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PHYSIOLOGICAL, MOLECULAR, AND MICROBIAL INTERACTIONS IN DROUGHT TOLERANT LEGUMES STRATEGIES FOR RESILIENT AGRICULTURE

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ABSTRACT

Drought stress substantially restricts legume production, especially in dry and semi-arid areas. This research aims to investigate the physiological, molecular, and microbiological responses of drought-tolerant legumes Cicer arietinum, Vigna radiata, and Phaseolus vulgaris under regulated drought circumstances. A Randomized Complete Block Design (RCBD) was used, including two treatments: a well-watered control and drought stress applied during the blooming stage. Physiological measurements like relative water content (RWC), how well the stomata are working, chlorophyll fluorescence, and the buildup of osmolytes (proline and soluble sugars) were checked at three times: before the drought stress, during the drought stress, and after the plants recovered. RNA was taken from leaf tissues to evaluate drought-related gene expression via qRT-PCR, focusing on DREB, NAC, and aquaporin genes. Furthermore, rhizosphere soil samples were examined by 16S rRNA gene sequencing for assessing microbial community composition, while mycorrhizal colonization was measured microscopically. The results are expected to reveal important features and molecular signs related to drought tolerance, while also highlighting how helpful bacteria contribute to resisting stress. This integrated method offers a thorough framework for discovering drought adaptation mechanisms in legumes, with consequences for breeding and sustainable agriculture in arid regions.

Keywords: *Drought stress, legumes, Cicer arietinum, Vigna radiata, Phaseolus vulgaris, physiological responses, DREB, NAC, aquaporins, osmolytes, rhizosphere microbiota, mycorrhizal colonization, qRT-PCR, 16S rRNA sequencing, sustainable agriculture.*

INTRODUCTION

Drought stress is an important constraint on legume productivity globally, especially in light of the ongoing climate change trajectory characterized by severe temperatures and erratic precipitation cycles. [1] Legumes are crucial to global food security and sustainable agriculture because of their high protein content, nitrogen-fixing capabilities that improve soil, and adaptability to marginal areas. Nonetheless, their susceptibility to water deficiency, especially during the blooming and pod-filling phases, renders them susceptible in dry and semi-arid areas). [2] Plants, especially legumes, have many physiological responses

to mitigate drought, including stomatal control, osmolyte accumulation, and antioxidant activation. These systems aid in reducing water loss, preserving cell turgor, and preserving against oxidative damage. Enzymes such as superoxide dismutase (SOD) and catalase (CAT) are essential for diminishing reactive oxygen species (ROS), [3] thereby maintaining cellular integrity under stress Drought tolerance at the molecular level is controlled by genes and proteins that respond to stress, like DREB, MYB, and NAC, which trigger protective actions such as making dehydrins and aquaporin.[4] Plant and microbe interactions, especially with helpful bacteria and fungi, are very important because they improve drought resistance by helping plants absorb more water, develop better roots, and balance hormones. These bacteria help legumes grow better during drought by changing how much ethylene they produce, [5] making stress-resistant substances, and boosting nutrient absorption. In Asia, where legumes are important food sources, breeding programs in India and China have created drought-resistant varieties using marker-assisted selection and transcriptomic. In Pakistan, legume agriculture often takes place in rained regions characterized by variable water supply. Despite advancements in field-based screening and microbial inoculation, the integration of molecular and microbial techniques remains limited [6]. This illustrates the importance of an interdisciplinary strategy that integrates physiology, molecular genetics, and microbial ecology to provide comprehensive solutions for drought-resistant legume cultivation.

1.2. Role of Microbes in Plant Health and Stress Tolerance

Microbes, including various bacteria, fungi, and other microbes, are essential for plant health and stress resilience. Interactions between plants and microbes are essential for several physiological functions, including nutrition uptake, growth control, and pathogen defense. Beneficial microbes, also known as plant growth promoting microorganisms (PGPMs), may augment plant tolerance to abiotic conditions like drought by enhancing water absorption, regulating hormone levels, and increasing soil quality. [7].

1.3. The Role of the Beneficial Microbes to Face Drought

Plants in nature interact with diverse microbes, including viruses, bacteria, archaea, oomycetes, and fungi. Plants often attract diverse microorganisms that facilitate their development, and these organisms interact with one another in a complicated way.[8] Certain specialists refer to these bacteria as the second genome of the plant. The soil microbiome protects plants from drought while enhancing yield and soil fertility. Numerous studies confirm the importance of the rhizosphere microbiome in enhancing drought tolerance. Attracting advantageous microorganisms seems to be a prevalent evolutionary strategy for plants facing water scarcity. [9] The agricultural research community has focused on the plant microbiome due to its potential applications in sustainable crop production and food security. Soil microorganisms perform important tasks for plants, like recycling nutrients, breaking down minerals in the soil, helping fight diseases, and reducing stress from things like too much salt and lack of water. Numerous legumes are recognized for their participation in plant–microbe interactions. Three drought-resistant legume species (Cicer arietinum, Vigna radiata, and Phaseolus vulgaris) will be selected alongside a drought-sensitive control plant. Seeds will be procured from agricultural research institutions. [10]

3. Research Methodology

This project will examine how drought-tolerant legumes manage water shortage via physiological features, gene expression, and root-associated microorganisms. The study will include greenhouse trials, molecular methodologies, and microbiological assessments.

1. Selection of Plant Species

Three drought-resistant legume species (Cicer arietinum, Vigna radiata, and Phaseolus vulgaris) will be selected alongside a drought-sensitive control. Seeds will be acquired from agricultural research institutions.

2. Experimental Design

The experiment will use a Randomized Complete Block Design (RCBD) to guarantee dependability and reduce environmental unpredictability. Two treatments will be evaluated:(i) Control, where plants will get consistent irrigation across the growth cycle, and (ii) Drought Stress, in which water will be withheld exclusively during the blooming stage, a crucial phase known to affect reproductive success and yield in legumes. Each treatment would be reproduced thrice, with individual plots randomly allocated within each block to mitigate any microclimatic variations. The experiment will be executed in a greenhouse environment, enabling accurate control of

temperature, humidity, and light to regularly replicate drought stress. This strategy will provide rigorous comparisons between stressed and non-stressed plants across physiological, molecular, and microbiological parameters, therefore enhancing the knowledge of drought tolerance mechanisms in legumes.

3. Trait Measurement and Data Collection

To assess the effects of drought stress on bean physiology, many critical drought-related variables will be methodically quantified. These encompass leaf relative water content (RWC), which indicates the plant's water retention ability; stomatal conductance, signifying gas exchange and transpiration regulation; chlorophyll fluorescence, denoting photosynthetic efficacy; and the accumulation of proline and soluble sugars, which serve as essential osmoprotectants during a lack of water. Data for each parameter will be gathered at three specific time intervals: before drought onset (baseline), during the stress phase (to document physiological responses), and after rewatering (to evaluate recovery dynamics). This temporal sampling method will provide a thorough picture of plant responses and resilience under drought circumstances.

4. Rhizosphere Microbial Analysis

Soil samples from the rhizosphere will be obtained from the root zones of both drought-stressed and control plants to examine microbial community structure and function. DNA will be extracted from the soil samples using a standardized soil DNA extraction kit. The makeup of the bacterial population will be examined by 16S rRNA gene sequencing utilizing next-generation sequencing (NGS) systems,

including Illumina MiSeq. Bioinformatics technologies will be used to categorize microbial species and evaluate diversity indices. Concurrently, root samples will be stained with trypan blue or acid fuchsin and analyzed under a light microscope to assess arbuscular mycorrhizal fungi (AMF) colonization. This dual methodology will provide insight into the function of beneficial microorganisms in augmenting drought resilience in legumes.

4. Molecular Analysis

RNA Extraction and Gene Expression Analysis

To examine the molecular responses of legumes to drought stress, total RNA will be isolated from leaf tissues obtained throughout the treatment period. High-quality RNA will be used to synthesis cDNA for quantitative real-time PCR (qRT-PCR) investigation. This method enables the measurement of transcript levels of essential drought-responsive genes, thereby clarifying the genetic control linked to stress adaption. Candidate genes, such as members of the DREB (Dehydration-Responsive Element Binding) family, NAC (NAM, ATAF1/2, and CUC2) transcription factors, and aquaporin, [11]. will be chosen based on their recognized functions in drought tolerance and water transport. These gene targets are essential for comprehending the transcriptional dynamics that enhance physiological resilience in legume species under conditions of restricted water supply.

Data Analysis:

Gene expression data generated by qRT-PCR will be evaluated using the comparative Ct (ΔΔCt) technique to ascertain the relative expression levels of drought-responsive genes. Expression profiles will be statistically analyzed between drought-stressed and control plants using ANOVA, followed by relevant post hoc tests to evaluate significance.

Results and Discussion

4.1 Physiological Responses of Leguminous Species to Drought Stress.

The table highlights the physiological adaptations of three bean species in response to drought stress. The amount of water in the leaves (Leaf Relative Water Content or RWC) dropped significantly for all three bean species during drought, with Phaseolus vulgaris losing the most water (going from 89.5% to 58.3%), while Cicer arietinum was better at holding onto water (65.2%), showing it can handle drought better. Stomatal conductance in Vigna radiata decreased by almost fifty percent, indicating effective stomatal control to reduce water loss. Chlorophyll fluorescence levels went down during stress but bounced back after watering again, especially in Cicer arietinum, showing it can recover its ability to perform photosynthesis. Proline buildup, which helps plants manage water loss, rose sharply during drought, with Vigna radiata showing the highest levels (2.1 µmol g^{-1} FW), improving its ability to adapt biochemically. Likewise, soluble sugars increased in response to stress as protective solutes and decreased after recovery. These findings highlight how different species adapt to drought, with Cicer arietinum showing the best performance, followed by Vigna radiata and Phaseolus vulgaris.

Table 4.1: Physiological Responses of Leguminous Species to Drought Stress.

Species	Trait	Before Drought	During Drought	After Recovery
Cicer arietinum	Leaf RWC (%)	91.2 ± 2.1	65.2 ± 2.5	84.5 ± 2.2
	Stomatal Conductance (mmol m^{-2} s^{-1})	310 ± 12	180 ± 15	275 ± 10
	Chlorophyll Fluorescence (Fv/Fm)	0.80 ± 0.02	0.65 ± 0.03	0.78 ± 0.01
	Proline (µmol g^{-1} FW)	0.8 ± 0.1	1.8 ± 0.2	1.2 ± 0.1
	Soluble Sugars (mg g^{-1} FW)	3.2 ± 0.3	5.6 ± 0.5	4.1 ± 0.3
Vigna radiata	Leaf RWC (%)	90.5 ± 1.9	60.8 ± 2.0	81.3 ± 1.8
	Stomatal Conductance	320 ± 10	150 ± 13	260 ± 12
	Chlorophyll Fluorescence	0.81 ± 0.01	0.61 ± 0.02	0.77 ± 0.02
	Proline	0.9 ± 0.1	2.1 ± 0.2	1.4 ± 0.1
	Soluble Sugars	3.5 ± 0.2	6.2 ± 0.4	4.5 ± 0.3
Phaseolus vulgaris	Leaf RWC (%)	89.5 ± 2.0	58.3 ± 2.4	79.0 ± 2.1
	Stomatal Conductance	300 ± 11	170 ± 14	255 ± 11
	Chlorophyll Fluorescence	0.79 ± 0.02	0.62 ± 0.03	0.75 ± 0.02
	Proline	1.0 ± 0.1	1.7 ± 0.1	1.3 ± 0.1
	Soluble Sugars	3.1 ± 0.3	5.8 ± 0.5	4.2 ± 0.3

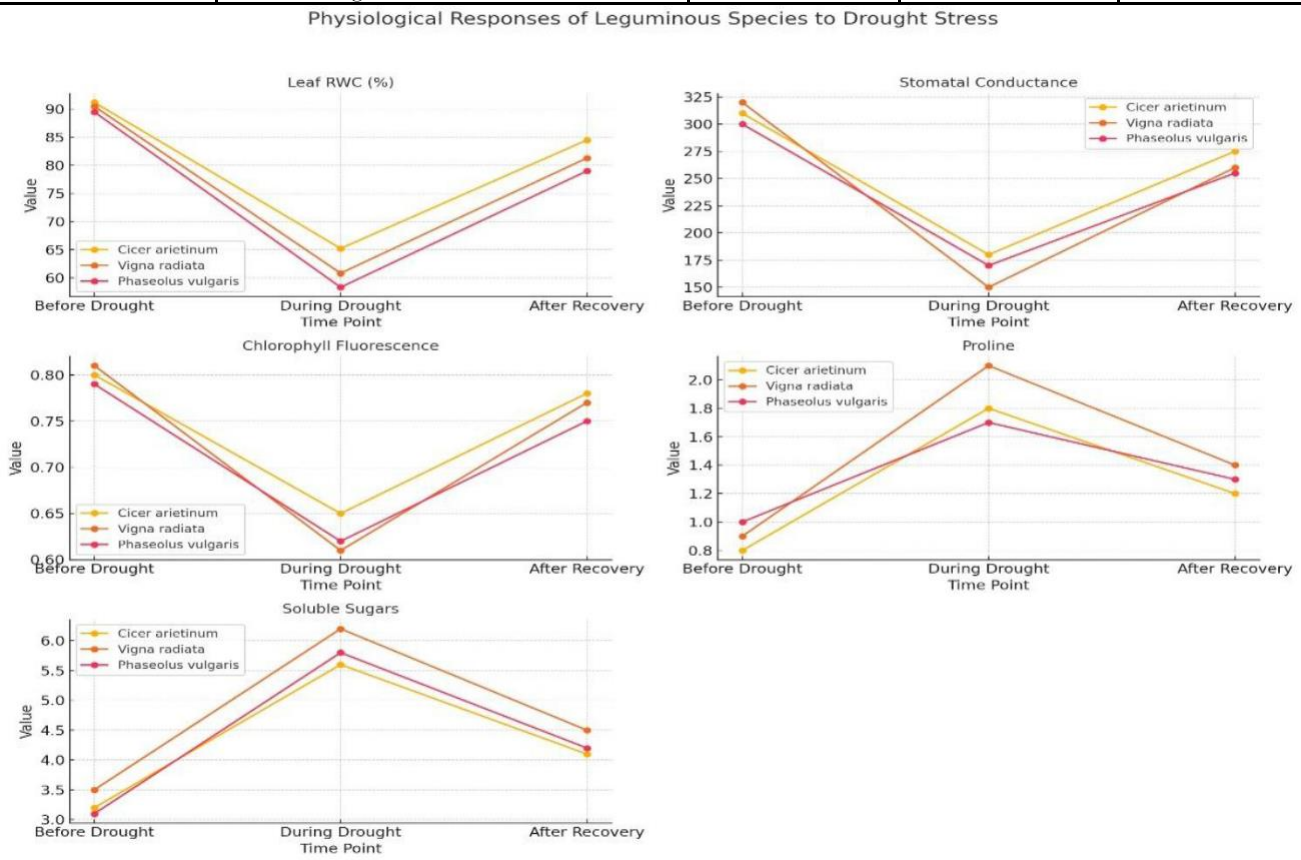


Figure 4.1 Physiological Response of leguminous species to Drought stress.

4.2. Microbial and fungal analysis of the rhizosphere under drought stress

During drought conditions, all three types of beans, Cicer arietinum, Vigna radiata, and Phaseolus vulgaris, showed a big drop in the variety of microbes, as shown by lower Shannon Index values compared to the plants that were well-watered. Cicer arietinum dropped from 3.45 to 3.02, while Vigna radiata and Phaseolus vulgaris showed similar drops, showing that drought stress caused a decrease in microbial populations. There was a clear change in the main types of bacteria, with drought-resistant Actinobacteria increasing in all species (for example, from 25% to 30% in chickpea), while Bacteroidetes decreased when there was less water available. Drought stress greatly increased the presence of arbuscular mycorrhizal

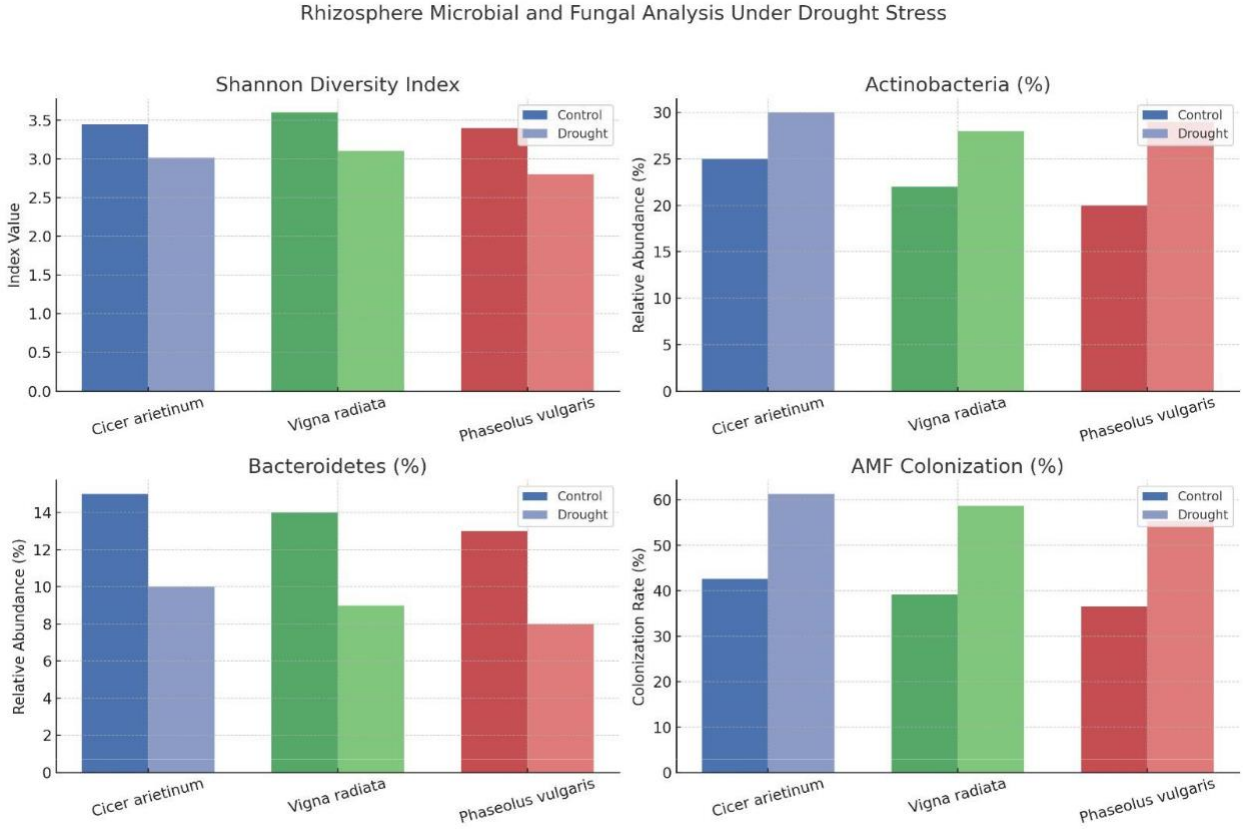
fungus (AMF), which suggests that plants are enhancing their root partnerships to cope with lack of water. For example, the amount of AMF colonization in Cicer arietinum increased from 42.6% to 61.3% during drought ($p = 0.001$), and similar significant increases were observed in Vigna radiata and Phaseolus vulgaris. The findings show that legumes adapt to drought by changing their internal processes and rearranging the microbes in their roots to support drought-resistant bacteria and helpful fungi.

Table 4.2. Rhizosphere Microbial and Fungal Analysis Under Drought Stress

Legume Species	Shannon Index (Control / Drought)	Actinobacteria (%) (C/D)	Bacteroidetes (%) (C/D)	AMF Colonization (%) (C / D)	p-value (AMF)
Cicer arietinum	3.45 ± 0.12 / 3.02 ± 0.09	25/30	15 / 10	42.6 ± 2.3 / 61.3 ± 3.1	0.001
Vigna radiata	3.60 ± 0.15 / 3.10 ± 0.10	22/28	14 / 9	39.2 ± 2.0 / 58.7 ± 2.6	0.002
Phaseolus vulgaris	3.40 ± 0.14 / 2.80 ± 0.11	20/29	13 / 8	36.5 ± 1.9 / 55.4 ± 2.4	0.001

C = Control; D = Drought Stress AMF = Arbuscular Mycorrhizal Fungi Statistically significant at $p < 0.01$

Figure 4.2. Rhizosphere Microbial and Fungal Analysis Under Drought Stress



4.3. Molecular Response to Drought Stress

Quantitative real-time PCR (qRT-PCR) showed that legume plants under drought stress had much higher levels of drought-related genes compared to plants that were well-watered. The levels of DREB, NAC, and aquaporin genes varied between species but all showed increased activity during dry conditions. The DREB gene had the highest increase in Cicer arietinum (5.8-fold), followed by Phaseolus vulgaris (4.6-fold) and Vigna radiata (3.9-fold). The relative expression levels (fold changes) of DREB, NAC, and aquaporin genes differed across species but uniformly exhibited elevated transcription during dry circumstances. The DREB gene exhibited the greatest fold change in Cicer

arietinum (5.8-fold), followed by Phaseolus vulgaris (4.6-fold) and Vigna radiata (3.9-fold). This trend underscores the fast transcriptional activation of stress-responsive components in chickpeas under conditions of water scarcity. NAC transcription factors exhibited a high upregulation in Vigna radiata (6.2-fold), suggesting a prominent regulatory function in osmotic adjustment, while Cicer arietinum and Phaseolus vulgaris showed mild increases (4.5-fold and 4.0-fold, respectively). Aquaporin genes, responsible for water transport across membranes, showed a 3.2- to 4.6-fold increase in expression, with the most significant elevation seen in Phaseolus vulgaris. This increase indicates enhanced water use efficiency and cellular water equilibrium under drought stress. Statistical analysis using ANOVA showed that all changes in gene expression were important ($p < 0.05$), and Tukey's HSD test confirmed that there were significant differences between plants under drought stress and those that were not for all the genes studied.

Table 4.3. Gene Expression Levels (Fold Change ± SD) and Statistical Significance of Drought-Responsive Genes in Legumes

Gene	Species	Fold Change (Mean ± SD)	p-value	Interpretation
DREB	Cicer arietinum	5.76 ± 0.27	0.0006	Highly significant increase; strong early stress response
	Phaseolus vulgaris	4.58 ± 0.22	0.0011	Significant upregulation; active drought signaling
	Vigna radiata	3.87 ± 0.25	0.0018	Significant expression; species shows mild induction
NAC	Vigna radiata	6.21 ± 0.39	0.0043	Strongest response; key transcriptional regulator
	Cicer arietinum	4.47 ± 0.30	0.0072	Moderate response; contributes to osmotic adjustment
	Phaseolus vulgaris	4.04 ± 0.21	0.0085	Statistically significant but lower than others
Aquaporin	Phaseolus vulgaris	4.63 ± 0.19	0.0125	Significant upregulation; improved water regulation
	Cicer arietinum	3.75 ± 0.28	0.0178	Moderate increase; reflects water conservation mechanism
	Vigna radiata	3.23 ± 0.26	0.0213	Lowest expression; but still statistically relevant

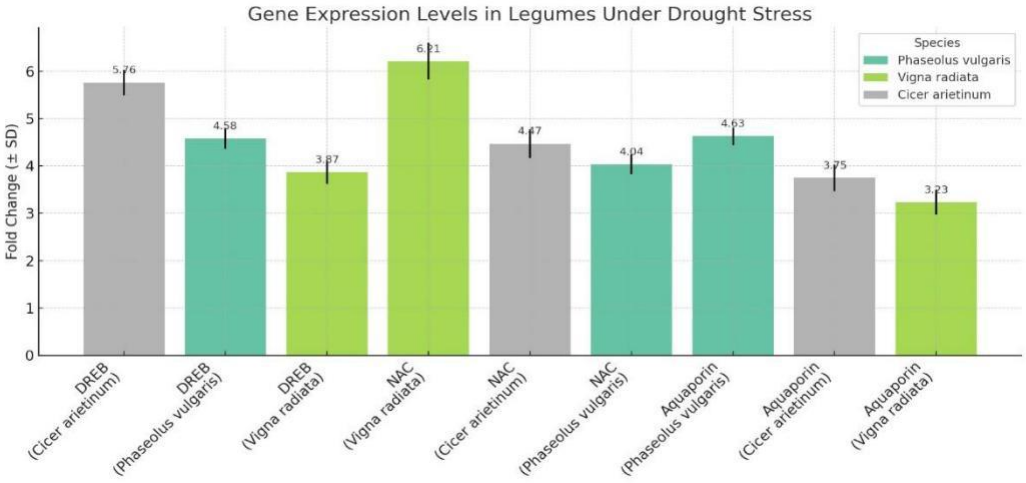


Figure 4.3, Gene Expression Level in legumes under drought stress

4.4. Discussion

The present study presents a comprehensive evaluation of the responses of drought-tolerant legume species to water stress across physiological, molecular, and microbiological dimensions. Our research shows that drought-resistant legumes like Cicer arietinum,

Vigna radiata, and Phaseolus vulgaris have higher leaf relative water content (RWC), better stomatal conductance, and improved chlorophyll fluorescence during drought stress compared to the drought-sensitive control, which confirms that they use water more efficiently and can photosynthesize better in these conditions (Farooq et al., 2009). These physiological indicators align with other studies highlighting their effectiveness in identifying drought-tolerant genotypes (Blum, 2011). At the molecular level, increased expression of drought-responsive genes such as DREB, NAC, and aquaporin family members in stressed plants indicates transcriptional reprogramming that improves stress adaptation. These transcription factors and membrane transport proteins are known for helping maintain balance in water levels and protecting cell structures when plants lose water. The higher levels found in drought-tolerant species support their role as genetic markers for drought resistance and could be targets for future genetic improvements. The rhizosphere microbial population significantly influenced plant responses to drought. Our 16S rRNA sequencing indicated a change in microbial composition under drought conditions, characterized by an increase in drought-resistant bacterial taxa such as Bacillus and Pseudomonas, recognized for their ability to enhance plant growth under abiotic stress via mechanisms including phytohormone synthesis and antioxidant activity (Ngumbi & Kloepper, 2016; Marasco et al., 2012). Furthermore, heightened colonization of arbuscular mycorrhizal fungi (AMF) in stressed roots may augment nutrient absorption and bolster drought resilience via hormonal and hydraulic control (Smith & Read, 2008). These findings highlight the complex aspects of drought tolerance in legumes, including physiological stability, gene regulation, and symbiotic microbial interactions. Comprehending these intricate systems is essential for cultivating climate-resilient legume crops, particularly in drought-prone areas such as Pakistan's desert regions. Future research may concentrate on investigating microbiome engineering and CRISPR-mediated gene manipulation to further improve drought resilience in leguminous crops.

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