples, or whether other taxa are diminishing. The imposition of drought is a highly selective force on the microbial community, possibly through encouraging the proliferation of a

few putatively tolerant species at the expense of other members of the community or a reduction in those intolerant members leaving only tolerant ones surviving without associated abundance shifts. The nature of amplicon sequencing cannot readily unpick the difference between these scenarios.

It was surprising that the fungus *Trichoderma* showed no drought response in the greenhouse study, though it was somewhat reduced in the Fe-supplemented drought treatment. Several studies have revealed the role of *Trichoderma* species in mobilizing Fe through increased siderophore production in different plants, including sorghum (Contreras-Cornejo et al. 2009; Kabir and Bennetzen 2024). Therefore, the disappearance of *Trichoderma* under elevated Fe may be attributed to the discouragement of mutualism by sorghum exposed to drought, as *Trichoderma*-based siderophore-bound Fe is not required by the plants in such conditions. However, the change in *Trichoderma* abundance in the roots of drought-exposed sorghum, supplemented with Fe, is in the opposite direction of total siderophore content in the rhizosphere. Siderophores are not only released by certain fungi but also by many bacteria (Timofeeva et al. 2022). Since Fe is essential for microbial metabolism, growth, and replication, the elevated levels of siderophores in the rhizosphere are likely caused by the availability of Fe in the soil provided by other microbes, rather than being related to the recruitment of *Trichoderma* by host genetic factors under drought conditions. Moreover, siderophores exhibit binding affinity not only for Fe but also for various toxic metals, although a higher binding capability is observed for Fe compared to toxic heavy metals (Baysse et al. 2000; Braud et al. 2009). Also, microbes capable of producing siderophores are known to facilitate plant growth, particularly under drought (Vivas et al. 2003; Breitkreuz et al. 2021). The promotion of Fe and other mineral uptake in drought-exposed sorghum through exogenous Fe appears to be intricately linked to microbial interactions and host gene expression in the roots of sorghum.

Streptomyces are known to play a crucial role in plant stress response by producing diverse siderophores (Jones et al. 2019; Cruz-Morales et al. 2017). Growing evidence shows that *Streptomyces* can promote plant growth or stress tolerance in many direct or indirect ways, for instance by triggering the secretion of plant growth regulators and/or siderophores (Myo et al. 2019; Sadeghi et al. 2012). Considering our somewhat unexpected results of little *Streptomyces* change under stress in our initial experiment, and the known significance of *Streptomyces* in various drought responses (Xu et al. 2021; Warrad et al. 2020), we conducted split-root assays to investigate the relationship between Fe dependency in this bacterial genus and drought tolerance in sorghum. This experiment aimed to ask whether *Streptomyces*, even in the absence of elevated Fe, can aid plants in coping with drought. Split-root assays are commonly utilized in plant research to explore signaling responses triggered by local treatments, encompassing nutrient availability, interaction with soil microbes, or exposure to abiotic stresses (Kafle et al. 2022; Saiz-Fernández et al. 2021). In our split-root assay, sorghum plants exhibited no significant improvement in chlorophyll score and shoot biomass when inoculated with *Streptomyces* in the absence of exogenous Fe. This suggests that elevated Fe is essential for the beneficial effects of *Streptomyces* in significantly mitigating drought stress in sorghum, even though an increased abundance of *Streptomyces* is not necessarily caused by elevated Fe levels.

Conclusion

High availability of Fe markedly enhances the health and productivity of drought-exposed sorghum, reinstating plant physiological and mineral status, associated with host regulatory response and endophytic microbial adaptation. This drought tolerance is associated with the induction of *SbFER1* and a reduction in H_2O_2, indicating that exogenous Fe facilitated by beneficial microbes helps accumulate root ferritin, safeguarding reactive Fe^{2+} and sustaining growth (Fig. 6). RNA-seq analysis identifies upregulated genes linked to elevated IAA and ABA in roots. In this study, the fungi *Curvularia* and *Pseudallescheria* decreased regardless of Fe under drought, while *Taralomyces* increased with drought but declined with added iron. *Trichoderma* remained unchanged under drought and control, but iron eliminated it. *Burkholderia*, especially *Trinickia*, increased during drought but was countered by Fe. *Streptomyces* remained stable during drought but decreased significantly with Fe in sorghum roots. Furthermore, *Streptomyces* was found to rely on sufficient Fe availability to enhance drought tolerance. In summary, Fe-mediated drought tolerance in sorghum involves ferritin-driven redox regulation and the alteration of microbial communities within the root microbiome. These findings can inspire agronomic strategies and biofertilizer industries to formulate effective microbiome inoculants to combat drought in sorghum and other crops.

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Competing interest

We have no conflict of interest.

Author contributions

AHK conceived the idea for the study, conducted the physiological assay, RNA-seq analysis, critically interpreted microbiome data, conducted a split-root assay, and prepared the draft manuscript. PBJ prepared the DNA library and analyzed the amplicon sequencing data. MA crosschecked amplicon sequencing data and critically reviewed the discussion. SPLT provided crucial contributions to the manuscript. JB supervised the work, provided intellectual contributions, and revised the manuscript.

Data availability

Illumina sequencing data was submitted to NCBI under the following accession numbers: PRJNA1128172 (RNA-seq), PRJNA1128337 (16S) and PRJNA1128338 (ITS).

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Table 1. Changes in leaf gas-exchange parameters in sorghum cultivated with different drought and Fe treatments. Different letters in superscript within a row (i.e., phenotype) indicate significant differences between means ± SD of three independent biological replicates (n = 3) at $p < 0.05$. Plants were cultivated in a growth chamber for 6 weeks of differential treatment.

Parameters	Control	Drought	Drought + Fe
Photosynthetic rate (μmol m^{-2} s^{-1})	19 ± 1.3^{a}	5 ± 0.52^{b}	16 ± 2.2^{a}
Transpiration rate (μmol m^{-2} s^{-1})	0.002 ± 0.0009^{a}	0.0005 ± 0.0001^{b}	0.0008 ± 0.0001^{c}
Stomatal conductance (μmol m^{-2} s^{-1})	0.09 ± 0.01^{a}	0.005 ± 0.003^{b}	0.02 ± 0.003^{c}
Water use efficiency (intrinsic) (μmol mol^{-1})	195 ± 12.8^{a}	72 ± 2.9^{b}	174 ± 13.1^{c}

Table 2. Changes in root phytohormone concentrations in sorghum cultivated with different drought and Fe treatments. Different letters in superscript within a row (i.e., phenotype) indicate significant differences between means ± SD of three independent biological replicates (n = 3) at $p < 0.05$. Plants were cultivated in a growth chamber for 6 weeks of differential treatment.

Plant hormone	Control	Drought	Drought + Fe
IAA (ng g^{-1})	8.3 ± 1.5^{a}	4.4 ± 1.0^{b}	12.7 ± 1.6^{c}
ABA (ng g^{-1})	3.7 ± 1.4^{a}	78.6 ± 28.9^{b}	148.6 ± 39.5^{c}
JA (ng g^{-1})	34.5 ± 1.4^{a}	9.2 ± 2.5^{b}	2.3 ± 0.70^{c}
GA3 (ng g^{-1})	10.5 ± 2.4^{a}	9.3 ± 2.2^{a}	8.5 ± 3.7^{a}

Table 3. Gene ontology and enrichment analysis of pathways classified as molecular function (MF), biological process (BP), and cellular component (CC). Padj(query_1) refers to the adjusted p-value corresponding to the gene enrichment analysis. These pathways or terms within the list of genes differentially (>Log2 fold, p value <0.05) upregulated in 'drought+Fe vs drought' conditions in roots of sorghum.

ID	Source	Term ID	Term Name	P_{adj}(query_1)
1	GO:MF	GO:0003824	Catalytic activity	3.650×10^{-10}
2	GO:MF	GO:0046906	Tetrapyrrole binding	4.458×10^{-5}
3	GO:MF	GO:0016788	Hydrolase activity, acting on ester bonds	2.225×10^{-3}

4	GO:MF	GO:0005506	Iron ion binding	1.368×10^{-2}
5	GO:BP	GO:0019748	Secondary metabolic process	4.383×10^{-7}
6	GO:BP	GO:0045229	External encapsulating structure organization	2.571×10^{-2}
7	GO:BP	GO:0071554	Cell wall organization or biogenesis	4.208×10^{-2}
8	GO:CC	GO:0071944	Cell periphery	9.236×10^{-6}
9	GO:CC	GO:0005576	Extracellular region	3.053×10^{-4}

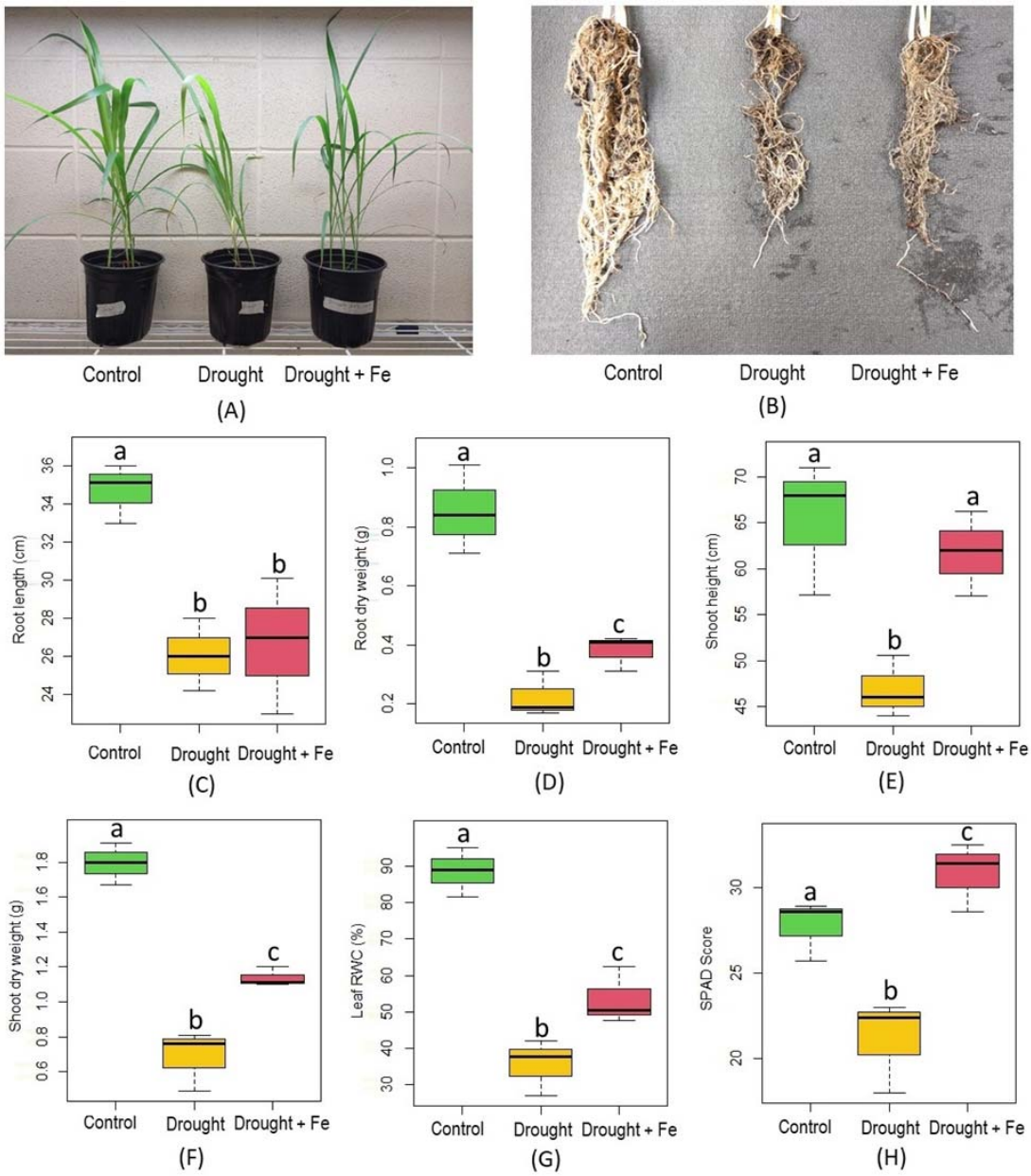

Fig. 1. Changes in morphological and physiological attributes in sorghum cultivated with or without drought and Fe supplementation. Shoot phenotype (A), root phenotype (B), root length (C), root dry weight (D), shoot height (E), shoot dry weight (F), leaf relative water content (G), and leaf SPAD score (H) in sorghum cultivated in the absence or presence of drought and Fe supplementation (Fe-EDTA). The data presented are means with standard deviations (n = 3) of individual replicates). Statistically significant differences among the treatments at a $p < 0.05$ level are indicated by different alphabetical letters above the bars, where applicable. Plants were cultivated in a growth chamber for 6 weeks.

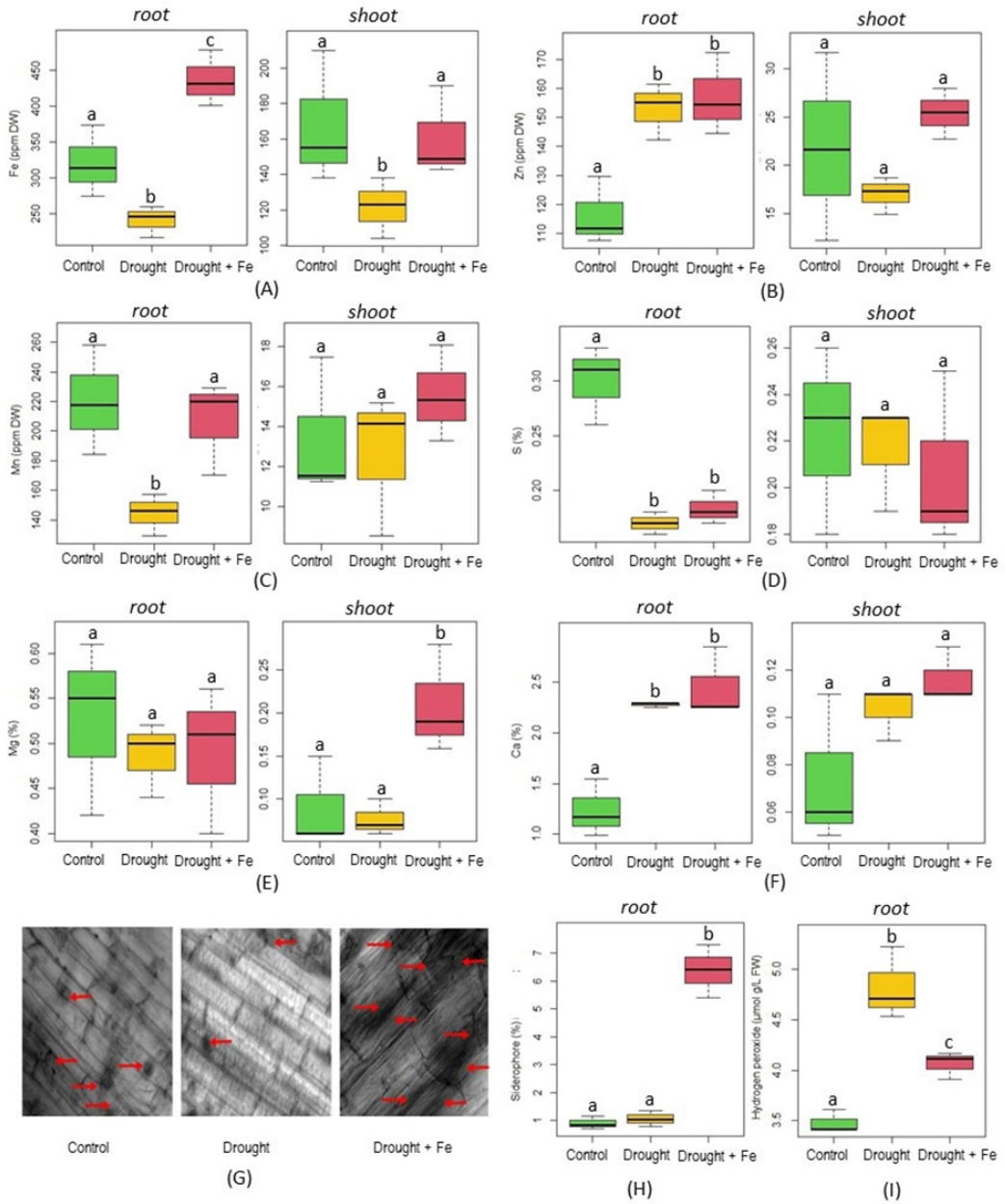

Fig. 2. Changes in mineral accumultation, siderophore levels and H_2O_2 concentrations in roots and/or shoots of sorghum cultivated with or without drought and/or Fe supplementation. Fe (A), Zn (B), Mn (C), S (D), Mg (E), Ca (F), root Fe localization using Perl-DAB staining (G), root siderophore levels (H) and root H_2O_2 concentration (I) in sorghum cultivated in the absence or presence of drought and Fe supplementation (Fe-EDTA). The data presented are means with standard deviations (n = 3) of individual replicates. Statistically significant differences among the treatments at a $p < 0.05$ level were indicated by different alphabetical letters above the bars, where applicable. Plants were cultivated in a growth chamber for 6 weeks.

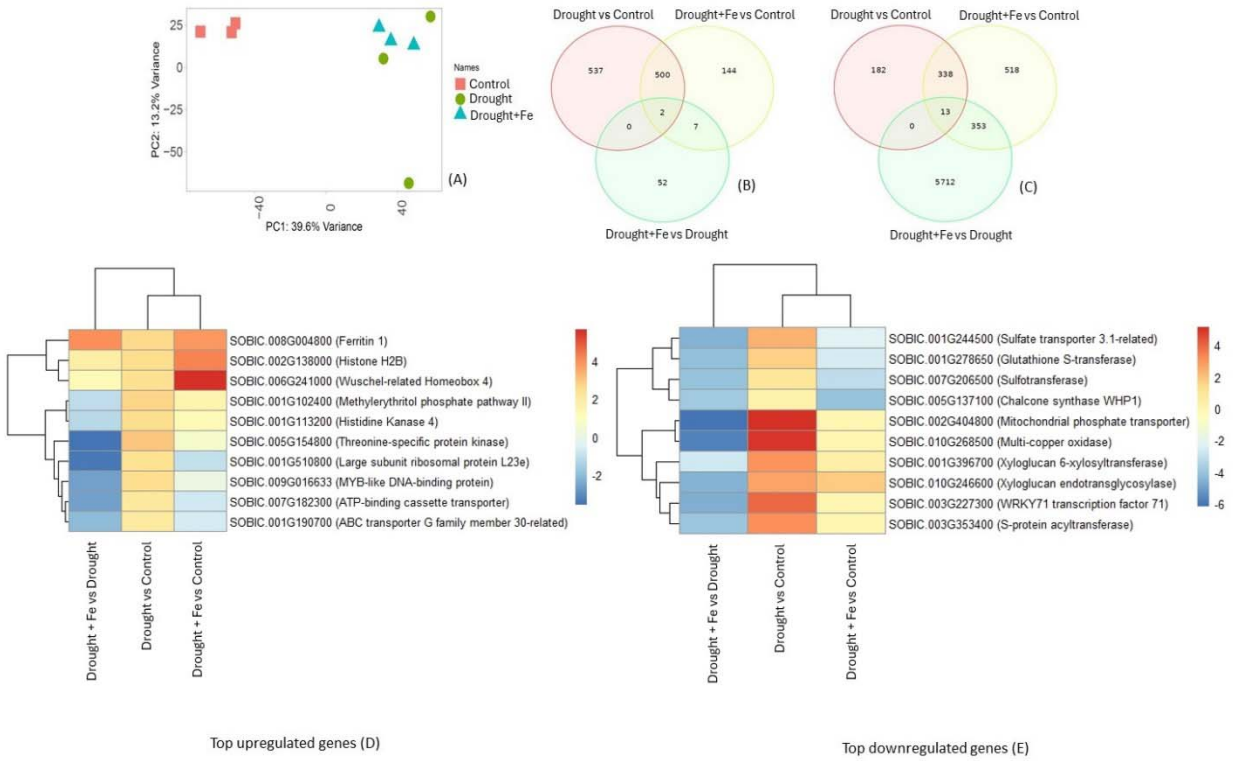

Fig. 3. Differentially expressed genes (> Log2 fold, p value < 0.05) in the roots of sorghum cultivated with or without drought and/or Fe supplementation. Principal component analysis of samples (A), Venn diagrams of upregulated genes (B), Venn diagrams of downregulated genes (C), (D) heatmap of top 10 upregulated genes and top 10 downregulated genes (E) differentially expressed (> Log2 fold, p value < 0.05) in the roots of sorghum plants cultivated in the absence or presence of drought and/or Fe-EDTA. A minimum of 20 million reads on average, with more than 33% read quality per sample, were utilized per treatment. Plants were cultivated in a growth chamber for 6 weeks of differential treatment.

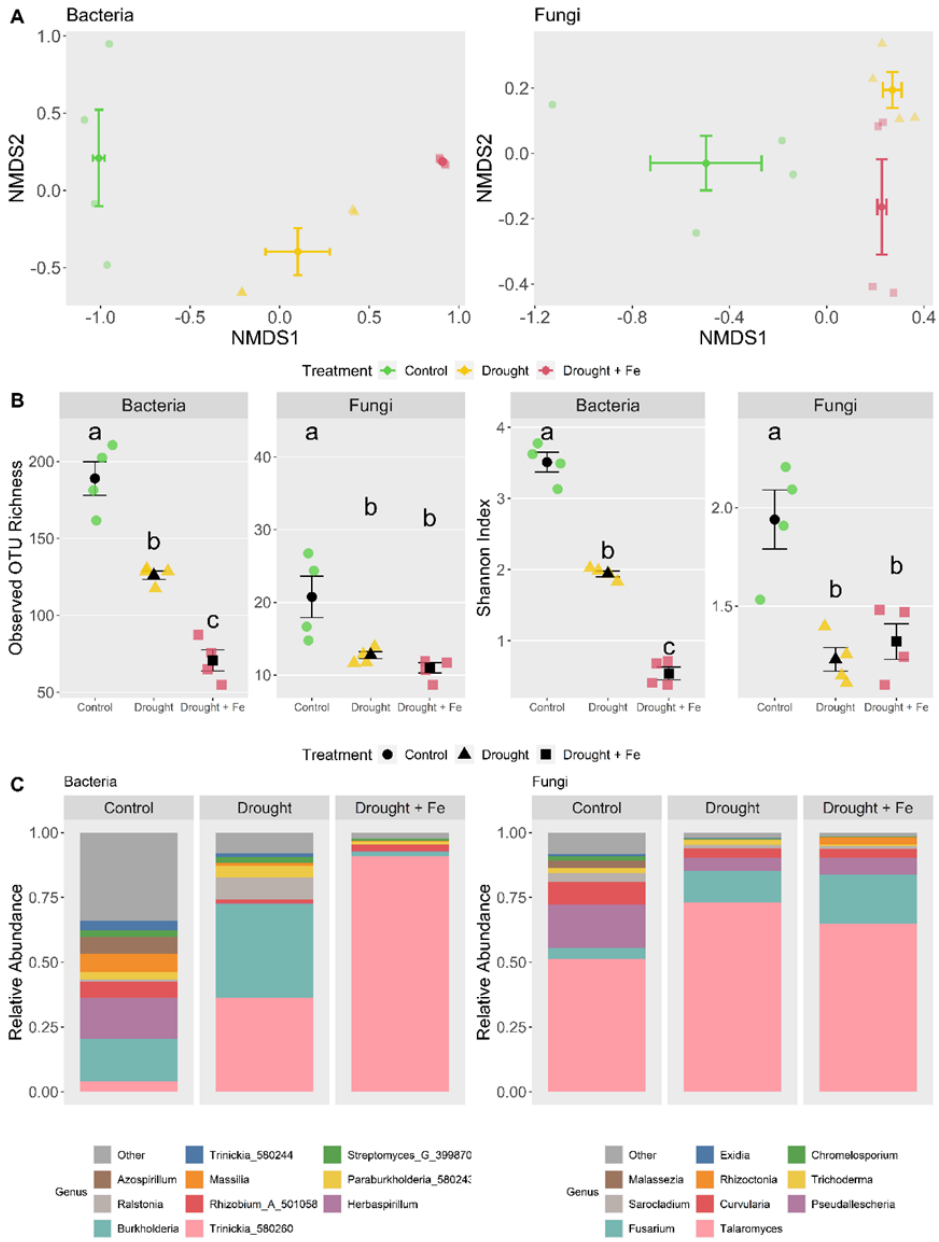

Fig. 4. Panel A) Non-metric dimensional scaling (NMDS) ordination that displays community dissimilarity between samples for bacterial and fungal communities among the treatments. Mean centroids are displayed as diamonds ± standard errors in the NMDS1 and NMDS2 axes to demonstrate the dispersal of data points around the mean within each treatment group. Individual sample shape and color are determined by experimental treatment (green / circle = control, yellow / triangle = drought, red / square = drought + Fe-EDTA). Panel B) Observed OTU richness and Shannon index values for bacterial and fungal communities. Mean values ± SE for each treatment is displayed in black, and individual sample values are colored by treatment. Letters denote distinct statistical groupings of treatments determined through post-hoc pairwise testing. Panel C) Stacked barplots displaying the mean relative abundance of the top 10 most

abundant genera for bacteria and fungi. The 'Other' category includes both lower abundance genera and taxa that were unclassified at the genus level.

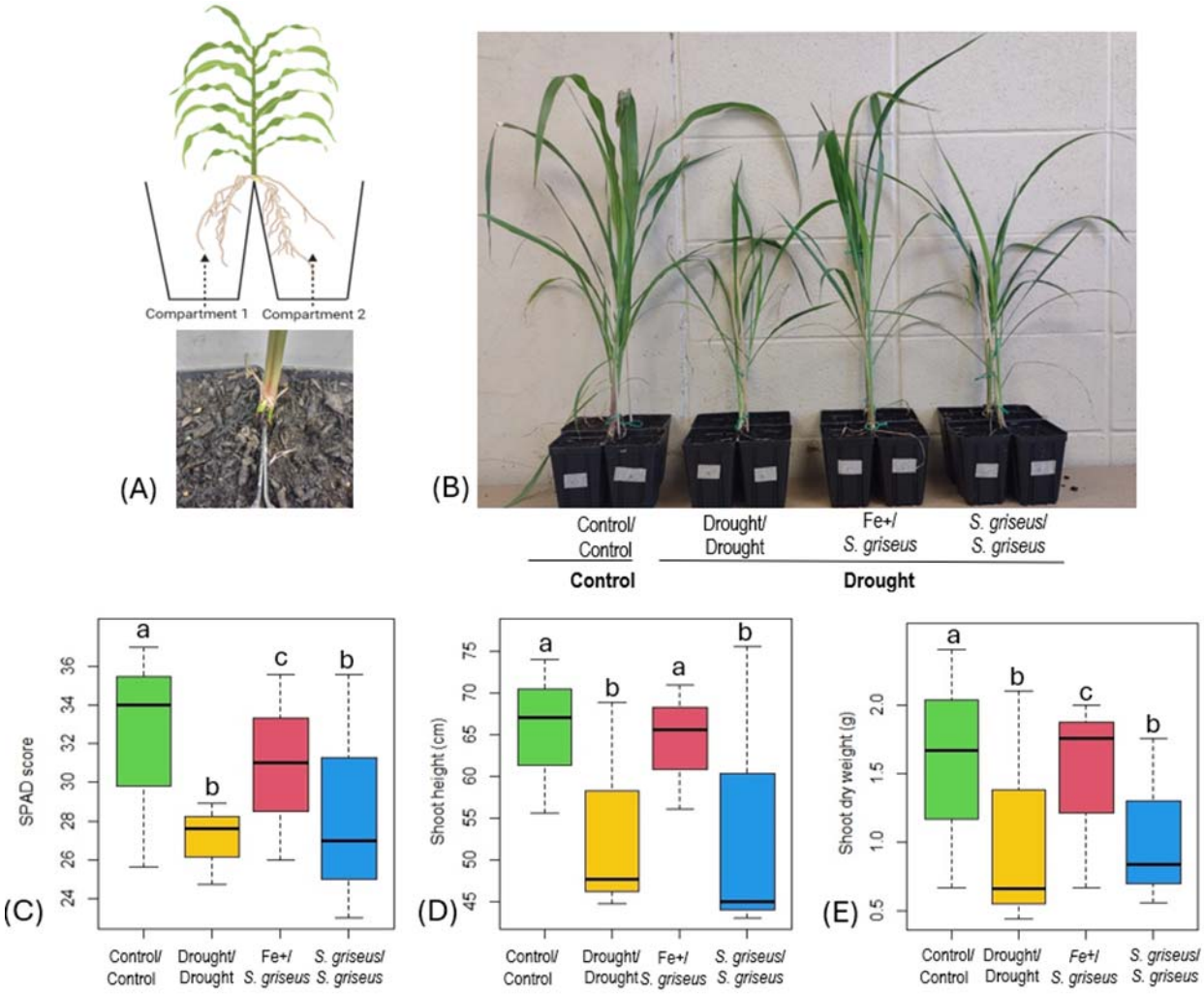

Fig. 5. Split-root assay: Schematic diagram of the split-root technique (A), shoot phenotypes (B), leaf SPAD scores (C), shoot heights (D), and shoot dry weights (E) of sorghum cultivated in a soil-based split-root system in two compartments in the absence or presence of Fe-EDTA and *S. griseus* under control and drought. The data presented are means with standard deviations (n = 3) of individual replicates. Statistically significant differences among the treatments at $p < 0.05$ were indicated by different alphabetical letters above the bars, where applicable. Data were collected on 7-week-old plants.

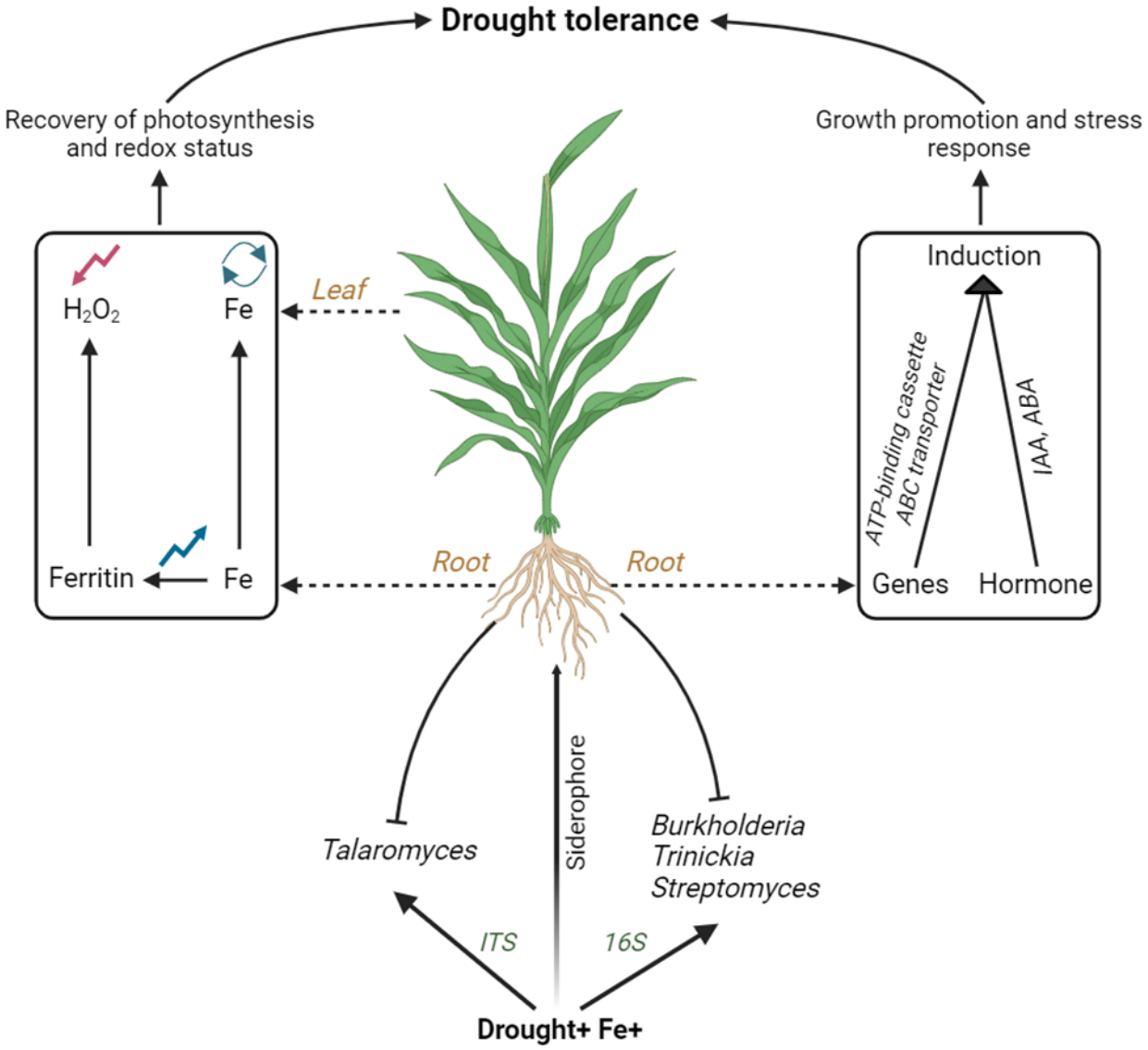

Fig. 6. Model of interaction between plant host and Fe inducing drought tolerance in sorghum. The addition of Fe to the soil induces *FER1* (ferritin) expression in the roots as well as the recovery of Fe status in the shoot. The upregulation of ferritin preventing H_2O_2 accumulation and maintenance of adequate Fe restore photosynthetic activity and redox status to help plants cope with drought. Furthermore, the elevated Fe induces the accumulation of rhizosphere siderophore as well as the expression of two genes responsible for auxin transport (ATP-binding cassette transporter, ABC transporter) and phytohormone (IAA, ABA) in the roots. Of the microbiomes, certain fungal (*Talaromyces*) and bacterial (*Burkholderia*, *Trinickia*, *Streptomyces*) substantially decreased in the roots of sorghum under drought augmented with Fe.