Fe deficiency causes transcriptional shift in roots leading to disruption of drought tolerance in soybean

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Abstract

Iron (Fe) deficiency in alkaline soils, exacerbated by drought, collectively affects soybean health. This study aimed to evaluate the physiological and transcriptional changes in Fiskeby IV, a drought-tolerant genotype that loses its tolerance when exposed to simultaneous Fe deficiency and drought. In this growth incubator study, Fe deficiency and drought stress resulted in substantial reductions in plant biomass, photosynthetic efficiency, and nutrient uptake in Fiskeby IV. Despite these disruptions, the photochemical efficiency of photosystem II remained stable, suggesting the activation of protective mechanisms to maintain essential photosynthetic functions. RNA-seq analysis highlighted a complex response, showing the upregulation of ethylene-responsive genes (*Ethylene-response sensor 2*, *Ethylene-responsive TF018*, *Ethylene-responsive TF5*) as well as the genes related to rhizosphere acidification (*ATPase 1*) and redox homeostasis (*Glutaredoxin-3*). It suggests that ethylene signaling and rhizosphere acidification may be responsive in coordinating Fe homeostasis and drought adaptation in soybean. On the flip side, combined stresses caused the downregulation of several genes related to nutrient uptake (*nicotianamine transporter YSL1*, *ammonium transporter 2*, *sulfate transporter 3.4*, and *major facilitator family protein*). In a targeted study, supplementation with 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor, led to substantial improvements in morpho-physiological traits and Fe status under combined stress conditions. This ACC treatment enhanced root flavonoid content and rhizosphere siderophore levels accompanied by restoration of 16S and ITS microbial community under Fe deficiency and drought. It underscores the potential of targeting ethylene signaling that may facilitate Fe mobilization and microbial interactions to enhance soybean tolerance to concurrent Fe deficiency and drought. This is the first report on the transcriptional response and requirement of Fe status underlying drought tolerance, potentially guiding future strategies for improving combined stress resilience in soybean.

Keywords: Combined stress, ethylene signaling, legume, siderophore, symbiosis.

Running title: Effect of combined Fe deficiency and drought in soybean

1 Introduction

Global agricultural sustainability faces increasing challenges due to climate change, which exacerbates abiotic stressors such as drought and nutrient deficiencies. Drought stress severely limits crop productivity by disrupting physiological processes essential for growth and development (Barnabas et al., 2008). Drought stress reduces soil moisture, limiting the availability and uptake of essential minerals by plant roots (Li et al., 2023). In plants, drought response involves intricate regulatory networks that coordinate responses at molecular, physiological, and morphological levels (Geng et al., 2024; Seleiman et al., 2021). However, recent research highlights that the presence of additional stress factors, such as Fe (Fe) deficiency, can significantly alter these mechanisms, potentially negating the benefits of drought-tolerant plant species/cultivars (Afshar et al., 2012; Elanz et al., 2011). Fe deficiency is a common nutritional disorder affecting plants, characterized by impaired chlorophyll synthesis and reduced photosynthetic efficiency (Li and Lan, 2017). Beyond its direct impacts on plant health, Fe deficiency interacts with other stressors, altering gene expression patterns and metabolic pathways critical for stress adaptation (Liang, 2022). Understanding how Fe deficiency modulates drought tolerance mechanisms in plants is therefore pivotal for devising strategies to enhance crop resilience under multifaceted stress environments.

Plants employing Strategy I responses to Fe deficiency enhance Fe uptake through several mechanisms to help manage and mitigate the deficiency. They increase proton extrusion via *ATPase* 1 to acidify the rhizosphere, improving Fe solubility (Kabir et al. 2012). Additionally, they boost the activity of ferric-chelate reductase enzymes to reduce Fe(III) to the more readily absorbed Fe(II) form (Morrissey and Guerinot, 2009). Plants also enhance the expression of Fe transporter proteins to facilitate Fe uptake into root cells (Kroh and Pilon, 2019). In addition, drought tolerance in plants encompasses a diverse array of adaptive strategies aimed at maintaining cellular hydration, sustaining metabolic processes, and minimizing damage during water deficit conditions (Volaire, 2018; Kumar et al., 2021). Furthermore, plants exhibit dynamic changes at the molecular level, involving the activation of stress-responsive genes and signaling pathways such as abscisic acid (ABA) signaling, which coordinates responses to water stress by regulating stomatal closure and promoting stress-responsive gene expression (Chaves et al., 2003; Ali et al., 2020). Also, ethylene plays a pivotal role in mediating plant responses to both drought stress and Fe deficiency, highlighting its multifaceted involvement in stress tolerance mechanisms (Fatma et al., 2022). Ethylene signaling influences the plant's ability to cope with low Fe availability by modulating the expression of genes involved in Fe uptake and utilization (Kabir et al., 2012; Lucena et al., 2015). Furthermore, plants employ a variety of strategies to cope with drought stress, among which the Strategy I response in dicot plants plays a crucial role in ensuring adequate mineral nutrition, particularly under water-deficit conditions (Khan et al., 2023; D'Oria et al., 2022). Strategy I refers to the mechanism by which plants acquire Fe from the soil through the reduction of Fe(III) to Fe(II) at the root surface, facilitated by proton extrusion and subsequent uptake into root cells via specific transporters (Kabir et al., 2012; Kobayashi and Nishizawa, 2012). These adjustments are crucial for ensuring sufficient Fe acquisition despite limited water availability, thereby safeguarding essential metabolic functions and overall plant growth and productivity. Flavonoids released by plants have been identified as pre-symbiotic signaling molecules in microbial-plant symbiosis (Lidoy et al., 2023; Schliemann et al., 2008). When plants are deficient in Fe, there is

typically an increase in siderophores in the rhizosphere, secreted by both bacterial and fungal communities associated with host plants in a symbiotic relationship, to chelate Fe (Jin et al., 2010; Crowley et al., 2006). However, the role of microbial association and siderophores in the rhizosphere of legume crops exposed to combined abiotic stresses is understudied.

Among staple crops, soybeans (*Glycine max*) play a crucial role in global food security and industrial applications (Kulkarni et al., 2018). Efforts to enhance soybean resilience to drought have yielded promising cultivars, yet their efficacy can be compromised under concurrent stress conditions, such as Fe deficiency. However, the modulation of drought tolerance due to differential Fe availability in soybeans is still not well understood. Therefore, this study aims to unravel the physiological responses and transcriptome governing the interplay between Fe deficiency and drought tolerance in soybean. Furthermore, we studied the potential role of an ethylene precursor in mitigating the detrimental impacts of Fe deficiency and drought in soybeans. These insights may optimize breeding strategies for developing resilient crop varieties of soybeans capable of withstanding complex environmental challenges.

2 Materials and methods:

2.1 Plant cultivation and growth conditions

The seeds of soybean (var. Fiskeby IV: drought tolerant) were collected from USDA-GRIN. Before germination, seeds were surface sterilized with 4% sodium hypochlorite solution for 5 min, followed by three washes with sterile water. Subsequently, seeds were placed in a tray and incubated at 25°C in the dark. Once uniform and healthy seedlings emerged in 3d, they were transplanted into soil pots (500 g soil), consisting of a mixture of natural field soil and commercial potting mix in a 1:2 ratio, for two distinct treatments: control (soil without lime, pH ~6.5) and Fe-drought+ (soil amended with 4.5 g $NaHCO_3$ and 3.0 g $CaCO_3$, pH ~7.8). Throughout the cultivation period, 15 mM $NaHCO_3$ was added weekly to the Fe-deficient soil to maintain its pH to 7.8 to make the Fe unavailable for plants (Kabir et al., 2012). 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, 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 5 weeks before data measurement. In a targeted experiment, a precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) of the ethylene synthesis (McDonnell et al., 2009), was supplemented in soil (10 mM) to validate whether it can induce tolerance in 6-week-old plants in response to combined Fe deficiency and drought.

2.2 Determination of morphological and photosynthetic parameters

The height of each plant's shoot and longest root were measured. The shoots and roots were then dried for three days at 70°C in an electric oven before their dry weights were recorded. Furthermore, chlorophyll scoring and chlorophyll fluorescence kinetics (OJIP) measurements, including Fv/Fm (maximal photochemical efficiency of PSII) and Pi_ABS (photosynthesis performance index) were conducted on the uppermost fully developed leaves at three different locations using a handheld chlorophyll meter (AMTAST, United States) and FluorPen FP 110 (Photon Systems Instruments, Czech Republic), respectively. For OJIP analysis, the leaves were acclimated to darkness for 1 hour before data collection. 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.

2.3 RNA-seq analysis in roots

RNA-seq analysis was performed on root samples. The soybean roots were thoroughly cleaned by washing twice in sterile phosphate-buffered saline with 10 seconds of vortexing, followed by two rinses with sterile water. The cleaned roots were then ground into a powder with a pre-cooled pestle and mortar using liquid nitrogen. RNA was extracted from the powdered material using the SV Total RNA Isolation System (Promega, USA). For each sample, 1 µg of RNA with an RIN value above 8 was used for RNA-seq library preparations. The libraries were created with the KAPA HiFi HotStart Library Amplification Kit (Kapa Biosystems, USA) and sequenced on an Illumina NovaSeq 6000 instrument at the RTSF Genomics Core at Michigan State University. A total of 92.5% of reads passed filters with a quality score of ≥ Q30.

Bioinformatics analysis of raw FastQ files was performed using Partek Flow genomic analysis software (Partek, St. Louis, MO, USA). Adaptor sequences and low-quality reads were removed with Trimmomatic (Bolger et al., 2014) to obtain high-quality clean reads. The clean reads were aligned to the soybean reference genome, downloaded from HISAT2 (v2.0.5) (Yates et al., 2022; Kim et al., 2015). Gene read counts were determined using HTSeq (Anders et al., 2015). Differentially expressed genes (DEGs) were identified using the limma v.3.19 R package (Ritchie et al., 2015), with FPKM (fragments per kilobase of exon per million mapped fragments) calculations. DEGs were defined as those with an adjusted P-value < 0.05 and a fold change ≥ 2, using the false discovery rate (FDR) method. Venn diagrams and heatmaps were created with VennDetail (Hinder et al., 2017) and pheatmap (Hu et al., 2021) in R, respectively. Gene ontology and enrichment analyses of soybean DEGs were conducted using the SoyBase website developed by the USDA-ARS SoyBase and Legume Clade Database group at Iowa State University (Ames, IA, United States).

2.4 Determination of tissue nutrient elements.

Nutrient analysis was conducted on the roots and young leaves. The roots were detached, washed with running tap water, soaked in a 0.1 mM $CaSO_4$ solution for 10 min, and then rinsed with deionized water. The young leaves were washed separately with deionized water. All samples were placed in envelopes and dried in an electric oven at 75°C for three days. Elemental concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS) at the Agricultural and Environmental Services Laboratories at the University of Georgia. The concentrations were determined by comparing the intensity of the ion peaks in the spectrum to calibration standards.

2.5 Estimation of flavonoid content in roots

The total flavonoid content in soybean roots was measured as described by Piyanete et al., (2009). In brief, 0.5 mL of 70% ethanol extract from the roots was mixed with 2 mL of distilled water and

0.15 mL of a 5% $NaNO_2$ solution. After incubating for 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and left to stand for another 6 min. Then, 2 mL of a 4% NaOH solution was added, and the mixture was diluted to a final volume of 5 mL with methanol and thoroughly mixed. After a 15-minute incubation period, the absorbance was measured at 510 nm using a spectrophotometer against a blank. The total flavonoid concentration was expressed in milligrams per gram of dry weight, calculated using a quercetin standard curve.

2.6 Determination of siderophore in the rhizosphere

We quantified the siderophore content in the rhizosphere soil using a chrome azurol S (CAS) assay, following the procedure outlined by Himpsl and Mobley (2019). In summary, rhizosphere soil was homogenized with 80% methanol and subsequently centrifuged for 15 min at 10,000 rpm. The resulting supernatant (500 µl) was mixed with 500 µl of CAS solution and allowed to incubate at room temperature for 5 min. The absorbance of each reaction was then measured at 630 nm against a reference containing 1 ml of CAS reagent alone. The siderophore level per gram of soil sample was calculated using the formula: % siderophore unit = [(Ar - As) / Ar] x 100, where Ar represents the absorbance at 630 nm of the reference (CAS reagent only), and As represents the absorbance at 630 nm of the sample (CAS reagent + soil sample).

2.7 Determination of relative abundance of microbial community

Harvested roots were cleaned by vortexing in sterile phosphate-buffered saline (PBS) for 10 sec, 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. The DNA samples were then quantified and diluted before performing qPCR in a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using specific primers for bacterial (16S-341_F: CCTACGGGNGGCWGCAG, 16S-341_R: GACTACHVGGGTATCTAATCC) and ITS fungal (ITS1-F: TCCGTAGGTGAACCTGCGG, ITS4-R: TCCTCCGCTTATTGATATGC) community. The soybean *Tubulin* gene (Fw: GCTCCCAGCAGTACAGGACTCT, Rv: TGGCATCCCACATTTGTTGA) served as the internal control for relative quantification.

2.8 Data analysis

All data were collected from three independent biological replicates for each treatment. The statistical significance of each variable was assessed through Student's t-test. Graphical presentations were prepared using ggplot2 in the R program.

3 Results

3.1 Changes in morphological and traits and photosynthetic features

The plants grown under Fe deficiency and drought showed a significant decrease in root length, root dry, shoot height and shoot dry weight compared to the untreated controls (Figure 1A-1D). We observed a significant decrease in chlorophyll scores and Pi_ABS under Fe deficiency combined with drought compared to the controls (Figure 2A, 2C). However, Fv/Fm remained the same in the Fe-deficient and drought-stressed plants compared to the controls (Figure 2B).

3.2 Elemental changes in plant tissues

The ICP-MS analysis of soybean roots and shoots under control and Fe-drought+ conditions revealed significant differences in nutrient concentrations (Table 1). Fe and N levels significantly

decreased in both root and shoot under Fe-drought+ conditions compared to control. Zn, Mn and Ca levels showed no significant changes in the roots due to Fe-drought+ conditions but these minerals significantly declined in the shoot in such conditions relative to controls (Table 1). S and K levels significantly declined in the roots of soybean cultivated in response to combined Fe deficiency and drought compared to controls, while these minerals showed no significant changes in the shoot in such conditions. However, Mg levels showed no significant changes either in the root or shoot under Fe-drought+ conditions relative to untreated controls (Table 1).

3.3 RNA-seq analysis of differentially expressed genes

We conducted RNA-seq analysis to examine the changes in the transcriptome of soybean roots exposed to a combination of Fe (Fe) deficiency and drought. Principal component analysis (PCA) using three biological replicates revealed that the first two principal components, PC1 and PC2, accounted for 53.4% and 23.6% of the variance, respectively (Figure 3A). The PCA results indicated significant differences among the sample groups. Furthermore, the Venn diagram highlighted the genes that were significantly altered in response to the combined Fe deficiency and drought compared to non-treated controls (Figure 3B). Specifically, we identified 705 upregulated and 1,297 downregulated genes differentially expressed under Fe-deficient and drought conditions compared to the control. Additionally, the MA plot showed a higher number of downregulated genes than upregulated ones in response to dual stress (Figure 3C).

3.4 Heatmap analysis of top 15 differentially upregulated and downregulated genes in roots

From the RNA-seq analysis, we present the top 15 upregulated and downregulated genes that are differentially expressed (> Log2 fold, < P value 0.05) and associated with plant growth promotion and stress responses (Figure 4A, 4B). The expression of the top 15 key genes that were significantly upregulated in the roots of soybean treated with Fe deficiency and drought relative to controls includes: ethylene-responsive TF5, ABC transporter C family 10, ATPase 1, dehydration-responsive binding protein 2A, calcium-dependent protein kinase 6, copper ion transporter, sulfate transporter 1.1, ferredoxin, MATE efflux family protein, ethylene-responsive TF018, BZIP transcription factor ATB2, ABA-GTase, glutaredoxin-3, ethylene-response sensor 2, and heavy-metal-associated protein 26 (Figure 4A, Table 2). In contrast, the top 15 downregulated genes include nicotianamine transporter YSL1, multi-copper oxidase type I, anion transporter 6, sulfate transporter 3.4, heme-binding protein-related, auxin efflux carrier component 6, calcium transporting *ATPase 4*, jasmonic acid-amido synthesis *JAR1*, ammonium transporter 2, molybdate transporter 1, neoxanthin synthase, solute carrier family 40, major facilitator family protein, heavy metal transport protein, and peroxidase 66 (Figure 4B, Table 2).

3.5 Gene enrichment analysis

Gene enrichment analysis revealed the enrichment of several pathways, categorized into three groups: biological processes, cellular components, and molecular functions (Figure 5A-5B). The biological process category showed significant upregulation in translation, signal transduction, biosynthesis, cell cycle, transport, embryo development, flower development, cell differentiation, and cellular metabolic processes. In contrast, the cellular component category indicated significant upregulation of the nucleus, cytoplasm, mitochondria, membranes, plasma membrane, cytoskeleton, endoplasmic reticulum, and Golgi apparatus. Additionally, the molecular function category showed significant upregulation of protein binding, nucleic acid binding, RNA binding,

catalytic activity, sequence-specific DNA binding transcription factor activity, nucleotide binding, kinase activity, and transferase activity - specifically transferring phosphorus-containing groups. We also observed downregulation in several pathways compared to the control. In the biological process category, there was significant downregulation in signal transduction, translation, lipid metabolic processes, flower development, cell death, transport, carbohydrate metabolic processes, cell differentiation, protein modification processes, and reproductive structure development. The cellular component category showed significant downregulation of the nucleus, mitochondria, plasma membrane, thylakoid, cytoplasm, extracellular region, golgi apparatus, cytosol, and vacuole. Furthermore, the molecular function category exhibited significant downregulation of protein binding, catalytic activity, nucleic acid binding, RNA binding, DNA binding, sequence-specific DNA binding transcription factor activity, nucleotide binding, kinase activity, and chromatin binding compared to controls under Fe deficiency and drought conditions.

3.6 ACC (ethylene precursor) on promoting dual stress tolerance

Given that we observed the upregulation of several ethylene-responsive genes in RNA-seq, we further investigated whether ACC (an ethylene precursor) has significant biological effects on recovering dual stress in soybean cultivated for 6 weeks. In this study, the combined Fe deficiency and drought exhibited a significant decrease in chlorophyll score, plant height, plant dry weight, stem diameter and leaf RWC (%) in soybeans relative to untreated controls (Figure 6A-6F). ACC supplementation resulted in significant improvements in these traits compared to the Fe-Drought+ conditions without ACC (Figure 6A-6F). ICP-MS analysis showed a significant decrease in leaf Fe concentration due to Fe- drought+ conditions compared to controls. However, the addition of ACC under Fe- drought+ conditions caused a significant increase in Fe levels in the leaf compared to the plants in such conditions without ACC (Figure 6G). We found that Fe-drought+ conditions showed no significant changes in flavonoid content in the roots of soybean relative to controls. However, the addition of ACC under Fe-drought+ caused a significant increase in flavonoid content compared to those plants exposed to combined Fe deficiency and drought without ACC (Figure 6H). Interestingly, siderophore levels in the rhizosphere of soybean exposed to Fe-drought+ conditions showed a significant increase relative to controls (Figure 6I). Soybean plants exposed to combined Fe deficiency and drought supplemented with ACC showed a significant increase in rhizosphere siderophore compared to those plants exposed to Fe-drought+ conditions without ACC supplementation (Figure 6I).

We further determined the relative of 16S and ITS microbial communities in roots by DNA-based qPCR. We found that combined Fe deficiency and drought caused a significant decline in 16S bacterial abundance in roots relative to controls. However, the addition of ACC in Fe-Drought+ conditions showed a significant increase in 16S, which is similar to controls (Figure 7A). Similarly, the relative abundance of ITS fungal community significantly decreased in the roots due to Fe-Drought+ conditions in contrast to untreated controls. Interestingly, ITS fungal abundance showed a significant increase when ACC was added to soil exposed to Fe deficiency and drought. Also, the increase in ITS abundance was relatively higher than the plants cultivated in control conditions (Figure7B).

4 Discussion

This study reveals that drought tolerant cultivar (Fiskeby IV) exposed to combined Fe deficiency and drought stress experience significant reductions in morphological and photosynthetic

efficiency. Plants are constantly exposed to combined abiotic stresses, such as mineral deficiency and drought, in their natural environments. These simultaneous challenges can lead to an intensified effect, making it harder for plants to adapt and survive (Teshome et al., 2020). This is the first growth incubator study to reveal transcriptional changes, with many genes related to growth and stress responses being differentially expressed. We also observed the requirement for ethylene-mediated Fe homeostasis and possible indications of symbiosis that may be necessary for combined stress tolerance in soybean.

Morphological and physiological responses to combined stresses

The significant reduction in plant biomass subjected to Fe deficiency and drought stress, as observed in our study, highlights the detrimental impact of these combined abiotic stresses on soybean growth. These morphological changes are likely due to impaired cellular processes and metabolic activities resulting from inadequate Fe availability and water stress. Fe is a critical micronutrient involved in various physiological processes, including chlorophyll synthesis and electron transport in photosynthesis (Samira et al., 2013). The observed decrease in chlorophyll content and photosynthetic performance index under Fe deficiency and drought stress conditions underscores the compromised photosynthetic capacity of the plants. Interestingly, the maximum quantum efficiency of photosystem II (Fv/Fm) remained unaffected under Fe deficiency and drought stress suggesting that while the overall photosynthetic apparatus is impacted, the core photochemical efficiency of PSII remains intact. It is possible that the plants activate protective mechanisms to maintain the functional integrity of PSII under combined stress conditions, even though the overall photosynthetic capacity is reduced.

The elemental analysis of soybean tissues revealed significant alterations in nutrient concentrations under Fe deficiency and drought stress. The marked decrease in Fe and N levels in both roots and shoots highlights the impaired nutrient uptake and transport mechanisms under these stress conditions. The unchanged levels of Zn, Mn, and Ca in the roots, despite significant declines in the shoots under Fe deficiency and drought stress, suggest differential regulation of nutrient allocation and homeostasis between root and shoot tissues. The stability of these elements in the roots might be attributed to their relatively lower mobility and the plant's efforts to prioritize root functionality under stress. Furthermore, the significant reduction in S and K levels in the roots, coupled with no significant changes in the shoots, further underscores the differential nutrient management strategies adopted by the plants under Fe deficiency and drought stress. Sulfur is vital for the synthesis of amino acids and proteins, while potassium plays a crucial role in osmotic regulation and enzyme activation (Ren et al., 2022; Künstler et al., 2020). The observed decline in these nutrients in the roots indicates a potential trade-off between root and shoot nutrient allocation to mitigate stress effects.

Transcriptomic responses to combined Fe deficiency and drought stress

The transcriptomic analysis reveals a complex network of gene expression changes in response to combined Fe deficiency and drought stress in soybean roots. We found that upregulated genes are primarily associated with stress responses and adaptive mechanisms. Particularly, the upregulation of ethylene-responsive transcription factors (TF5 and TF018) and dehydration-responsive binding protein 2A highlights the activation of stress-related signaling pathways. The induction of several ethylene-responsive genes suggests a connection of ethylene signaling as part of the adaptive responses to combined Fe deficiency and drought stress in soybean. Ethylene is a critical plant

368 hormone involved in regulating various physiological and developmental processes, including
369 responses to biotic and abiotic stresses (Chen et al., 2021; Dubois et al., 2018). Ethylene has been
370 shown to be involved in the regulation of Fe homeostasis (Wu et al., 2011). Under Fe deficiency,
371 ethylene-responsive genes are induced to enhance the plant's ability to acquire and utilize Fe and
372 other nutrients efficiently (Müller and Munné-Bosch, 2015; Li and Lan, 2017). This is correlated
373 with the upregulation of sulfate transporter 1.1, heavy-metal-associated protein 26, and copper ion
374 transporter, indicating their efforts in maintaining cellular ion balance under stress conditions.
375 Ethylene has also been shown to interact with Fe deficiency signaling pathways, enhancing the
376 expression of Fe acquisition genes, including *ATPase 1* (Waters et al., 2007). In Strategy I plants,
377 *ATPase 1*, a plasma membrane proton pump, plays a significant role in Fe uptake (Zhang et al.,
378 2017). *ATPase 1* contributes to this by creating a proton gradient across the root plasma membrane,
379 which is essential for the acidification of the rhizosphere (Michalak et al., 2022). This acidification
380 helps solubilize Fe^{3+} making it more accessible for uptake by the plant (Kabir et al., 2012). Thus,
381 the upregulation of *ATPase 1* in roots suggests an enhanced effort to regulate rhizosphere
382 acidification which helps plants mobilize Fe under combined Fe deficiency and drought in
383 soybean.
384
385 Furthermore, studies suggest that ethylene biosynthesis is often upregulated, leading to changes in
386 the expression of drought-responsive genes that help plants conserve water and maintain cellular
387 homeostasis (Mohorović et al., 2023; Arraes et al., 2015). Naing et al., (2022) showed that
388 ethylene-insensitive mutants exhibited increased sensitivity to drought stress, indicating that
389 ethylene-responsive genes contribute to drought adaptation by modulating stomatal conductance
390 and enhancing root growth. Thus, our findings imply that the induction of ethylene-responsive
391 genes may act as a central regulator, integrating signals from both Fe deficiency and drought stress
392 to orchestrate an adaptive response in soybean. Ethylene signaling may facilitate crosstalk between
393 Fe deficiency and drought stress pathways. This crosstalk can regulate the reactive oxygen species
394 (ROS) scavenging enzymes can help mitigate oxidative stress, which is common under both Fe
395 deficiency and drought conditions (Zhang et al., 2016; Kabir et al., 2012). In support of this
396 possible phenomenon, we observed the upregulation of glutaredoxin-3 in the roots of soybean.
397 Glutaredoxin-3 is involved in the assembly and maintenance of Fe-S clusters, which are crucial
398 for the function of many enzymes, including those involved in ROS detoxification and other
399 metabolic processes (Couturier et al., 2015). In addition, the induction of ferredoxin among the
400 upregulated genes also points to the importance of redox homeostasis in stress adaptation.
401 Ferredoxin is involved in maintaining cellular redox homeostasis by ROS, particularly under
402 drought, salinity, and high light-intensity stresses in plants (Huang et al., 2020; Lehtimäki et al.,
403 2010). Taken together, the top upregulated genes found in this study can be leveraged to develop
404 soybean varieties with enhanced resilience to concurrent abiotic stresses, potentially leading to
405 improved crop yields and sustainability.
406
407 Our RNA-seq analysis exhibited that several downregulated genes are associated with various
408 physiological processes, including nutrient transport, hormone signaling, and stress responses due
409 to combined Fe deficiency and drought. Fe deficiency and/or drought can cause significant
410 nutritional imbalances in plants, disrupting essential metabolic processes and leading to stunted
411 growth and poor health (Kumari et al., 2022). In this study, the downregulation of nicotianamine
412 transporter *YSL1* and anion transporter 6 reflects a possible disruption in nutrient uptake and
413 translocation in soybean (Fiskeby IV) exposed to Fe deficiency and drought. Furthermore, the

414 decreased expression of jasmonic acid-amido synthetase *JAR1* and auxin efflux carrier component
415 6 suggests an alteration in hormonal balance, which could be one of the factors disrupting the
416 drought tolerance ability of the genotype Fiskeby IV to combat dual stresses on soybean.
417 Decreased *JAR1* expression can impair drought resilience by weakening the jasmonic acid
418 signaling pathways that regulate stomatal closure and stress-responsive gene activation (Ali et al.,
419 2020). Another study demonstrates that over-expression of *JAR1)* enhances drought tolerance in
420 *Arabidopsis thaliana* by increasing jasmonyl-L-isoleucine production, which leads to better water
421 retention (Mahmud et al., 2022). Although Fe is not a direct cofactor for the enzymes specific to
422 jasmonic acid signaling, it is crucial for the biosynthesis of JA, as it is required for the functioning
423 of lipoxygenases and other enzymes that convert fatty acids into jasmonic acid (Singh et al., 2022;
424 Oliw 2022). In contrast, jasmonate-deficient *cpm2* mutant revealed that jasmonates inhibit the
425 expression of several Fe deficiency-inducible genes associated with Feuptake in rice (Kobayashi
426 et al., 2016). Particularly, auxin plays a critical role in mediating adaptive responses to improve Fe
427 acquisition and drought tolerance in plants (Liu et al., 2024; Iqbal et al., 2022; Kabir et al., 2012).
428 In this study, we also found the downregulation of neoxanthin synthase and solute carrier family
429 40 highlights disruptions in carotenoid biosynthesis and solute transport, which is consistent with
430 the decreased photosynthetic efficiency and nutrient uptake in Fiskeby IV exposed to concurrent
431 Fe deficiency and drought. Under abiotic stress, such as drought or nutrient deficiency, plants often
432 experience disruptions in chlorophyll and carotenoid biosynthesis resulting in decreased energy
433 production and plant growth (Muhammad et al., 2021; Dong et al., 2007). Carotenoid synthesis
434 may also be affected, impacting plant protection against oxidative damage and influencing overall
435 stress tolerance (Giani and Martínez-Espinosa 2020). Thus, phytohormone and other
436 downregulated genes can be targeted for genetic engineering or breeding strategies aimed at
437 enhancing stress resilience in soybean.
438
439 **Ethylene enhances dual stress tolerance in soybean**
440 In light of the RNA-seq analysis revealing upregulation of several ethylene-related genes, we
441 conducted a targeted study using an ethylene precursor to determine if it could eliminate the
442 impairment in drought-tolerant Fiskeby IV exposed to combined Fe deficiency and drought. The
443 results of our study underscore the potential of ACC, an ethylene precursor, in enhancing dual
444 stress tolerance in soybean. In this study, supplementation with ACC led to significant
445 improvements in these morpho-physiological traits, suggesting that ethylene signaling helps
446 restore key growth parameters along with leaf Fe status disrupted by combined stress conditions.
447 Precious studies indicating the up-regulation of genes (i.e. *1-aminocyclopropane-1-carboxylic*
448 *acid synthase (ACS)*, and *1-aminocyclopropane-1-carboxylic acid oxidade*) involved in ethylene
449 synthesis further support the increased ethylene production as part of the adaptive responses under
450 Fe deficiency in plants (García et al., 2015; Sauter et al., 2013). In most cases, ethylene, whose
451 production increases under nutrient deficiency in genotypically efficient genotypes, acts as an
452 activator of stress responses in plants (Dubois et al., 2018). Thus, it appears that ethylene-related
453 or Fe-uptake genes could be potential targets for improving stress tolerance in soybean exposed to
454 Fe deficiency and drought.
455
456 Our extended study, which revealed a correlation between root flavonoid content and rhizosphere
457 siderophore levels induced by the presence of an ethylene precursor, further underscores the
458 significance of microbial associations in soybean subjected to combined Fe deficiency and
459 drought. While 'Fe- drought+' stress conditions alone did not affect root flavonoid content, ACC

supplementation significantly increased flavonoid levels. This enhancement in flavonoids might contribute to improved stress resilience in soybean as flavonoids are known to play roles in symbiotic associations (Wang et al., 2022). Flavonoids play an essential role in rhizobium-legume symbiosis as chemoattractants and *Nod* gene inducers, and they are also believed to stimulate fungal spore germination and hyphal growth (Abdel-Lateif et al., 2012; Hassan et al., 2012). Similarly, siderophore levels in the rhizosphere were elevated under 'Fe-drought stress', and ACC further boosted siderophore production. This indicates that ACC not only improves plant tolerance to concurrent Fe deficiency and drought but also potentially aids in Fe acquisition by increasing siderophore-mediated Fe mobilization in the rhizosphere. Microbial siderophores are compounds secreted by both bacterial and fungal microbial communities that bind and solubilize Fe, making them more accessible for uptake by plants (Singh et al., 2022). These siderophores can enhance plant Fe acquisition, particularly in Fe-deficient soils, by facilitating the transfer of Fe from the soil to plant roots (Kabir and Bennetzen, 2024; Radzki et al., 2013). Our qPCR experiment further demonstrated the restoration of 16S bacterial and ITS fungal community when ACC was augmented in soybeans exposed to dual stresses, compared to plants exposed to Fe-drought+ conditions. Ethylene plays a complex yet crucial role in enhancing microbial symbiosis in plants by modulating defense responses and facilitating beneficial interactions (Shekhawat et al. 2022; Ravanbakhsh et al. 2018). In legume-rhizobia symbiosis, ethylene levels influence root nodule formation essential for nitrogen fixation (Guinel, 2015). Similarly, in mycorrhizal associations, ethylene can modulate root architecture and colonization (Khatabi et al. 2012). Thus, it appears that ethylene signaling may indirectly promote microbial symbiosis, thereby recovering Fe status and drought tolerance in Fiskeby IV exposed to combined Fe deficiency and drought. This study may inspire future research on the composition of microbial dynamics, including rhizobia, to uncover the interactions between ethylene signaling and microbiome to possess plant growth-promoting and stress resilience functions. This could lead to improved stress tolerance in soybeans in response to Fe deficiency and drought.

5 Conclusion

This study evaluated the physiological and transcriptional changes in the drought-tolerant soybean genotype Fiskeby IV, which loses its tolerance when exposed simultaneously to Fe deficiency and drought. Exposure to these combined stresses resulted in significant reductions in plant biomass, photosynthetic efficiency, and nutrient uptake. Despite these disruptions, the core photochemical efficiency of photosystem II remained stable, suggesting protective mechanisms at play. RNA-seq analysis highlighted the upregulation of genes related to ethylene signaling, rhizosphere acidification, and redox homeostasis (Figure 8). This indicates that ethylene is crucial for coordinating Fe homeostasis and drought adaptation pathways. Conversely, combined Fe deficiency and drought led to the downregulation of genes related to nutrient uptake. Supplementation with an ethylene precursor, improved morpho-physiological traits and Fe status under combined stress, enhanced root flavonoid content, and increased rhizosphere siderophore levels and microbial community abundance. These findings underscore the potential of targeting ethylene signaling and microbial interactions to enhance soybean tolerance to concurrent Fe deficiency and drought. Future research should focus on these mechanisms to develop strategies that improve stress resilience and sustainability in soybean crops.

506 **Data availability statement**
507 Raw RNA-seq data was submitted to NCBI under the following accession number:
508 PRJNA1140285.
509
510 **Author contributions**
511 MRH cultivated the plants, conducted most of the experiments, performed RNA-seq analysis, and
512 prepared the draft manuscript. AT extracted the RNA from root samples. MGM helped with data
513 interpretations and critically revised the manuscript. AHK conceived the idea, supervised the
514 whole work, and revised the manuscript.
515
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519
520 **Conflict of Interest Statement**
521 We have no conflict of interest.
522
523 **References**
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Table 1. ICP-MS analysis of nutrients in the root and shoot of soybean grown in control and Fe-drought+ conditions. Different letters indicate significant differences between the two treatment groups at a P <0.05 level. The data represents the means ± SD of three independent biological samples (n = 3).

Elements	Root		Shoot	
	Control	Fe- Drought+	Control	Fe- Drought+
Fe (ppm)	460.8±32.5a	339.9±28.8b	95.3±8.6a	66.7±4.9b
Zn (ppm)	34.4±1.36a	34.2±1.64a	40.5±6.0a	28.6±3.1b
Mn (ppm)	24.1±4.0a	22.1±2.9a	49.2±8.5a	25.9±4.4b
N (%)	2.48±0.19a	1.77±0.19b	3.79±0.01a	3.45±0.07b
S (%)	0.23±0.02a	0.14±0.04b	0.25±0.01a	0.25±0.03a
K (%)	0.95±0.11a	0.70±0.05b	3.50±0.13a	3.51±0.21a
Ca (%)	0.64±0.06a	0.065±0.02a	1.02±0.17a	0.56±0.02b
Mg (%)	0.59±0.13a	0.40±0.07a	0.36±0.03a	0.40±0.04a

Table 2. The top 15 differentially expressed genes in roots of soybean exposed to combined Fe deficiency and drought relative to untreated controls. Values marked with an asterisk are significant (> Log2 fold, < P value 0.05).

Upregulated genes	Fe- Drought+ vs Control (fold change)
Glyma.04G089000 (MATE efflux family protein)	2.0
Glyma.20G202200 (Ethylene-response sensor 2)	2.1
Glyma.07G122500 (Copper ion transporter)	2.0
Glyma.15G180000 (Ethylene-responsive TF018)	2.5
Glyma.11G091400 (Heavy-metal-associated protein 26)	2.4
Glyma.13G087200 (Sulfate transporter 1.1)	2.1
Glyma.19G130800 (Ferredoxin)	2.4
Glyma.07G215500 (ABA-GTase)	4.8
Glyma.10G227400 (ABC transporter C family 10)	3.0
Glyma.13G088100 (Ethylene-responsive TF5)	3.1
Glyma.03G262600 (ATPase 1)	2.1
Glyma.14G071400 (BZIP transcription factor ATB2)	2.2
Glyma.02G261700 (Dehydration-responsive binding protein 2A)	3.0
Glyma.14G000800 (Calcium-dependent protein kinase 6)	2.4
Glyma.13G180400 (Glutaredoxin-3)	2.3
Downregulated genes	
Glyma.03G134200 (Calcium transporting ATPase 4)	-3.0
Glyma.17G203500 (Molybdate transporter 1)	-4.4
Glyma.03G212400 (heavy metal transport protein)	-2.6
Glyma.10G240300 (Peroxidase 66)	-4.1
Glyma.17G087500 (Heme-binding protein-related)	-3.1
Glyma.04G019500 (Multi-copper oxidase type I)	-2.7
Glyma.07G057900 (Jasmonic acid-amido synthetase JAR1)	-3.1
Glyma.13G162900 (Anion transporter 6)	-2.7
Glyma.07G006500 (Sulfate transporter 3.4)	-2.5
Glyma.10G172900 (Nicotianamine transporter YSL1)	-4.2
Glyma.13G038300 (Auxin efflux carrier component-6)	-3.4
Glyma.18G204800 (Ammonium transporter 2)	-3.0
Glyma.10G146600 (Solute carrier family 40)	-4.0
Glyma.13G315200 (Neoxanthin synthase)	-3.9
Glyma.05G211300 (Major facilitator family protein)	-4.0

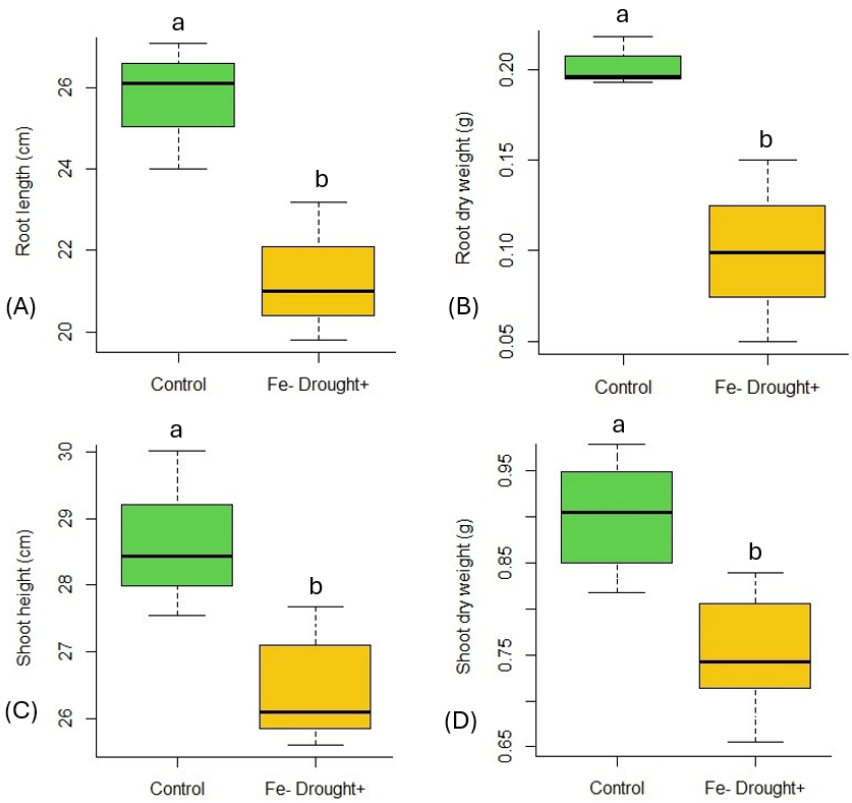

Figure 1. Changes in root length (A), root dry weight (B), shoot height (C), and shoot dry weight (D) in soybean in 'control' and 'Fe-drought+' conditions. Different letters above the bar indicate significant differences between treatment groups at a P <0.05 level. The data represents the means ± SD of three independent biological samples (*n* = 3).

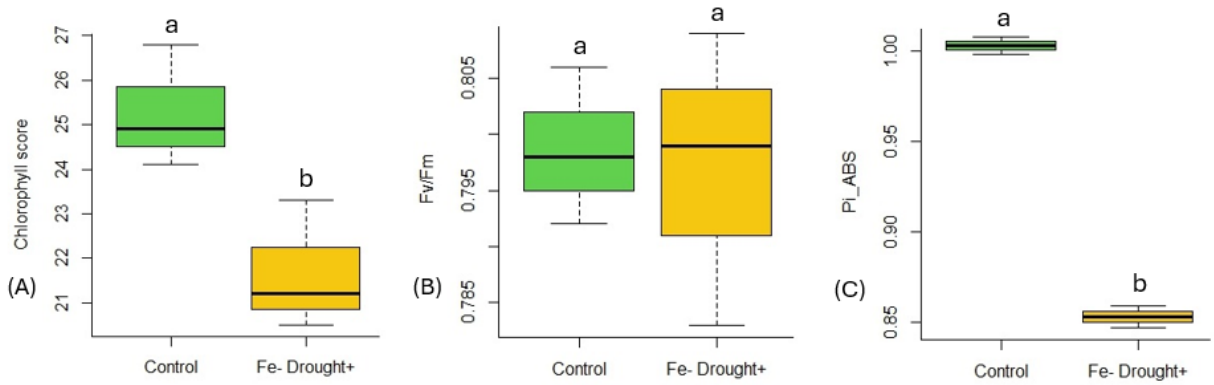

Figure 2. Changes in chlorophyll score (A), Fv/Fm: maximal quantum yield of PS II (B), and Pi_ABS: photosynthesis performance index (C) in the leaves of soybean in 'control' and 'Fe-drought+' conditions. Different letters above the bar indicate significant differences between treatment groups at a P <0.05 level. The data represents the means ± SD of three independent biological samples (*n* = 3).

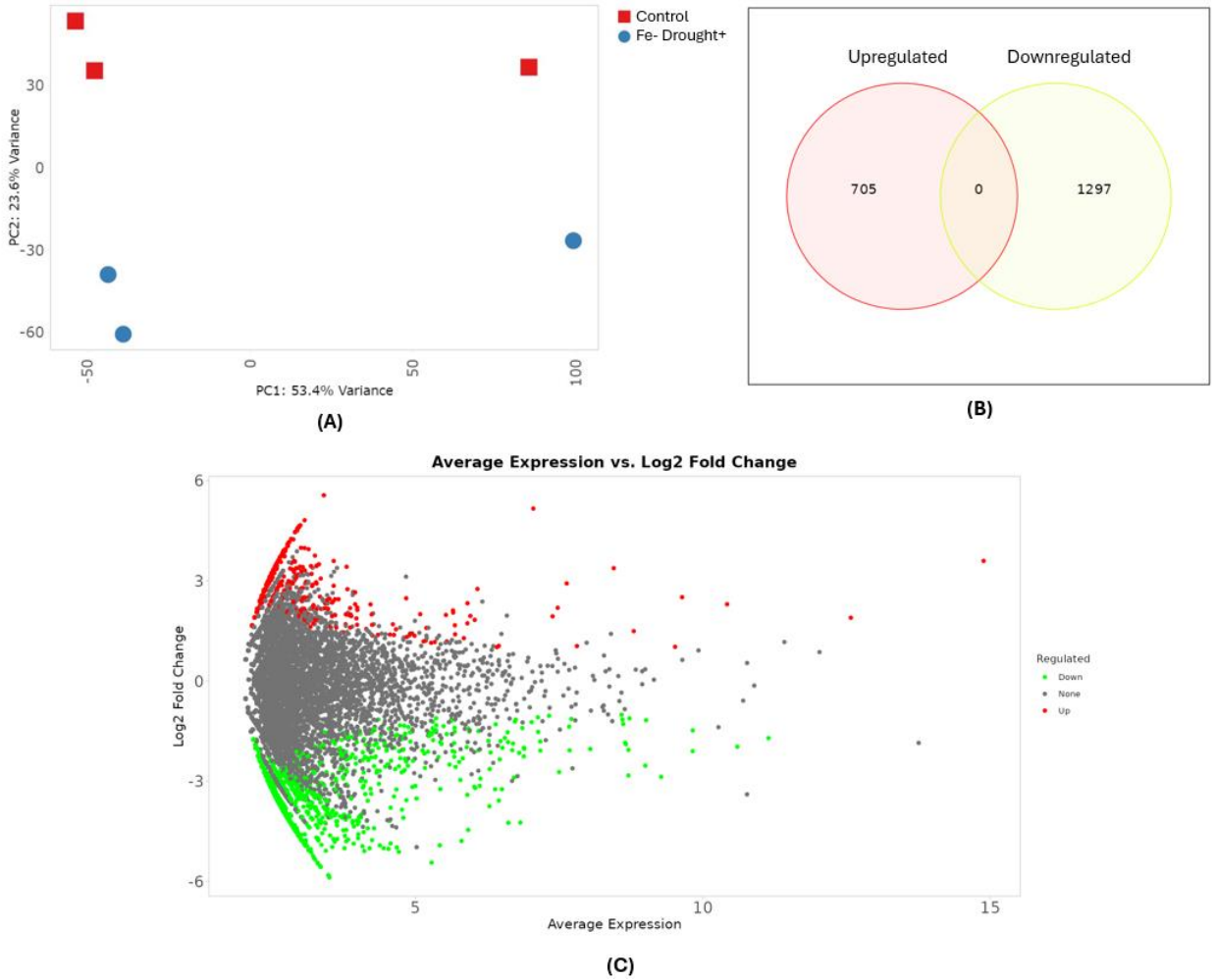

Figure 3. Analysis of RNA-sequencing data for differentially expressed genes (DEGs): principal component analysis of DEGs (A), vein diagram of upregulated and downregulated genes (>Log2 fold, $p<0.05$) grown in 'Fe-drought+' vs. 'control' (B) and MA plot of genes expressed in 'Fe-drought+' vs. 'control' conditions (C).

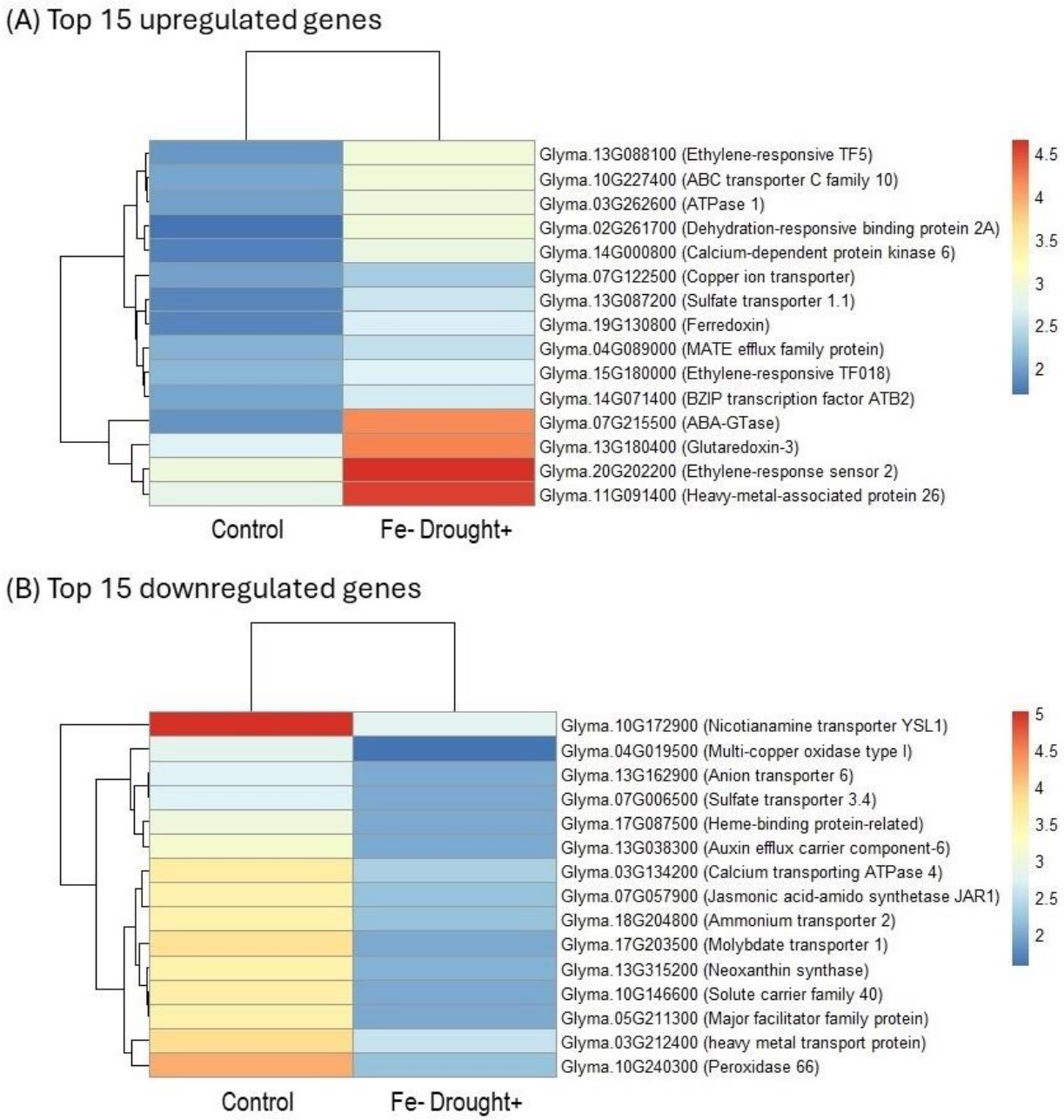

Figure 4. Heatmap of top 15 differentially expressed (>Log2 fold, $p<0.05$) upregulated (A) and downregulated (B) genes in roots of Fiskeby IV in 'Fe-drought+' vs 'control' conditions.

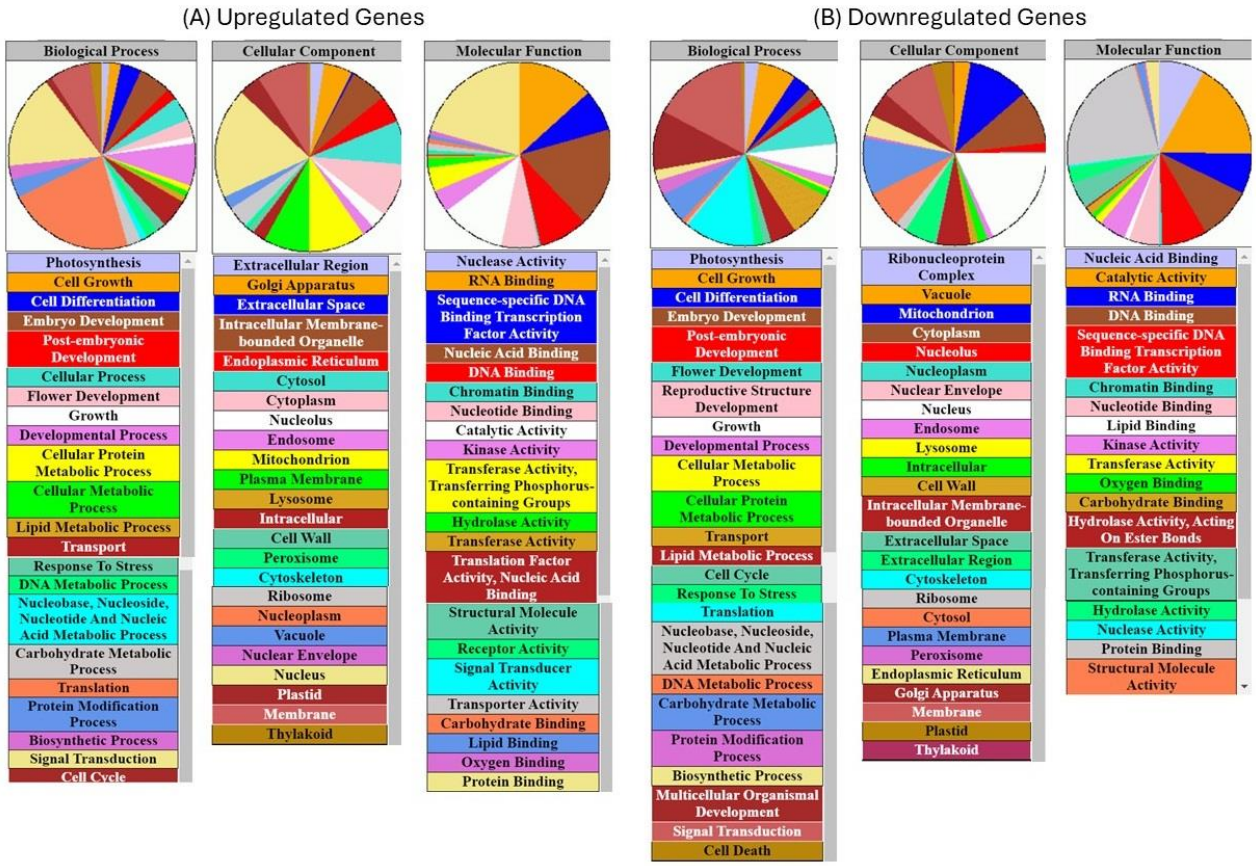

Figure 5. Gene enrichment analysis for the upregulated (A) and downregulated genes (B) in roots of Fiskeby IV cultivated in 'Fe-drought+' and 'control' conditions for biological processes, cellular components, and molecular functions.

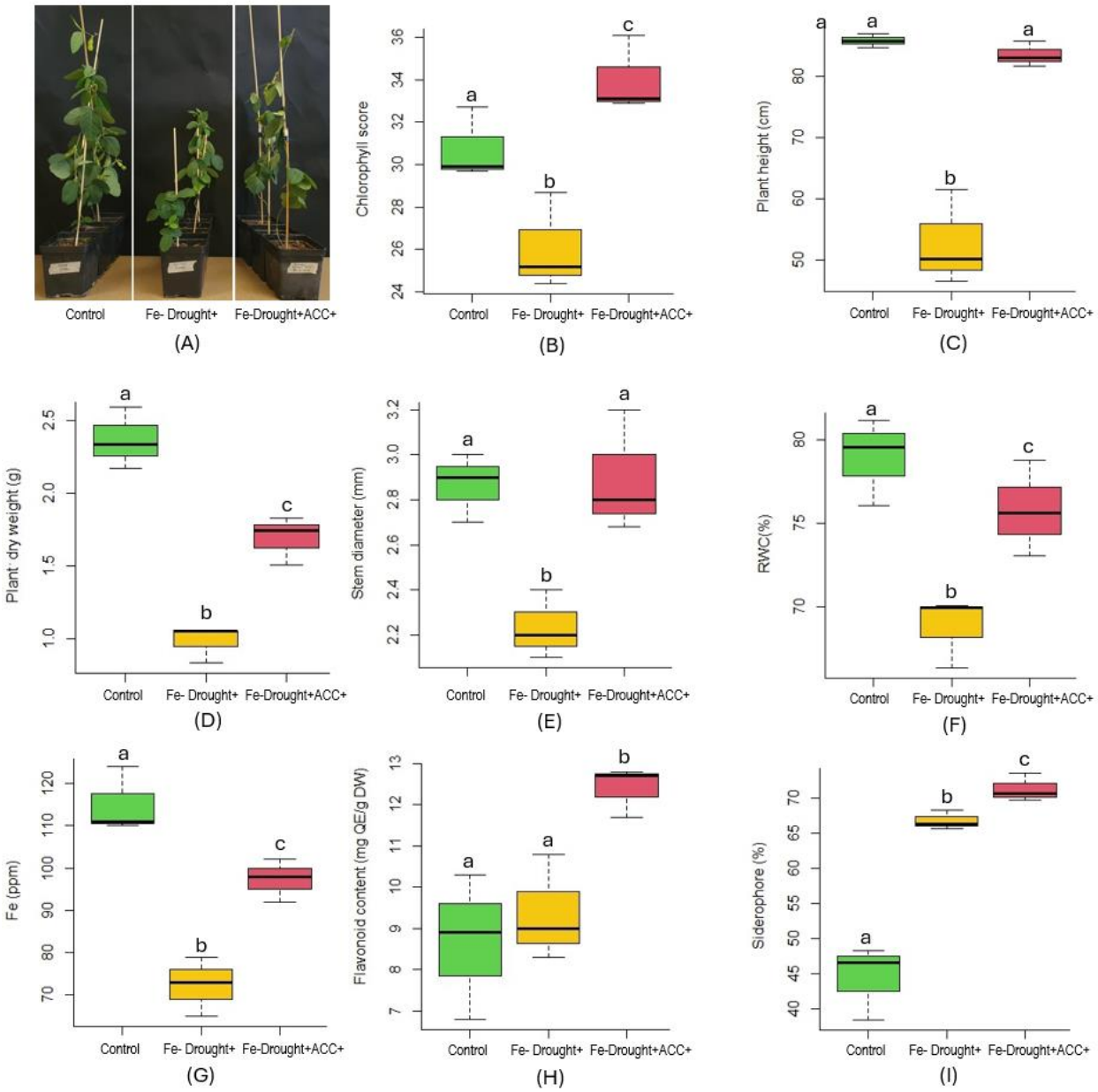

Figure 6. Effect of ACC on plant phenotype (A), chlorophyll score (B), plant height (C), plant dry weight (D), stem diameter (E), relative water content (F), leaf Fe (G), flavonoid content (H) and rhizosphere siderophore (I) in soybean plants cultivated with or without Fe-drought+ conditions. Different letters above the bar indicate significant differences between treatment groups at a P <0.05 level, where applicable. The data represents the means ± SD of three independent biological samples (n = 3).

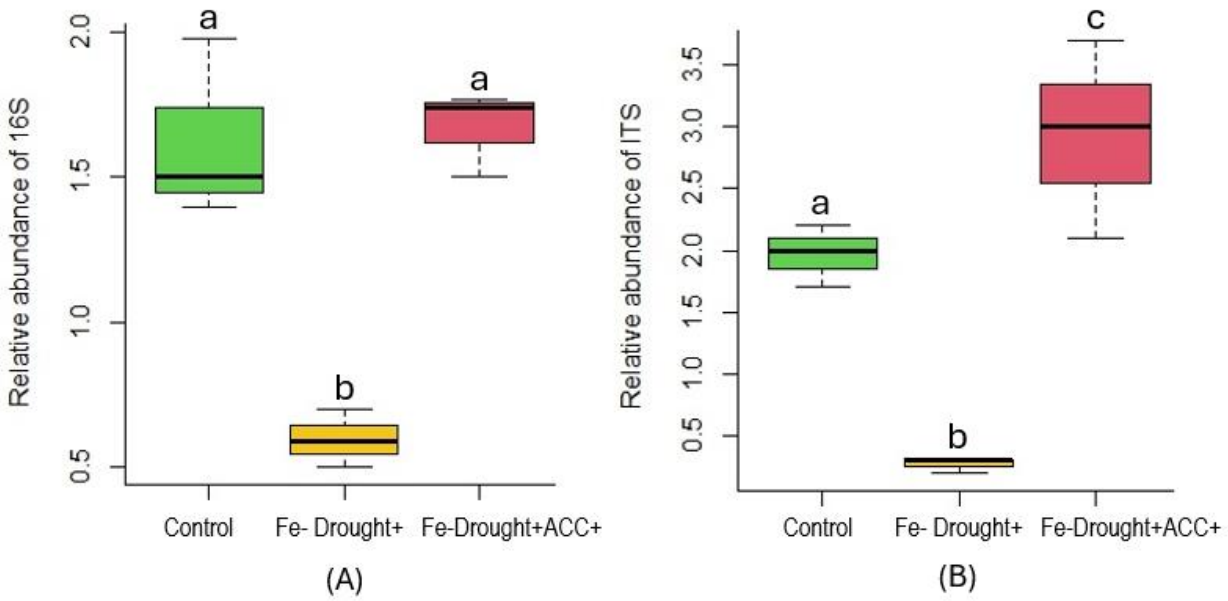

Figure 7. Effect of ACC on the relative abundance of 16S (bacteria) and ITS (fungi) communities in roots of Fiskeby IV exposed to combined Fe deficiency and drought. Different letters above the bar indicate significant differences between treatment groups at a $P < 0.05$ level, where applicable. The data represents the means ± SD of three independent biological samples ($n = 3$).

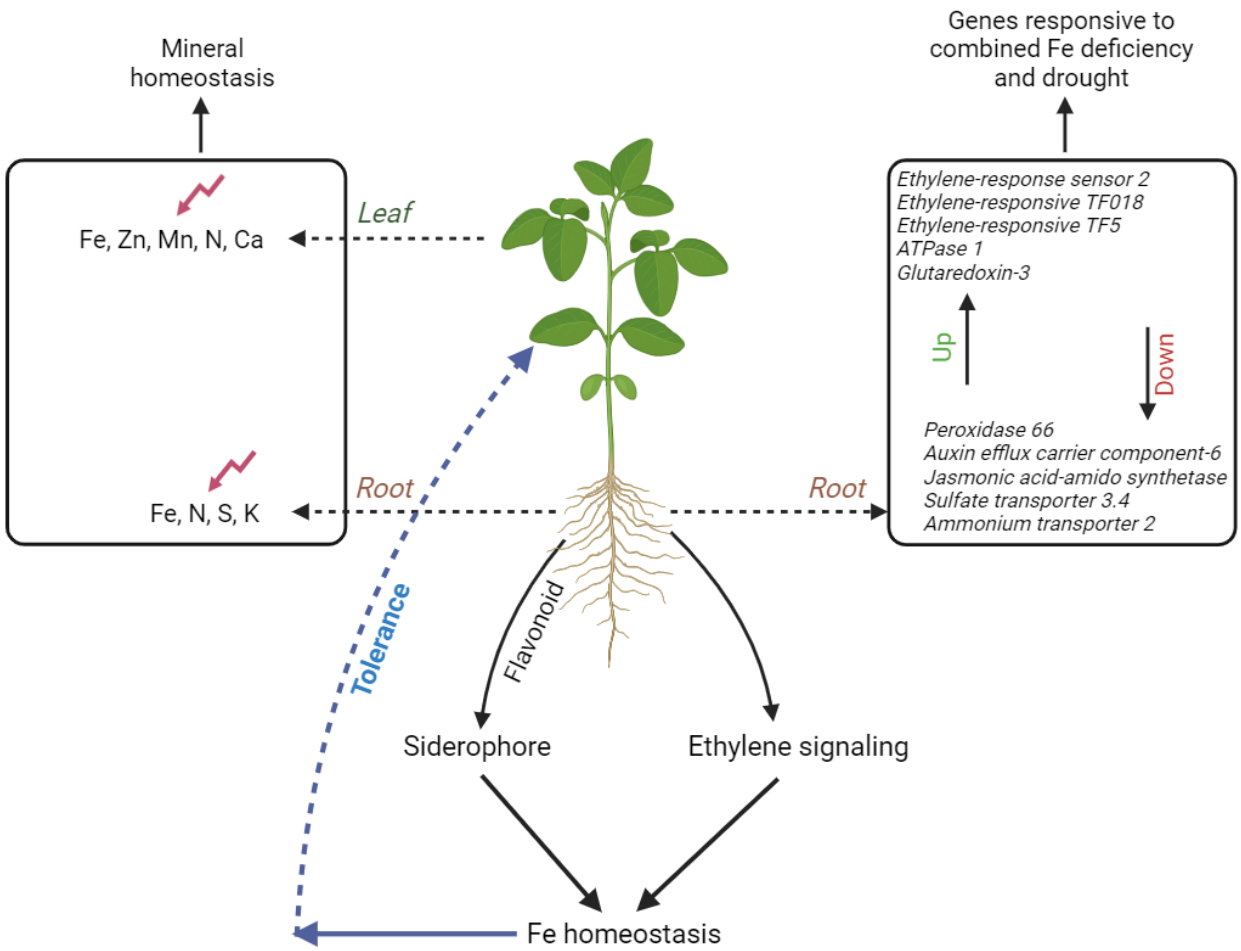

Figure 8. Physiological and transcriptional responses of soybean plants exposed to concurrent Fe deficiency and drought. The combined Fe deficiency and drought caused the upregulation of genes related to ethylene signaling (*Ethylene-response sensor 2*, *Ethylene-responsive TF018*, *Ethylene-responsive TF5*), proton extrusion (*ATPase 1*) and redox homeostasis (*Glutaredoxin-3*) while the expression of genes associated with peroxidase activity (*Peroxidase 66*), phytohormone synthesis (*Auxin efflux carrier component-6*, *Jasmonic acid-amido synthetase*) and nutrient transport (*Sulfate transporter 3.4*, *Ammonium transporter 2*) showed downregulation. 'Fe-Drought+' conditions caused a decline in mineral elements in both root and leaf. Interestingly, ethylene precursor (ACC) improved Fe homeostasis either directly or indirectly through root flavonoid and rhizosphere siderophore to induce drought tolerance.