Role of iron status and ethylene signaling in stress resilience of soybean exposed to combined iron deficiency and drought

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Abstract

Iron (Fe) deficiency in alkaline soils, exacerbated by drought, collectively affects soybean health. However, the availability of Fe and the transcriptional changes associated with drought tolerance remain unclear in soybean. This study aimed to evaluate the physiological and transcriptional changes in Fiskeby IV, a drought-tolerant genotype that loses its resilience under simultaneous Fe deficiency and drought. In this growth incubator study, Fe deficiency and drought stress led to significant 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, soybean plants supplemented with 1-aminocyclopropane-1-carboxylic acid (an ethylene precursor) showed significant improvements in morpho-physiological traits and Fe status under combined stress conditions. Furthermore, the ethylene precursor enhanced root flavonoid and rhizosphere siderophore levels, along with restoring the overall abundance of bacterial and fungal microbial cells under Fe deficiency and drought. It implies that elevated ethylene may modulate root exudates, enhance microbial recruitment, and promote a stress-resilient rhizosphere environment to help soybean plants cope with combined stresses. It underscores the role of targeting ethylene signaling that may facilitate Fe mobilization and microbial interactions to induce soybean tolerance to concurrent Fe deficiency and drought. This is the first report on the transcriptional response and Fe status requirement underlying drought tolerance in soybean, potentially guiding future strategies to improve combined stress resilience in legume crops.

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). The lack of soil moisture causes drought stress, 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 deficiency, can significantly alter these mechanisms, potentially negating the benefits of drought-tolerant plant species/cultivars (Afshar et al., 2012; Elanz et al., 2011). However, Fe bioavailability in soil is often limited, especially in alkaline or calcareous soils common to many agricultural regions (Kabir et al., 2012; 2015; Li and Lan, 2017). Fe deficiency is a common nutritional disorder affecting plants, characterized by impaired chlorophyll synthesis and reduced photosynthetic efficiency (Li and Lan, 2017). Furthermore, drought stress reduces soil moisture and restricts Fe solubility, further exacerbating Fe deficiency and negatively impacting crop yield (García et al., 2012). 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). Especially in legume crops, Fe is essential for nitrogen fixation and yield productivity (Briat et al., 2015). Therefore, understanding how Fe deficiency modulates drought response in plants is pivotal for devising strategies to enhance crop resilience under multifaceted stress environments.

In plants, two primary strategies are used to absorb Fe from the soil: Strategy I and Strategy II. Strategy I is utilized by non-graminaceous (non-grass) plants, such as dicots (e.g., soybean, pea) and most non-grass monocots (Liang, 2022). 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 (Kobayashi and Nishizawa, 2012; Morrissey and Guerinot, 2009). Plants also enhance the expression of Fe transporter proteins to facilitate Fe uptake into root cells (Kroh and Pilon, 2019). Furthermore, 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). In addition, microbes also play a crucial role in stress resilience in plants. 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).

In response to drought, plants possess a diverse array of adaptive strategies aimed at maintaining cellular hydration, sustaining metabolic processes, and minimizing damage during water deficit

conditions. These strategies include morphological adjustments such as root architecture modification to enhance water uptake and leaf rolling to reduce water loss, alongside physiological mechanisms like osmotic adjustment, where compatible solutes accumulate to protect cellular structures (Volaire, 2018; Kumar et al., 2021; Khan et al., 2023; D'Oria et al., 2022). On a molecular level, plants undergo dynamic changes involving the activation of stress-responsive genes and signaling pathways. Among these, abscisic acid (ABA) signaling plays a central role by coordinating responses to water stress; ABA mediates stomatal closure, conserves water and activates a cascade of stress-responsive genes to help the plant acclimate to drought conditions (Chaves et al., 2003; Ali et al., 2020). Additionally, ethylene contributes significantly to drought tolerance, not only by regulating drought-responsive genes but also by influencing cell wall remodeling and antioxidant responses, which are essential for minimizing oxidative damage under stress (Fatma et al., 2022). Ethylene's role is further highlighted in conditions of Fe deficiency, where it activates pathways that modify root architecture to improve nutrient acquisition, underscoring its multifaceted involvement in stress tolerance mechanisms (Sánchez-Rodríguez et al., 2011; Barnaby et al., 2019).

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. While the individual roles of Fe deficiency and drought stress in limiting plant growth are well-documented, the interplay between Fe availability and drought tolerance remains underexplored. Particularly, the modulation of transcriptional changes and 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 examined the role of ethylene signaling and potential crosstalk with other hormones, providing insights into adaptive responses that may improve soybean resilience in response to combined 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 soybean 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 the length of the longest root were measured using a digital caliper. The shoots and roots were then dried for three days at 75°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 75ºC. 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 collected from 6-week-old plants. 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 ShinyGO 0.81 (Ge et al., 2020).

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-min incubation, 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). Briefly, the rhizosphere soil was collected by washing off the soil tightly attached to the root surface. The rhizosphere soil was then 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 cells

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 16S bacteria (16S-341_F: CCTACGGGNGGCWGCAG, 16S-341_R: GACTACHVGGGTATCTAATCC) and ITS fungi

(ITS1-F: TCCGTAGGTGAACCTGCGG, ITS4-R: TCCTCCGCTTATTGATATGC). The soybean *Beta-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 using Student's *t*-test and one-way ANOVA, followed by Duncan's Multiple Range Test (DMRT) where applicable. Graphical presentations were prepared using ggplot2 in the R program.

3 Results

3.1 Changes in morphological traits and photosynthetic features

We measured the morphological and photosynthetic attributes to study the responses of plants exposed to combined iron deficiency and drought. 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 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 separation along PC1 and PC2 indicates distinct grouping patterns among the samples, suggesting that treatment groups led to substantial differences in gene expression. 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). 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).

3.5 Gene enrichment analysis

Gene enrichment analysis revealed the enrichment of several pathways, categorized into three groups: biological processes and molecular functions (Figure 5A-5B). The upregulated genes showed significant enrichment in major biological processes, including sequestering of actin monomers, cell redox homeostasis, negative regulation of actin filament polymerization, cellular homeostasis, and the oxidation-reduction process (Figure 5A). Among the molecular functions, oxidoreductase activity exhibited the highest fold enrichment, followed by actin monomer binding, protein disulfide isomerase activity, phosphorelay sensor kinase activity, and disulfide oxidoreductase activity (Figure 5A). Conversely, the top enriched biological processes among downregulated genes included defense response to insects, which showed the highest fold enrichment, followed by regulation of vacuole fusion, DNA replication, and cell redox homeostasis (Figure 5B). Additionally, the top enriched molecular functions among downregulated genes included beta-galactosidase activity, beta-amylase activity, galactosidase activity, amylopectin maltohydrolase activity, and oxidoreductase activity (Figure 5B).

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 (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 cells 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 abundance of ITS fungal cells 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.

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 the drought-tolerant soybean genotype. 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 non-photochemical quenching helps dissipate excess light energy as heat to prevent damage to PSII reaction centers (Ruban et al., 2016; Demmig-Adams et al., 2014). Furthermore, enhanced scavenging of reactive oxygen species (ROS) may also contribute to the stability of PSII by neutralizing ROS generated under stress (Foyer & Noctor, 2011). We observed the upregulation of glutaredoxin-3 in the roots of soybean. Glutaredoxin-3 is involved in the assembly and maintenance of Fe-S clusters, which are crucial for the function of many enzymes, including those involved in ROS detoxification and other metabolic processes (Couturier et al., 2015).

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. Also, the decline in these nutrients in the roots indicates a potential trade-off between root and shoot nutrient allocation to mitigate stress effects. However, 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 (Ren et al., 2022; Künstler et al., 2020). Furthermore, potassium regulates stomatal exposure, helping to reduce water loss through transpiration and maintain turgor pressure under drought conditions (Wang et al., 2013). K also activates numerous enzymes, including those involved in photosynthesis, protein synthesis, and carbohydrate metabolism, helping plants sustain metabolic functions under stress (Ho et al., 2022; Jin et al., 2011). Additionally, K is critical for maintaining ion homeostasis and enhancing the plant’s ability to uptake other essential nutrients (Lian et al., 2023). Hence, the retention of K levels in the shoot may therefore reflect the soybean plants’ attempt to optimize water usage and nutrient uptake under combined Fe deficiency and drought stress. Furthermore, the retention of sulfur levels in the shoot aligns with the gene enrichment in oxidoreductase activity, indicating a significant role in redox reactions and sulfur metabolism in soybeans exposed to the combined effects of Fe deficiency and drought. Several studies demonstrated that redox reactions and sulfur metabolism play a critical role in responses to abiotic stress plants (Mittler, 2002; Kopriva & Rennenberg, 2004).

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. In Strategy I plants, *ATPase 1*, a plasma membrane proton pump, plays a significant role in Fe uptake (Zhang et al., 2017). *ATPase 1* contributes to this by creating a proton gradient across the root plasma membrane, which is essential for the acidification of the rhizosphere (Michalak et al., 2022). This acidification helps solubilize Fe^{3+} making it more accessible for uptake by the plant (Kabir et al., 2012). Thus, the upregulation of *ATPase 1* in roots suggests an enhanced effort to regulate rhizosphere acidification which helps plants mobilize Fe under combined Fe deficiency and drought in soybean. In addition, the induction of ferredoxin among the upregulated genes also points to the importance of redox homeostasis in stress adaptation. Ferredoxin is involved in maintaining cellular redox homeostasis by ROS, particularly under drought, salinity, and high light-intensity stresses in plants (Huang et al., 2020; Lehtimäki et al., 2010). Our gene enrichment analysis also indicates an upregulation of cell redox homeostasis, highlighting the importance of redox regulation required for soybean plants to cope with combined Fe deficiency and drought. Furthermore, soybean plants exposed to concurrent Fe deficiency and drought showed upregulation of the *ABA-Gtase* gene. It serves as an ABA receptor and is also a key component of the ABA signaling pathway in plants (Xu et al., 2002). Although ABA is a well-known stress hormone, particularly involved in drought responses, it can also induce the

expression of Fe-deficiency-responsive genes, suggesting its role in regulating Fe uptake and transport under Fe-limiting conditions (Lei et al., 2014; García et al., 2011). This crosstalk between ABA and Fe deficiency is particularly important in drought conditions, where the plant's need for Fe might increase due to compromised root functionality.

Interestingly, we found the upregulation of several ethylene-responsive genes, such as ethylene-responsive transcription factors (TF5 and TF018) and dehydration-responsive binding protein 2A, in response to combined Fe deficiency and drought in soybean. This may imply the activation of stress-related signaling pathways and the role of ethylene signaling in soybean's adaptive response to combined Fe deficiency and drought. Under Fe deficiency, ethylene-responsive genes are induced to enhance the plant's ability to acquire and utilize Fe and other nutrients efficiently (Müller and Munné-Bosch, 2015; Li and Lan, 2017; Wu et al., 2011). This is correlated with the upregulation of sulfate transporter 1.1, heavy-metal-associated protein 26, and copper ion transporter, indicating their efforts in maintaining cellular ion balance under stress conditions. Interestingly, ethylene may interact with Fe deficiency signaling pathways to enhance ATPase 1 activity (Waters et al., 2007), a finding that aligns with our RNA-seq results. Additionally, ethylene biosynthesis may induce drought-responsive genes that help plants conserve water and maintain cellular homeostasis (Mohorović et al., 2023; Arraes et al., 2015). Naing et al., (2022) showed that ethylene-insensitive mutants exhibited increased sensitivity to drought stress, indicating that ethylene-responsive genes contribute to drought adaptation by modulating stomatal conductance and enhancing root growth. Ethylene signaling may facilitate ROS scavenging enzymes, helping mitigate oxidative stress, which is common under both Fe deficiency and drought conditions (Zhang et al., 2016; Kabir et al., 2012), and aligns with the upregulation of glutaredoxin-3 in the roots of soybean. In most cases, ethylene, whose production increases under nutrient deficiency in genotypically efficient genotypes, acts as an activator of stress responses in plants (Dubois et al., 2018). Precious studies indicating the upregulation of *1-aminocyclopropane-1-carboxylic acid synthase (ACS)*, and *1-aminocyclopropane-1-carboxylic acid oxidade* genes involved in ethylene synthesis further support the increased ethylene production as part of the adaptive responses under Fe deficiency in plants (García et al., 2015; Sauter et al., 2013). Thus, it appears that ethylene-related or Fe-uptake genes could be potential targets for improving stress tolerance in soybean exposed to Fe deficiency and drought.

To further elucidate the role of ethylene, we conducted a targeted study using an ethylene precursor (ACC) to determine if it could eliminate the impairment in drought-tolerant Fiskeby IV exposed to combined Fe deficiency and drought. In this study, supplementation with ACC led to significant improvements in these morpho-physiological traits, suggesting that ethylene signaling helps restore key growth parameters along with leaf Fe status disrupted by combined stress conditions. Interestingly, we observed a correlation between root flavonoid content and rhizosphere siderophore levels induced by the presence of an ACC, further underscoring the significance of microbial associations in soybean subjected to combined Fe deficiency and 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). In this study, the decline in microbial cells due to Fe deficiency and drought could be attributed to the combined stress effects on soil and plant environments. Fe deficiency reduces the availability of essential nutrients and impacts plant root exudation, which serves as a primary energy source for microbes (Chen and Liu, 2024; Pascale et al., 2020). Additionally, drought conditions limit soil moisture, further restricting microbial activity and growth due to reduced mobility and access to nutrients. Furthermore, drought conditions may limit soil moisture, further restricting microbial activity and growth due to reduced mobility and access to nutrients (Qu et al., 2024). Interestingly, we observed the restoration of the overall 16S bacterial and ITS fungal abundance when ACC was augmented in soybeans exposed to dual stresses, compared to plants exposed to Fe-drought+ conditions. 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. Previous studies demonstrated that 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). Also, plants exposed to abiotic stresses often produce elevated levels of ethylene to influence root architecture and the composition of root exudates (Glick, 2014). These exudates, which include organic acids, amino acids, and secondary metabolites, act as signaling molecules and attractants for beneficial microbes, such as rhizobacteria and mycorrhizal fungi (Vacheron et al., 2013). Thus, our findings imply that the induction of ethylene-responsive genes may act as a central regulator, integrating signals from both Fe deficiency and drought stress to orchestrate an adaptive response in soybean. 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.

Our RNA-seq analysis exhibited that several downregulated genes are associated with various physiological processes, including nutrient transport, hormone signaling, and stress responses due to combined Fe deficiency and drought. Fe deficiency and/or drought can cause significant nutritional imbalances in plants, disrupting essential metabolic processes and leading to stunted growth and poor health (Kumari et al., 2022). In this study, the downregulation of nicotianamine transporter *YSL1* and anion transporter 6 reflects a possible disruption in nutrient uptake and translocation in soybean (Fiskeby IV) exposed to Fe deficiency and drought. Furthermore, the decreased expression of jasmonic acid-amido synthetase *JAR1* and auxin efflux carrier component 6 suggests an alteration in hormonal balance, which could be one of the factors disrupting the drought tolerance ability of the genotype Fiskeby IV to combat dual stresses on soybean. Decreased *JAR1* expression can impair drought resilience by weakening the jasmonic

acid signaling pathways that regulate stomatal closure and stress-responsive gene activation (Ali et al., 2020). Another study demonstrates that over-expression of *JAR1)* enhances drought tolerance in *Arabidopsis thaliana* by increasing jasmonyl-L-isoleucine production, which leads to better water retention (Mahmud et al., 2022). Also, JA has been shown to promote root hair development, which helps increase the plant's ability to acquire Fe (Kobayashi et al., 2016. Kobayashi and Nishizawa, 2012). Although Fe is not a direct cofactor for the enzymes specific to jasmonic acid signaling, it is crucial for the biosynthesis of JA, as it is required for the functioning of lipoxygenases and other enzymes that convert fatty acids into jasmonic acid (Singh et al., 2022; Oliw 2022). In contrast, jasmonate-deficient *cpm2* mutant revealed that jasmonates inhibit the expression of several Fe deficiency-inducible genes associated with Feuptake in rice (Kobayashi et al., 2016). Particularly, auxin plays a critical role in mediating adaptive responses to improve Fe acquisition and drought tolerance in plants (Liu et al., 2024; Iqbal et al., 2022; Kabir et al., 2012). In this study, we also found the downregulation of neoxanthin synthase and solute carrier family 40 highlights disruptions in carotenoid biosynthesis and solute transport, which is consistent with the decreased photosynthetic efficiency and nutrient uptake in Fiskeby IV exposed to concurrent Fe deficiency and drought. Under abiotic stress, such as drought or nutrient deficiency, plants often experience disruptions in chlorophyll and carotenoid biosynthesis resulting in decreased energy production and plant growth (Muhammad et al., 2021; Dong et al., 2007). Carotenoid synthesis may also be affected, impacting plant protection against oxidative damage and influencing overall stress tolerance (Giani and Martínez-Espinosa 2020). Thus, phytohormone and other downregulated genes can be targeted for genetic engineering or breeding strategies aimed at enhancing stress resilience in soybean.

5 Conclusion

Fe deficiency, intensified by drought in alkaline soils, compromises soybean health by reducing biomass, photosynthetic efficiency, and nutrient uptake. Despite these stresses, Fiskeby IV maintained stable photosystem II efficiency, indicating protective adaptations. RNA-seq revealed upregulation of ethylene-responsive genes, rhizosphere acidification, and redox homeostasis factors, suggesting these mechanisms aid in coordinating Fe and drought resilience (Figure 8). Conversely, key nutrient transporters were downregulated under combined stress. Supplementation with an ethylene precursor improved physiological traits, Fe status, root flavonoid, and siderophore levels. 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 abundance. These findings reveal 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.

Data availability statement

Raw RNA-seq data was submitted to NCBI under the following accession number: PRJNA1140285.

Author contributions

MRH cultivated the plants, conducted most of the experiments, performed RNA-seq analysis, and prepared the draft manuscript. AT extracted RNA from root samples and assisted with plant phenotyping. MGM helped with data interpretations and critically revised the manuscript. AHK conceived the idea, supervised the whole work, and revised the manuscript.

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Conflict of Interest Statement

We have no conflict of interest.

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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 statistically significant differences between the treatment groups, as determined by a *t*-test at the $P < 0.05$ 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	0.65±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

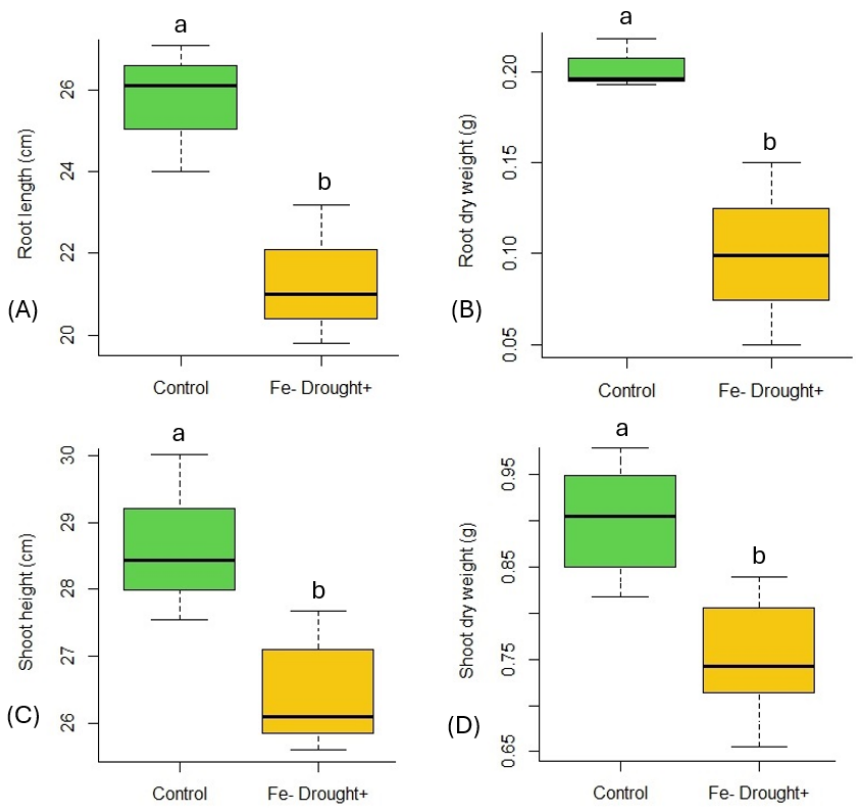

Figure 1. Changes in root length (A), root dry weight (B), shoot height (C), and shoot dry weight (D) in soybean in 'control' and 'Fe-drought+' conditions. Different letters above the bar indicate significant differences by a *t*-test between treatment groups at a P <0.05 level. The data represents the means ± SD of three independent biological samples (n = 3).

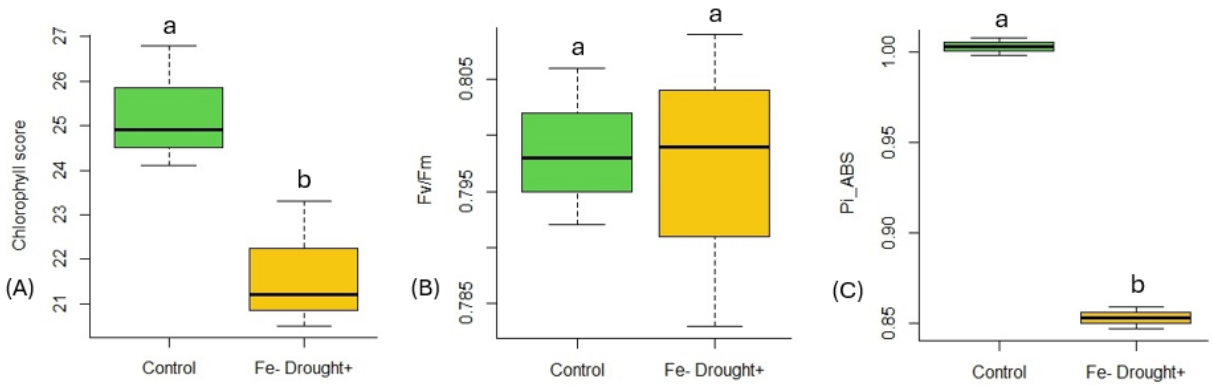

Figure 2. Changes in chlorophyll score (A), Fv/Fm: maximal quantum yield of PS II (B), and Pi_ABS: photosynthesis performance index (C) in the leaves of soybean in 'control' and 'Fe-drought+' conditions. Different letters above the bar indicate significant differences by a *t*-test between treatment groups at a P <0.05 level. The data represents the means ± SD of three independent biological samples (n = 3).

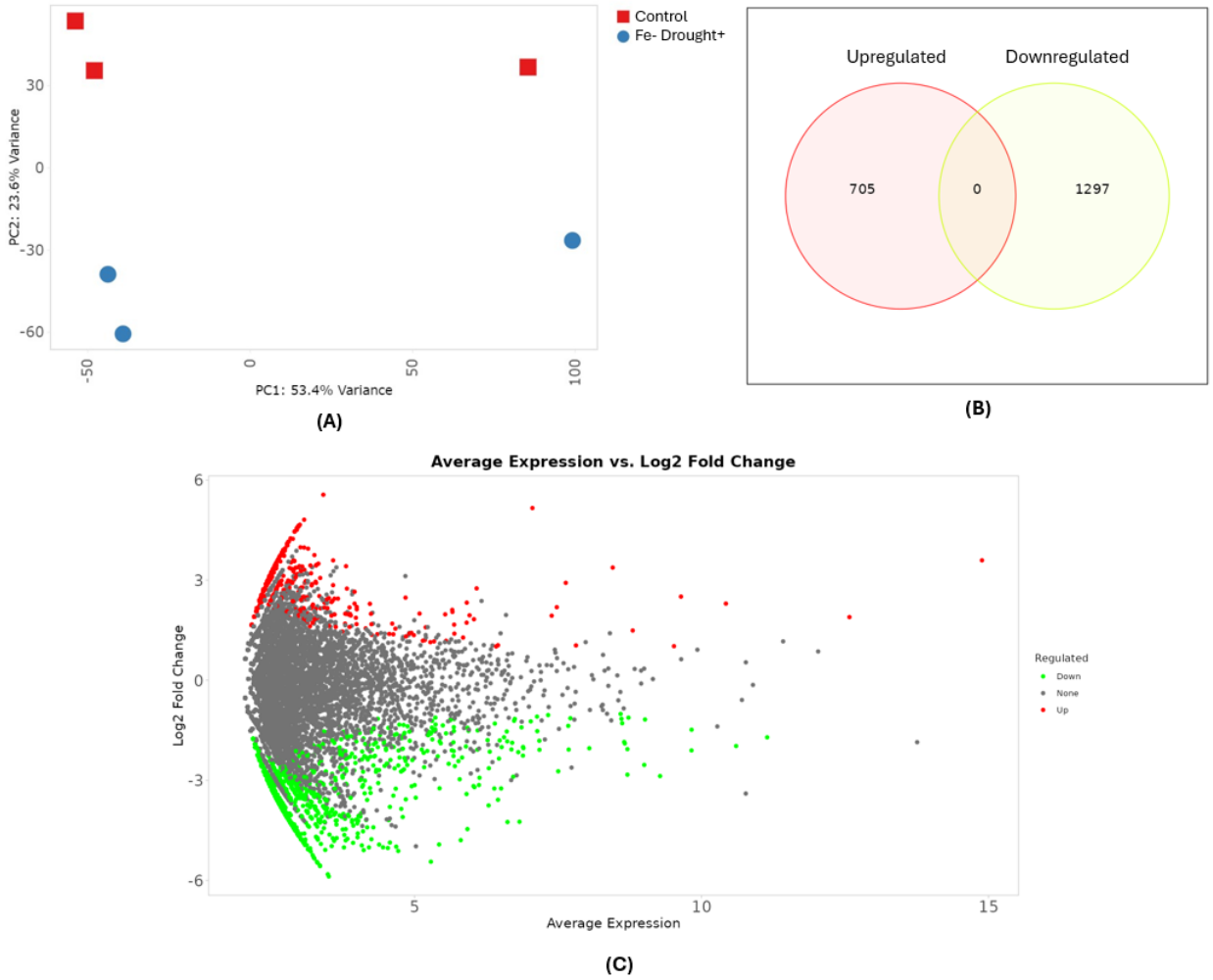

Figure 3. Analysis of RNA-sequencing data for differentially expressed genes (DEGs): principal component analysis of DEGs (A), vein diagram of upregulated and downregulated genes (>Log2 fold, P < 0.05) grown in 'Fe-drought+' vs. 'control' (B) and MA plot of genes expressed in 'Fe-drought+' vs. 'control'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

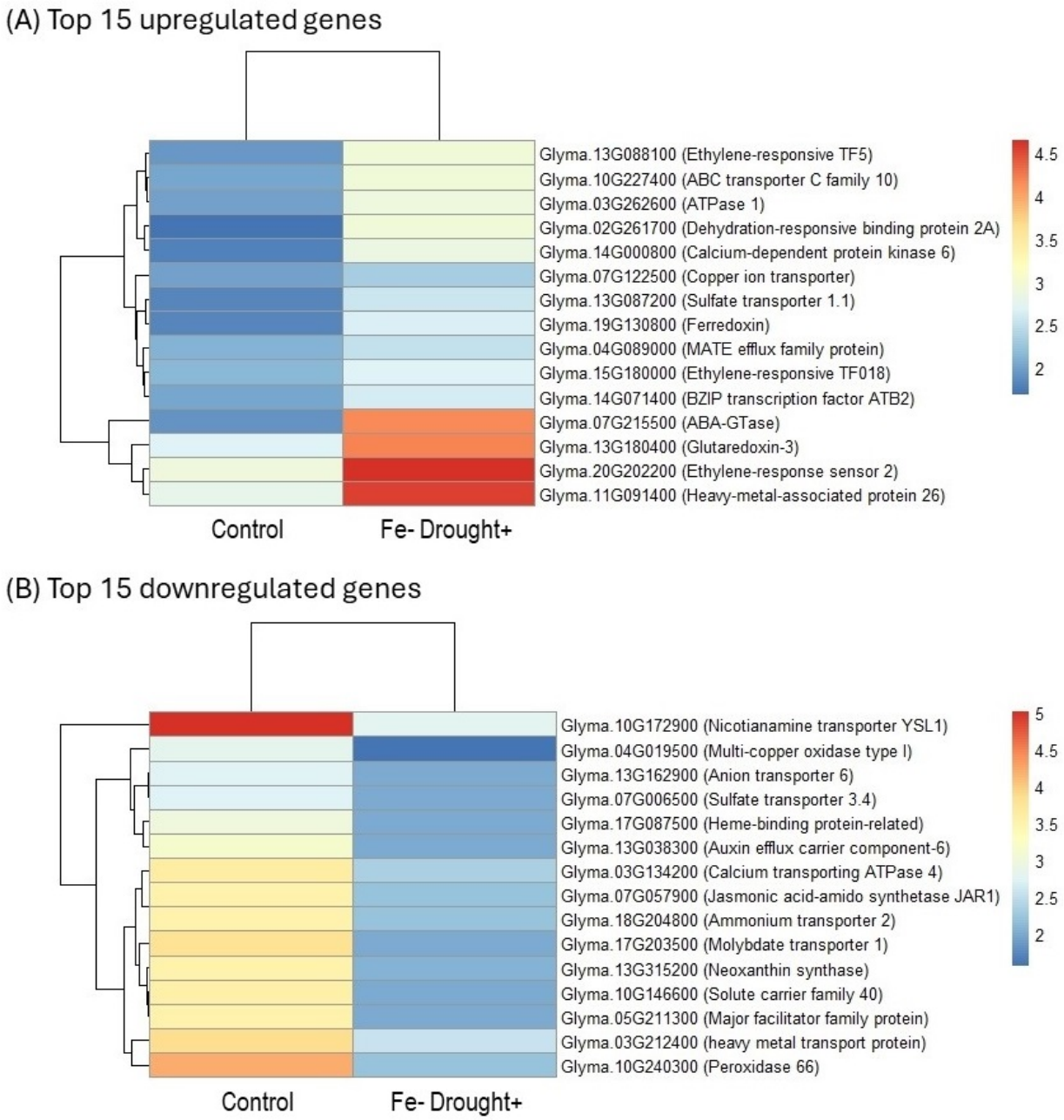

Figure 4. Heatmap of the top 15 differentially expressed genes (Log2 fold change > 1, $P < 0.05$) in the roots of Fiskeby IV exposed to combined Fe deficiency and drought relative to untreated controls. (A) Top 15 upregulated genes. (B) Top 15 downregulated genes.

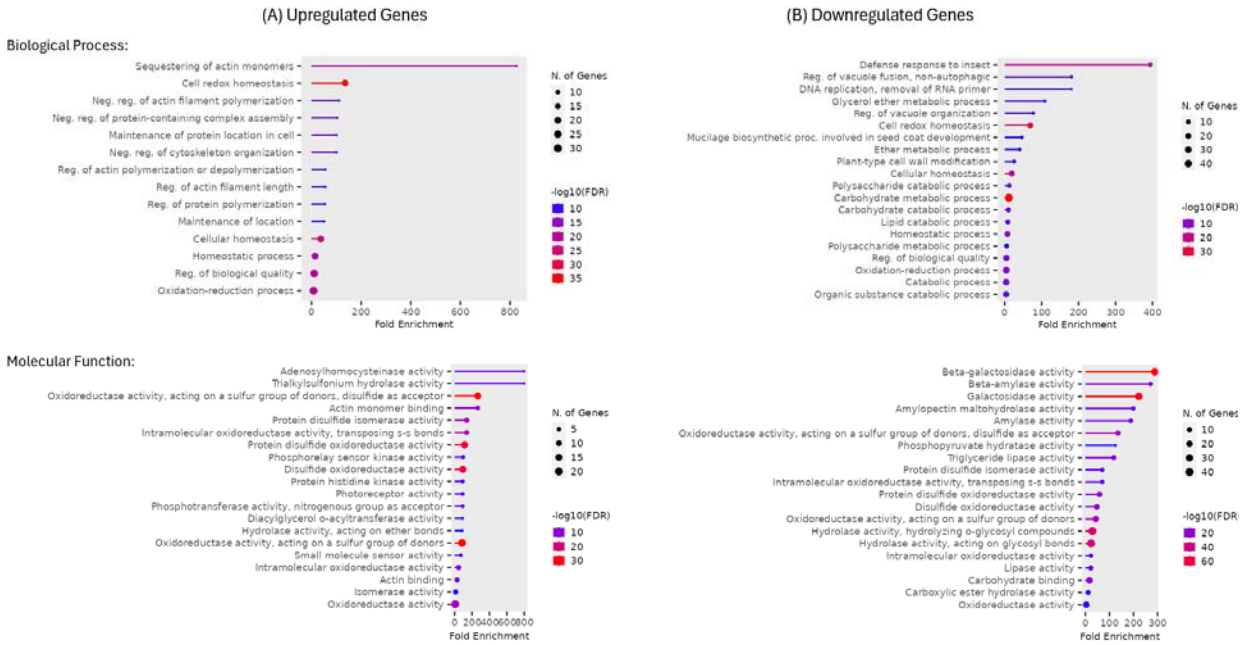

Figure 5. Gene enrichment analysis for the upregulated (A) and downregulated genes (B) in roots of Fiskeby IV cultivated in 'Fe-drought+' and 'control' conditions for biological processes, and molecular functions. The size and color of the data points indicate the number of genes involved and the significance level, respectively, with larger points and deeper colors representing more substantial enrichment and higher significance.

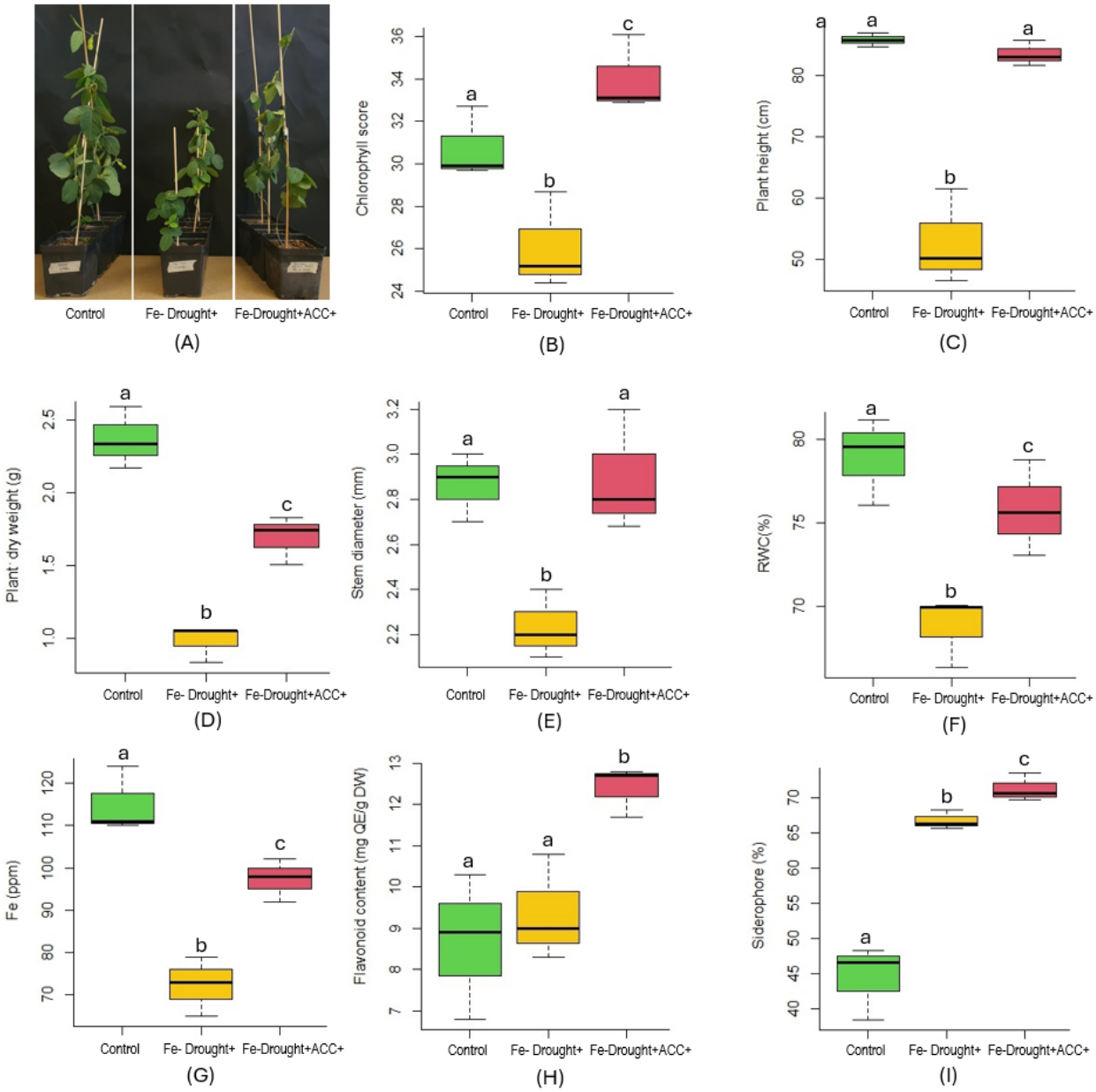

Figure 6. Effect of ACC on plant phenotype (A), chlorophyll score (B), plant height (C), plant dry weight (D), stem diameter (E), relative water content (F), leaf Fe (G), flavonoid content (H) and rhizosphere siderophore (I) in soybean plants cultivated with or without Fe-drought+ conditions. Different 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).

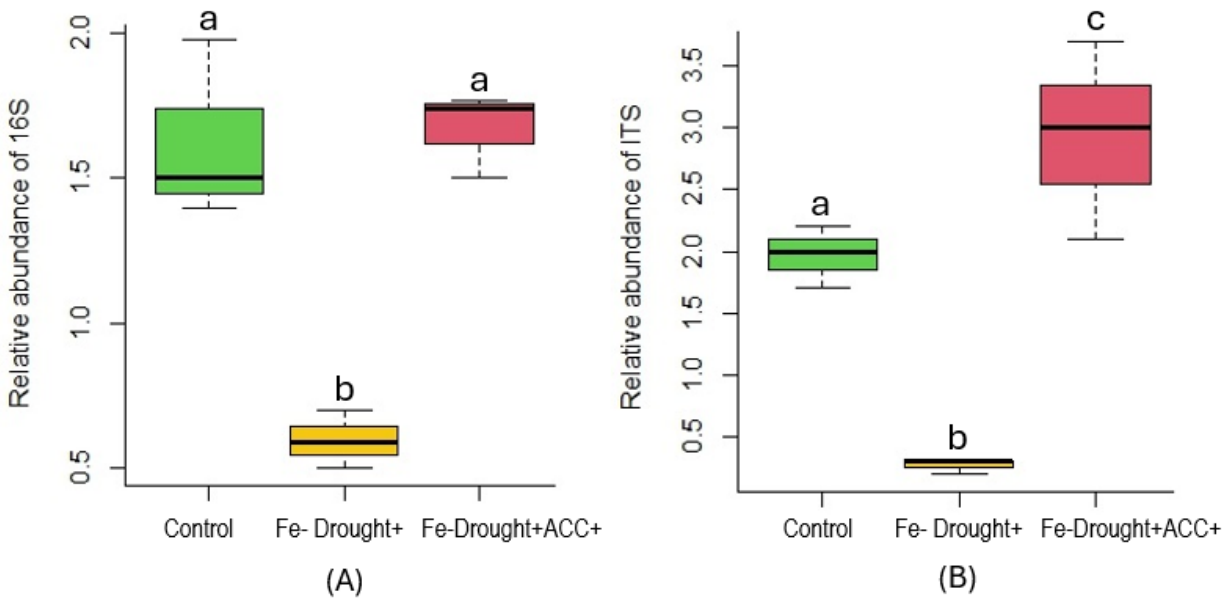

Figure 7. Effect of ACC on the relative abundance of 16S (bacteria) and ITS (fungi) cells in roots of Fiskeby IV exposed to combined Fe deficiency and drought. Different letters above the bar indicate significant differences between treatment groups at a P <0.05 level, where applicable. The data represents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

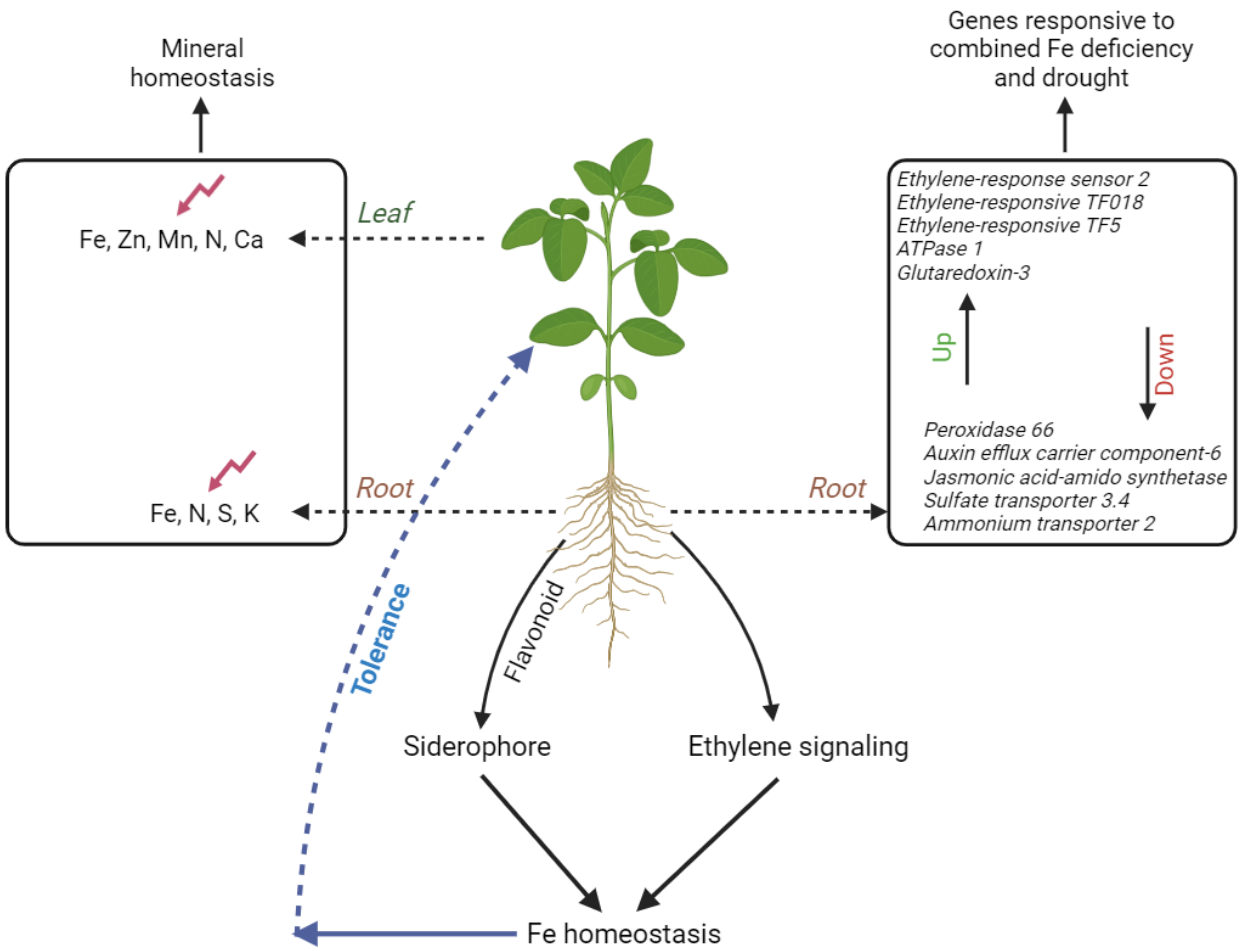

Figure 8. Physiological and transcriptional responses of soybean plants exposed to concurrent Fe deficiency and drought. The combined Fe deficiency and drought caused the upregulation of genes related to ethylene signaling (*Ethylene-response sensor 2*, *Ethylene-responsive TF018*, *Ethylene-responsive 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 leading to drought resilience either directly or indirectly through root flavonoid and rhizosphere siderophore to induce drought tolerance.