ESTIMATING WATER STRESS TOLERANCE GENE EXPRESSION IN COWPEA GENOTYPES †

[ESTIMACIÓN DE LA EXPRESIÓN GÉNICA DE TOLERANCIA AL ESTRÉS HÍDRICO EN GENOTIPOS DE CAUPÍ]

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SUMMARY

Background. This study examines cowpea (*Vigna unguiculata* L.) genotypes' water stress resistance molecular and physiological processes. It analyzes the association between *VuNCED1* and *P5CS* gene expression, antioxidant enzyme activities (SOD, CAT, APX, GR), and growth traits under different irrigation levels. **Objective**. Gene expression analysis and physiological responses to water stress will be used to assess the drought tolerance of Local, Ramshorn, and Black Crowder cowpea genotypes. **Methodology**. Split-plot within randomized complete block design was used in Babil Governorate, Iraq. Main plots had three irrigation levels (50 %, 65 %, and 80 % of available water depletion), which represent low, moderate, and high-water stress levels, respectively, to mimic field conditions in arid regions. While subplots had three cowpea genotypes. Important measurements were *VuNCED1* and *P5CS* gene expression, antioxidant enzyme activity (SOD, CAT, APX, GR), and growth-related characteristics. **Results**. *VuNCED1* and *P5CS* genes activated antioxidant defenses and maintained cellular homeostasis, thereby improving drought tolerance. Black Crowder and Ramshorn genotypes exhibited 30 % and 25 % higher gene expression levels, respectively, compared to the local genotype. Similarly, antioxidant enzyme activity was 35 % and 28 % greater in Black Crowder and Ramshorn genotypes, respectively, relative to the local genotype. Yield increases of 40 % and 25 % were observed in Black Crowder and Ramshorn genotypes, respectively, over the local genotype. Moreover, Black Crowder had 45 % more chlorophyll than Ramshorn and 30 % more than the local genotype, based on average measurements under identical conditions. **Implications**. Genetic and physiological modifications improve drought resilience, according to the study. The study reveals that Black Crowder and Ramshorn genotypes may be suitable for arid environments due to their better yields and stress resistance. **Conclusion**. Gene expression and physiological changes are crucial to crop water stress management, according to this study. The Black Crowder and Ramshorn genotypes are promising drought-resistant crop producers due to their flexibility and improved performance under low water conditions.

Key words: Cowpea; water stress; *VuNCED1*; *P5CS*; antioxidant enzymes; drought tolerance; irrigation levels.

RESUMEN

Antecedentes. Este estudio examina los procesos moleculares y fisiológicos de resistencia al estrés hídrico en genotipos de caupí (*Vigna unguiculata* L.). Analiza la asociación entre la expresión de los genes *VuNCED1* y *P5CS*, las actividades de las enzimas antioxidantes (SOD, CAT, APX, GR) y las características de crecimiento bajo diferentes niveles de riego. **Objetivo**. La evaluación de la tolerancia a la sequía de los genotipos Local,

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Ramshorn y Black Crowder mediante el análisis de la expresión génica y las respuestas fisiológicas al estrés hídrico. **Metodología**. Se utilizó un diseño de parcelas divididas en bloques completamente aleatorizados en la Gobernación de Babil, Irak. Las parcelas principales incluyeron tres niveles de riego (50 %, 65 % y 80 % de agotamiento de agua disponible), representando niveles bajos, moderados y altos de estrés hídrico, respectivamente, para simular condiciones de campo en regiones áridas. Las subparcelas incluyeron tres genotipos de caupí. Las mediciones clave fueron la expresión de los genes *VuNCED1* y *P5CS*, la actividad de las enzimas antioxidantes (SOD, CAT, APX, GR) y las características relacionadas con el crecimiento. Resultandos. Los genes *VuNCED1* y *P5CS* activaron las defensas antioxidantes y mantuvieron la homeostasis celular, mejorando así la tolerancia a la sequía. Los genotipos Black Crowder y Ramshorn exhibieron niveles de expresión génica un 30 % y 25 % superiores, respectivamente, en comparación con el genotipo local. De manera similar, la actividad de las enzimas antioxidantes fue un 35 % y 28 % mayor en los genotipos Black Crowder y Ramshorn, respectivamente, en relación con el genotipo local. Se observaron aumentos de rendimiento del 40 % y 25 % en los genotipos Black Crowder y Ramshorn, respectivamente, en comparación con el genotipo local. Además, el genotipo Black Crowder tuvo un 45 % más de clorofila que Ramshorn y un 30 % más que el genotipo local, basado en mediciones promedio bajo condiciones idénticas. **Implicaciones**. Las modificaciones genéticas y fisiológicas mejoran la resiliencia a la sequía, según el estudio. Se revela que los genotipos Black Crowder y Ramshorn podrían ser adecuados para entornos áridos debido a su mejor rendimiento y resistencia al estrés. **Conclusión**. Este estudio destaca que los cambios en la expresión génica y las respuestas fisiológicas son cruciales para la gestión del estrés hídrico en cultivos. Los genotipos Black Crowder y Ramshorn son prometedores como productores de cultivos resistentes a la sequía gracias a su flexibilidad y mejor desempeño bajo condiciones de agua limitada.

Palabras clave: Caupí; estrés hídrico; *VuNCED1*; *P5CS*; enzimas antioxidantes; tolerancia a la sequía; niveles de riego.

INTRODUCTION

Particularly in arid and semi-arid regions where limited water resources and the expanding consequences of climate change cause substantial problems, cowpea (*Vigna unguiculata* L.) is one of the key crops utilized in sustainable agriculture. Since cowpea can endure drought and strong weather, it has become a possible agricultural alternative in many nations, particularly in Africa and Asia where it supplies the main protein source. Still one of the key factors hindering agricultural output, though, is negative environmental conditions including drought. In this sense, research on the molecular and physiological mechanisms under control of drought tolerance in plants becomes vital to improve crops to be more resilient in these conditions (Nkomo *et al*., 2021). Drought affects not only output but also important physiological and biochemical changes in plants, therefore influencing their rates of growth. The very complex reaction of the plant to water stress consists multiple biochemical pathways and genes interacting to enable the plant in survival in conditions of limited water resources. Plants must change how their genes are expressed if they are going to survive a drought (Khatun *et al*., 2021). Hasan *et al*., (2023) assert that plant response to drought directly influences gene control that either increases or decreases oxidative damage or else helps to balance water. VuNCED1 is one key gene enabling Cowpea survival during a drought. Writing the coding for 9-cis-epoxycarotenoid dioxygenase, this gene produces AVA (Abscisic aldehyde**)**. Water evaporation and stomata closure help the plant hormone ABA (abscisic acid) to regulate the response to aridity. AVA raises osmolytes—compounds like sugars and proline—that enable plants retain optimal water levels inside their tissues—under extreme stress. Studies reveal that *VuNCED1* regulates the AVA biosynthesis pathway; levels significantly rise during a drought, therefore allowing plants to adjust to decreased water availability and sustain cellular osmotic equilibrium (Ajayi *et al*., 2021). AVA is a signaling hormone as controlling molecular and cellular processes helps to reduce water loss and increase plant drought tolerance. AVA produced by Rising VuNCED1 blocks stomata to conserve water and reduce evaporation. AVA aids in osmolyte management, osmotic equilibrium, and handling of prolonged water stress (Nadeem *et al*., 2019). The *P5CS* gene also shapes plant drought resistance. Pyrroline-5-carboxylate synthase generates proline, an osmolyte that prevents plant cells from deicing (Yang *et al*., 2021). Proline, an antioxidant, balances cell osmosis and lowers reactive oxygen species (ROS) generated in response to environmental stress. Drought enhances *P5CS* expression in plant cells, hence producing more proline accumulation. This accumulation preservation of essential enzymes, proteins, and cellular structure enables the plant to survive during drought (Zegaoui *et al*., 2017). Raised proline levels brought about by higher *P5CS* output aid to reduce oxidative damage and dehydration.

Proline protects proteins, maintains osmotic balance, and keeps plant cells from being destroyed. Under taxing conditions, proline increases plant survival (Ansari *et al*., 2019). The *VuNCED1* and *P5CS* genes are important participants of a sophisticated genetic network regulating plant responses to drought. Gene expression factors enable plants to control water constraint by means of control of stomatal function, osmolyte accumulation, osmotic balance, and oxidative stress damage reduction. This adaptation increases food output and helps plants to more effectively manage aridity (Boukar *et al*., 2019). We investigate the expression of these genes among several cowpea genotypes to identify biological mechanisms related with drought tolerance in plants. Techniques of genetic research let one grow cowpeas without waterlogging. Genomic research and genetic modification help to let plants thrive in numerous environments and enhance farming. This research is fundamental for food security since it also supports environmental protection and agricultural development.

MATERIALS AND METHODS

A field study was carried out in the Al-Muradiyyah Research Station in the Babil Governorate, Iraq (32°18'22"N, 44°23'36") over the seasons of 2023. The study sought to determine whether *VuNCED1* and *P5CS* genes might improve cowpea tolerance to water stress in several genotypes. Three irrigation levels [WA50%, WA65%, and WA80% of available water (WA)] were allocated as main plots, and three cowpea genotypes (Local, Ramshorn, and Black Crowder) as subplots, the experiment was set up as a split-plot arrangement within a randomized complete block design (RCBD). So, the experiment comprised nine treatments overall, each repeated three times, producing 27 experimental units.

Soil samples were collected from the experimental site before planting. Composite samples of 1 kg were taken from multiple locations within the site at two depths: 0–20 cm and 20–40 cm, to ensure representativeness. A total of 1 kg was collected from each depth (0–20 cm and 20–40 cm). The samples were then analyzed in the laboratories of the Field Crops Department, College of Agriculture, University of Karbala, Iraq, to determine selected physical and chemical properties (Table 1). The soil was tilled twice in perpendicular directions, and mineral fertilizers were added, including triple superphosphate (46 % P_2O_5), potassium sulfate (50 % K_2O), and urea (46 % N). Fertilizers were applied in two stages. The first stage, pre-planting, involved applying the entire amount of triple superphosphate (46 %) at a rate of 80 kg/ha in the form of P_2O_5, and the entire amount of potassium sulfate (50 %) at a rate of 80 kg/ha in the form of K_2O. The second stage, post-planting, included applying 80 kg/ha of nitrogen fertilizer (46 % urea) in three doses: the first dose of 32 kg/ha was applied two weeks after seedling emergence, the second dose of 32 kg/ha at the onset of pod formation, and the third dose of 16 kg/ha two weeks after the second application (MAAR, 2019).

Table 1. Selected physical and chemical properties of the field soil (0–40 cm depth).

Property	Value	Unit
Sand	176	g/kg
Silt	484	g/kg
Clay	340	g/kg
Texture	-	Clay loam
Bulk density	1.24	Mg/m^3
Organic matter	4.60	g/kg
Available nitrogen	73.20	mg/kg
Available phosphorus	8.70	mg/kg
Available potassium	276	mg/kg
Electrical conductivity (EC)	2.73	dS/m
pH	7.14	-
Volumetric moisture content at tensions		
- 33 kPa	0.3998	cm^3/cm^3
- 100 kPa	0.2638	cm^3/cm^3
- 300 kPa	0.2054	cm^3/cm^3
- 500 kPa	0.1905	cm^3/cm^3
- 1500 kPa	0.1865	cm^3/cm^3

After leveling and softening the soil, it was divided into experimental units based on three replicates, with 1 m between replicates. Each replicate contained nine experimental units with dimensions of 3 m × 3.5 m. Each experimental unit included four rows, spaced 70 cm apart, with plants spaced 20 cm apart within rows. Each row was 3 m long. Cowpea seeds were sown on March 20, 2023, on one side of the furrow in the upper third. Three seeds were placed in each hole at a depth of 7 cm, covered with a light layer of soil. Ten days after planting, replanting was conducted for non-germinated holes, while thinning was performed for holes with more than one seedling, leaving one plant per hole. Each experimental unit contained 56 plants, with an individual plant area of 0.19 m^2 and an experimental unit area of 10.5 m^2. Post-germination, all agronomic practices, including

replanting, hoeing, weeding, and pest control, were carried out in accordance with standard protocols for cowpea cultivation. The crop was harvested on October 28, 2023.

Irrigation Scheduling and Monitoring Depletion Levels

The volumetric method was adopted to measure soil moisture, monitor moisture changes, and determine irrigation timing based on the depletion levels specified for the irrigation treatments according to the soil moisture tension curve (Figure 1). Soil samples were collected from depths of 0–20 cm and 20–40 cm, and the water quantities added during irrigation were calculated for the last depth based on the depletion levels applied in the study (50 %, 65 %, and 80 % of available water), with amounts ranging from 214, 275, and 269 liters per experimental unit**.**

Moisture Content Estimation

Soil moisture content was estimated by drying soil samples in a microwave oven at 105°C for 12 minutes. The temperature and drying duration were calibrated with a conventional oven following the method proposed by Zein (2002). Samples were weighed while wet in aluminum containers, and then reweighed after drying to calculate the moisture content using the following equation:

$$Qv = Qw \times \partial b$$

Where:
Qv: Volumetric moisture content.
Qw: Gravimetric moisture content.
∂b: Soil bulk density (Mg/m³).

Irrigation Method

Irrigation was conducted using flexible hoses connected to a pump mounted on a 3 m³ water tank, equipped with a flow meter to measure the water quantities added to each experimental unit. Equal amounts of irrigation water were applied during planting to achieve field capacity and ensure seed germination. Subsequently, plants were irrigated when 50 % of available water was depleted, targeting depths of 0–20 cm and 20–40 cm.

Measuring soil moisture allowed irrigation scheduling to decide the right timing and to estimate the water depth required to restore ideal moisture levels. These judgments were guided by the soil moisture tension curve, and the equation put forward by Allen *et al*., (1998) was utilized to determine the necessary water depth to replace lost moisture.

$$d = (\theta fc - \theta I) \times D$$

Where:
d: Depth of water added (mm).
θfc: Volumetric moisture content at field capacity (cm³ cm-³).
θI: Volumetric moisture content before irrigation (cm³ cm-³).
D: Soil depth equivalent to the effective root zone depth (mm).

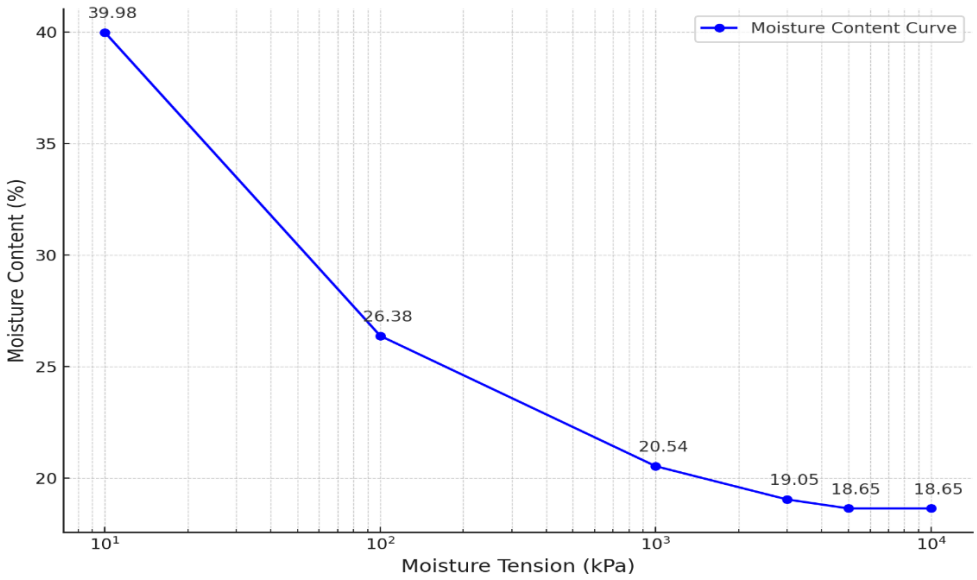

Figure 1. Soil Moisture Retention Curve for the Field Soil.

Data Collection

Expression of *VuNCED1* and *P5CS* Genes

After the fourth irrigation, all 27 experimental units gathered leaf samples from three irrigation treatments, three genotypes, and three replicates per treatment. To reduce RNA degradation, samples were collected early in the morning, flash-frozen in liquid nitrogen, and then macerated using a Micro-Tube Homogenizer to break down the tissue. The samples were stored at -80°C until RNA extraction. Following the manufacturer's instructions, the Add Bio RNA extraction kit was used to retrieve RNA from all 27 leaf samples. The isolated RNA was of high quality for downstream analysis, as its integrity and concentration were assessed using a NanoDrop spectrophotometer.

Reverse transcription-quantitative PCR (RT-qPCR) was used to measure *VuNCED1* and *P5CS* gene expression using *GAPDH* as the reference gene. Publicly available sequences were used to construct target and reference gene primers. For each RT-qPCR reaction, 20 µL was used with 1 µg of total RNA, 0.5 µM primers, and GoTaq® Probe RT-qPCR Master Mix (Promega). The thermal cycling RT-qPCR program contained:

cDNA synthesis: 50° C for 20 minutes.

Initial denaturation: 95° C for 10 minutes.

Amplification (40 cycles):
- Denaturation: 95° C for 45 seconds.
- Annealing: 60° C for 45 seconds.
- Extension: 72° C for 1 minute.

Fluorescence data were collected during the extension phase of each cycle, ensuring real-time monitoring of amplification. Relative gene expression levels of *VuNCED1* and *P5CS* were quantified using the $2^{\wedge(-\Delta\Delta Ct)}$ method to assess changes in expression relative to the control group.as described by Livak and Schmittgen (2001). The following equations were used:

$$\Delta ct = ct_{\text{target gene}} - ct_{\text{reference gene}}$$
$$\Delta\Delta ct = \Delta ct_{\text{Test}} - \Delta ct_{\text{Control}}$$
$$\text{Gene expression} = 2^{-\Delta\Delta ct}$$

Where:
$ct_{\text{target gene}}$ is the cycle threshold of the target gene (*VuNCED1* and *P5CS* genes),
$ct_{\text{reference gene}}$ is the cycle threshold of the *GAPDH* gene,
Δct_{Test} is the cycle threshold difference for the target gene sample,
$\Delta ct_{\text{Control}}$ is the cycle threshold difference for the control sample of the target gene.

Gene expression data is normalized to the reference gene and treatment and control samples are compared reliably using this method. The analysis covered all experimental units and examined gene expression variations under different irrigation regimes and genotypes.

Plant height (cm)

The plants' height was measured after the growing season. From the plant's base soil to its growing tip, measurements were gathered. The heights of five randomly selected plants from each experimental unit were measured in cm. The average plant height was estimated from these measurements.

Leaf area (dm² plant^{-1})

Cowpea leaf area was calculated during flowering using the formula:

$$\text{Leaf area } (\text{dm}^2\ \text{plant}^{-1}) = \text{Number of leaves per plant} \times \text{Leaf area per leaf}$$

Five randomly selected plants from each experimental unit were counted and measured for leaf area using Digimizer software. To represent experimental unit values, the average leaf area per plant was computed.

Number of branches (branches plant^{-1})

The number of main branches per plant was counted for five randomly selected plants from each experimental unit at the end of the growing season. The average number of main branches per plant was calculated for each experimental unit to represent the results statistically.

Chlorophyll content (mg 100g^{-1} FW)

Fresh leaf samples were used to evaluate chlorophyll concentration during flowering. A spectrophotometer was used to estimate according to Arnon (1949). Leaves were homogenized in 80 % acetone and centrifuged for 10 minutes at 10,000 rpm. The supernatant was collected and spectrophotometer absorbance measured at 645 and 663 nm. The chlorophyll content was calculated using the following equations:

$$Chlorophyll\ a\ = (12.7 \times A_{663}) - (2.69 \times A_{645})$$

$$Chlorophyll\ b = (22.9 \times A_{645}) - (4.68 \times A_{663})$$

$$Total\ Chlorophyll = Chlorophyll\ a + Chlorophyll\ b$$

The results were expressed as milligrams of chlorophyll per 100 grams of fresh weight (FW).

Yield per plant (g plant^{-1})

The yield per plant was estimated by dividing the cumulative yield of each experimental unit by its plant count. Total cumulative yield was calculated by harvesting all plants in the experimental unit and represented in grams per plant for standardized comparison between treatments and units.

Antioxidant Enzyme Activity Estimation (U min^{-1} g^{-1} FW)

The Pyrogallol Autoxidation Assay, using the technique developed by Marklund and Marklund (1974), evaluated SOD activity. Mixed in a mild phosphate buffer solution (pH 7.8), leaf samples were spun at 10,000 rpm for 15 minutes at a cool 4° C under a touch of EDTA. The supernatant produced enzyme extract. Measuring pyrogallol autoxidation, the enzyme extract test found inhibition. The mix calls for 50 mM phosphate buffer at pH 7.8, 1 mM EDTA, and 10 mM pyrogallol. The reaction started with the addition of enzyme extract; a spectrophotometer tracked the absorbance fluctuation at 420 nm every 30 seconds for five minutes. Enzyme ability to prevent pyrogallol autoxidation by 50 %, standardized to fresh weight of leaf samples, determined SOD activity. Data reported in units per minute per gram of fresh weight (U min^{-1} g^{-1} FW). This approach quantified SOD activity, which is essential to understand the natural defensive mechanisms of the plant.

Ascorbate Peroxidase (APX) Activity

APX activity was evaluated using Nakano and Asada (1981) techniques. 1mL EDTA, 1mL ascorbic acid, and one percent (w/v) polyvinylpyrrolidone were combined in a mild phosphate buffer solution (50 mM, pH 7.0). Made from the homogenate supernatant, enzyme extract was created using 4° C and 10,000 rpm centrifugation for 15 minutes. Enzyme extract, 0.5 mM ascorbic acid, 0.1 mM H_2O_2, 50 mM phosphate buffer (pH 7.0) comprised the 3 mL reaction mixture. Starting with H_2O_2, the spectrophotometer tracked the drop in absorbance at 290 nm every 10 seconds for one minute, therefore indicating ascorbate oxidation. Units per minute per gram of fresh weight (U min^{-1} g^{-1} FW) for APX activity were computed using ascorbate's molar extinction coefficient ($2.8\times10^{3-1}$ FW). This approach accurately measured APX activity, an enzyme used in plants to help remove reactive oxygen species.

Catalase (CAT) Activity

CAT activity was judged using the Aebi (1984) approach. Leaf samples were combined in a mild 50 mM phosphate buffer (pH 7.0) including 1 mM EDTA and 1 % (w/v) PVP. Following 15 minutes of homogenate supernatant centrifugation at 10,000 rpm and 4° C, the enzyme extract was made. Three milliliters of reaction mixture comprised 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and enzyme extract. The process started with the enzyme extract, and a spectrophotometer noted the breakdown of H_2O_2 by measuring the drop in absorbance at 240 nm. Over one minute, measurements were noted every ten seconds. Using the molar extinction coefficient of H_2O_2 (43.6 M^{-1}) the CAT activity was computed as U min^{-1} g^{-1} FW. Reflecting the plant's natural response to detoxifying hydrogen peroxide under stress, this approach accurately measured CAT activity.

Glutathione Reductase (GR) Activity

GR activity was measured using the Foyer and Halliwell (1976) technique. Using a mild 50 mM phosphate buffer (pH 7.5), leaf samples were mixed with 1 mM EDTA and 1 % (w/v) PVP. Made from the homogenate supernatant, enzyme extract was created using 4° C and 10,000 rpm centrifugation for 15 minutes. Enzyme extract, 0.5 mM GSSG, 0.1 mM NADPH, and 50 mM phosphate buffer (pH 7.5) comprised the 3 mL reaction mixture. Starting with the addition of NADPH, a spectrophotometer tracked the drop in absorbance at 340 nm to indicate the oxidation of NADPH. Over one minute, absorbance was measured every ten seconds. Expressing the GR activity as U min^{-1} g^{-1} FW, the molar extinction coefficient of NADPH ($6.22\times10^{1-1}$) was used for assessment. This approach precisely evaluates GR activity, a crucial component of the antioxidant system meant to recover lowered glutathione to maintain redox balance under stress.

Correlation Analysis

Pearson correlation analysis was conducted to evaluate the relationships among gene expression levels, antioxidant enzyme activities (SOD, CAT, APX, and GR), and physiological and growth parameters (plant height, leaf area, number of branches, chlorophyll content, and total yield) under different irrigation treatments. The analysis included data from all experimental units (27 units: three irrigation treatments × three genotypes × three replicates). The correlation coefficients were calculated using GenStat software version 12 to identify positive and negative associations, and their statistical significance was assessed at $p < 0.05$ (Al-Rawi and Khalafallah, 2000).

Statistical Analysis

To assess treatment effects, all quantitative cowpea attributes were analyzed using ANOVA. According to Al-Rawi and Khalafallah (2000), the least significant difference (LSD) test at 0.05 was used to compare and separate means. Statisticians used GenStat to compare arithmetic means to find significant changes between treatments. This method ensured robust experimental data analysis.

RESULTS AND DISCUSSION

Expression of *VuNCED1* and *P5CS* Genes Under irrigation Levels

As shown in Figure 2, the study examined the gene expression of the *VuNCED1* gene in three cowpea genotypes (Local, Ramshorn, and Black Crowder) at varying irrigation levels (WA50%, WA65%). All genotypes displayed equal gene expression level of 1 at the WA50% irrigation level, suggesting a same response under sufficient water circumstances. Gene expression grew progressively as water stress rose at WA65%; the Local genotype recorded 1.31, Ramshorn 1.37, and Black Crowder outperformed with an expression level of 1.87. Gene expression maintained growing in every genotype at WA80%, the highest water stress level. Black Crowder with an expression level of 2.23 was the most water stress tolerant. Second came Ramshorn in 1.75; third came local in 1.56. These results show that cowpea water stress is greatly influenced by the *VuNCED1* gene. This gene controls ABA needs by producing 9-cis-epoxycarotenoid dioxygenase (NCED). ABA helps to manufacture osmotic substances like proline and carbs and controls stomata closure to help to lower water loss. This reduces oxidative stress and lets the plant maintain its water balance (Ajayi *et al*., 2021). With the highest gene expression levels under all water conditions, the Black Crowder genotype performed especially well under very low water levels. This behavior begins with genetic mechanisms resistant to water stress, thereby boosting agricultural output in areas with low water supplies. Local and Ramshorn showed less evident effects, implying that genes regulate plant drought tolerance (Olajide and Ilori, 2017). While breeding for defense against water stress, the *VuNCED1* gene must be given more importance. By means of genetic engineering or conventional breeding, crops can be more resilient against climate change.

Figure 3 reveals that gene expression *P5CS* responses differed significantly between Local, Ramshorn, and Black Crowder cowpea genotypes under varied irrigation levels (WA50%, WA65%, WA80%). All genotypes had equal gene expression *P5CS* values of 1 at WA50% irrigation, indicating that sufficient water conditions did not cause the gene to adapt to water stress. But at WA65%, gene expression rose noticeably; the Local genotype

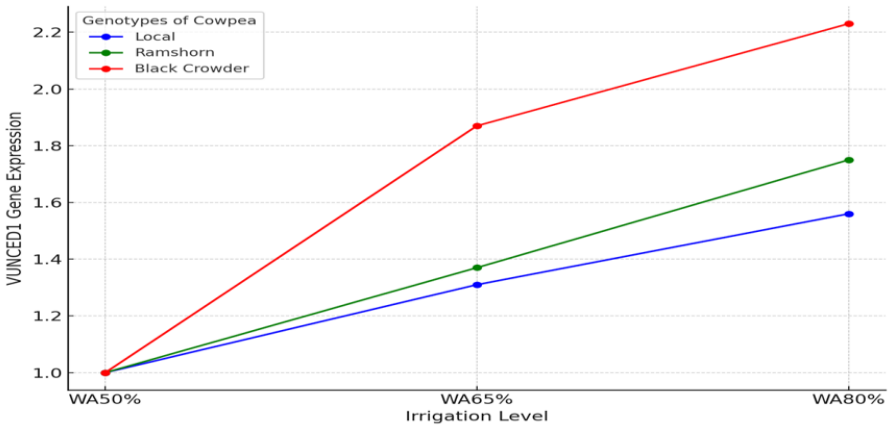

Figure 2. Impact of Irrigation levels on *VuNCED1* Gene Expression in Different Cowpea genotypes.

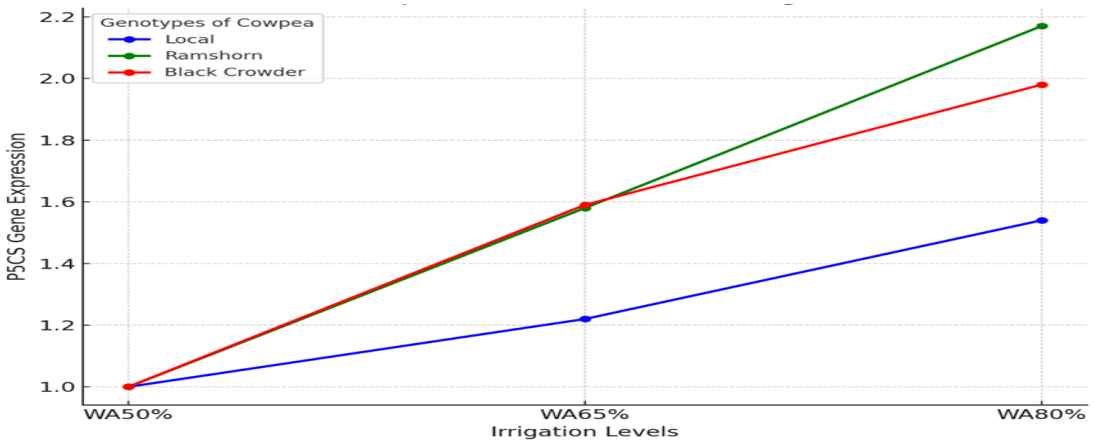

Figure 3. Impact of Irrigation levels on *P5CS* Gene Expression in Different Cowpea genotypes.

recorded 1.22, Ramshorn 1.58, and Black Crowder somewhat above it with 1.59, therefore showing the activation of genetic responses to water shortage.

Representing the maximum degree of water stress, WA80%, gene expression levels dropped dramatically in all genotypes. The local genotype reported 1.54; Ramshorn showed the greatest expression level at 2.17; Black Crowder came second at 1.98. These findings highlight Ramshorn's adaptability and capability to trigger genetic responses under extreme drought circumstances. These results indicate that resistance against water stress depends on the *P5CS* gene. The gene is crucial since proline is an antioxidant and osmolyte that controls cellular water balance. Reducing ROS accumulations lowers oxidative stress. Proline protects cell membranes and proteins; hence plants can survive in low-water conditions (Okeyo-Ikawa *et al*., 2016).

The study finds that Black Crowder is decent but ineffective; Ramshorn causes *P5CS* gene expression best. The local variant was unable to properly control water stress (Détain *et al*., 2022) having lowest gene expression. These results suggest that Black Crowder and Ramshorn are used in projects aimed at agricultural development with limited water resources. Furthermore, underlined is the genetic target for drought tolerance: the *P5CS* gene is Methods in agricultural methods or genetic engineering that increase its expression will help to raise crop yields in demanding conditions (Feng *et al*., 2022).

The study of *VuNCED1* and *P5CS* gene expression revealed amazing differences in how the three cowpea genotypes react to water stress, so suggesting which genotype is more drought resistant. Having the highest *VuNCED1* gene expression (2.23 at WA80%), the Black Crowder genotype lowers abscisic acid generation. ABA stimulates water stress responses and helps to slow water loss. Higher *P5CS* gene expression (1.98) associated with this genotype also helps store proline, therefore preserving water balance and shielding cells from oxidative stress (Goufo *et al*., 2017). At WA80% (2.17), the Ramshorn genotype displayed the highest *P5CS* expression, therefore shielding cellular membranes against proline, a major osmolyte insulator, and damage caused by dryness. Low *VuNCED1* expression at 1.75 to a mixed strategy for controlling water loss by stomatal control and proline accumulation (Jalal *et al*., 2023).

Instead, the local genotype exhibited the lowest expression levels for both genes at all irrigation levels. At WA80%, P5CS was 1.54 and *VuNCED1* 1.56. Low levels indicate a diminished ability to adapt to dry conditions, making it the least drought-resistant choice. These data suggest that Black Crowder has greater drought tolerance due to its powerful and coordinated response of both genes. The Ramshorn genotype has significant proline accumulation reactivity. Unlike the Local genotype, which responded only partially to water stress, these two genotypes present promising drought-prone regions.

Activity of Some Oxidoreductase Enzymes

Water stress levels clearly affected the activity of oxidative and reductive enzymes (SOD, APX, CAT, and GR) (Table 2). SOD activity increased with higher water stress, reaching 57.44 U min^{-1} g^{-1} FW at 50 % irrigation and 76.76 U min^{-1} g^{-1} FW at

Table 2. Effect of Irrigation Levels on the Estimation of the Activity of Some Oxidoreductase Enzymes in Three Genotypes of Cowpea.

Irrigation Levels		(SOD) Activity (U min^{-1} g^{-1} FW)	(APX) Activity (U min^{-1} g^{-1} FW)	(CAT) Activity (U min^{-1} g^{-1} FW)	(GR) Activity (U min^{-1} g^{-1} FW)
WA 50%		57.44	72.43	28.33	20.45
WA 65%		76.76	56.84	48.14	32.53
WA 80%		84.82	39.58	63.40	45.51
LSD $_{0.05}$		8.09	5.02	3.91	3.01
Genotypes		**(SOD) Activity** (U min^{-1} g^{-1} FW)	**(APX) Activity** (U min^{-1} g^{-1} FW)	**(CAT) Activity** (U min^{-1} g^{-1} FW)	**(GR) Activity** (U min^{-1} g^{-1} FW)
Local		65.55	51.18	38.23	25.38
Ramshorn		74.48	56.06	46.90	34.31
Black Crowder		78.99	61.60	54.74	38.80
LSD $_{0.05}$		6.17	4.10	2.57	2.19
Irrigation Levels	Genotypes	**(SOD) Activity** (U min^{-1} g^{-1} FW)	**(APX) Activity** (U min^{-1} g^{-1} FW)	**(CAT) Activity** (U min^{-1} g^{-1} FW)	**(GR) Activity** (U min^{-1} g^{-1} FW)
WA 50%	Local	51.44	67.00	25.89	18.01
	Ramshorn	58.20	71.10	28.11	20.34
	Black Crowder	62.70	79.20	31.00	23.00
WA 65%	Local	68.00	53.11	36.87	26.12
	Ramshorn	79.17	57.00	49.56	33.48
	Black Crowder	83.12	60.41	58.00	38.00
WA 80%	Local	77.23	33.45	51.93	32.03
	Ramshorn	86.09	40.10	63.05	49.11
	Black Crowder	91.15	45.19	75.22	55.40
LSD $_{0.05}$		12.55	8.23	5.07	4.76

65 %, indicating a clear response to water deficit (Table 2). This increase matches the plant's physiological defense mechanism activating to balance the accumulation of reactive oxygen species (ROS). Conversely, the activity of APX decreased with rising water stress (Barros *et al*., 2021), recording its highest value at 50% (72.43 U min^{-1} g^{-1} FW), dropping to 56.84 U min^{-1} g^{-1} FW at 65%, and reaching its lowest value at 80% (39.58 U min^{-1} g^{-1} FW). Under extreme dryness, this drop represents the loss of ascorbate as a fundamental substrate for enzyme function. Higher water stress greatly raised CAT activity, recording 28.33 U min^{-1} g^{-1} FW at 50%, rising to 48.14 U min^{-1} g^{-1} FW at 65%, and reaching its greatest value at 80% (63.40 U min^{-1} g^{-1} FW), so demonstrating its vital importance in eliminating hydrogen peroxide and shielding cells from oxidative damage. Reflecting its function in preserving cellular equilibrium by glutathione recycling, GR activity also steadily rose from 20.45 U min^{-1} g^{-1} FW at 50% to 32.53 U min^{-1} g^{-1} FW at 65%. Peak was 45.51 U min^{-1} g^{-1} FW at 80%. Among the genotypes, the Black Crowder genotype showed the highest average activity for all enzymes—78.99 U min^{-1} g^{-1} FW for SOD, 61.60 U min^{-1} g^{-1} FW for APX, 54.74 U min^{-1} g^{-1} FW for CAT, and 38.80 U min^{-1} g^{-1} FW for GR. This superiority increases antioxidant activity, so improving its great efficiency in handling water stress. With comparatively good activity, the Ramshorn genotype came second with 74.48 U min^{-1} g^{-1} FW for SOD, 56.06 U min^{-1} g^{-1} FW for APX, 46.90 U min^{-1} g^{-1} FW for CAT, and 34.31 U min^{-1} g^{-1} FW for GR, thereby showing a good but less efficient response compared to Black Crowder. Reflecting its limited capacity to cope with water stress, the local genotype displayed the lowest average activity for all enzymes; values of 65.55 U min^{-1} g^{-1} FW for SOD, 51.18 U min^{-1} g^{-1} FW for APX, 38.23 U min^{-1} g^{-1} FW for CAT, and 25.38 U min^{-1} g^{-1} FW for GR (Moloto *et al*., 2020; Tavares *et al*., 2021).

Analyzing the connection between water stress levels and variants, the Black Crowder genotype proved to be most flexible in all irrigation levels. It recorded the maximum activity for all the enzymes at the 50% irrigation level: SOD 62.70 U min^{-1} g^{-1} FW, APX 79.20 U min^{-1} g^{-1} FW, CAT 31.00 U min^{-1} g^{-1} FW, and GR 23.00 U min^{-1} g^{-1} FW. It kept reaching the greatest values of 83.12 U min^{-1} g^{-1} FW for SOD, 60.41 U min^{-1} g^{-1} FW for APX, 58.00 U min^{-1} g^{-1} FW for CAT, and 38.00 U min^{-1} g^{-1} FW for GR when water stress reached the 65% threshold. The activity peaked at the 80% irrigation level with SOD recorded 91.15 U min^{-1} g^{-1} FW,

APX recorded 45.19 U min^{-1} g^{-1} FW, CAT recorded 75.22 U min^{-1} g^{-1} FW, and GR recorded 55.40 U min^{-1} g^{-1} FW. With the local genotype showing the highest reduction, Black Crowder's performance demonstrates the ability of Black Crowder to activate antioxidant mechanisms more effectively than Ramshorn and the local genotype, which showed less efficient responses (Jayawardhane *et al*., 2022). These findings imply Black Crowder is the most drought tolerant as the antioxidant system there seems to be the most efficient in adjusting to water pressure. On the other hand, the local genotype is most impacted by strong environmental changes since it demonstrates a low capacity to manage stress. The diversity among the genotypes emphasizes the need of genetic adaptation and the function of antioxidant enzymes in raising plant tolerance to water stress.

Growth and Yield Traits

The study revealed a clear influence of water stress levels on growth and production (Table 3). Plants at the 50% irrigation level exhibited the highest values, including an average plant height of 87.00 cm, leaf area of 2240.08 dm^2 $plant^{-1}$, and total yield of 2.16 g $plant^{-1}$. As water stress increased to 65%, these parameters declined, with plant height dropping to 71.92 cm and yield to 1.51 g $plant^{-1}$. The most severe water stress at the 80% irrigation level led to a significant reduction in all measured traits, with plant height reaching 47.09 cm and total yield declining to 0.94 g $plant^{-1}$, highlighting the detrimental effects of prolonged water deficit. This drop in growth and productivity underscores the profound influence of water stress on plant physiology. Water scarcity leads to a cascade of physiological responses that limit plant performance. As water availability declines, plants experience reduced stomatal conductance, leading to a decrease in CO_2 uptake, which ultimately impairs photosynthesis and reduces carbohydrate production. Furthermore, prolonged water stress induces the accumulation of reactive oxygen species (ROS), which results in oxidative damage to plant cells, including lipids, proteins, and nucleic acids, affecting membrane integrity and enzyme activity. To counteract this, plants activate antioxidant

Table 3. Effect of Irrigation Levels, Cowpea genotypes, and Their Interaction on Some Growth and Yield Indicators.

Irrigation Levels		Plant height (cm)	Leaf area (dm^2 $plant^{-1}$)	Number of Branches (branch $plant^{-1}$)	Chlorophyll Content (mg 100 g^{-1})	Total yield (g $plant^{-1}$)
WA 50%		87.00	2240.08	13.40	2.71	2.16
WA 65%		71.92	1779.02	11.74	2.05	1.51
WA 80%		47.09	1007.94	7.91	1.36	0.94
LSD $_{0.05}$		5.12	77.28	1.04	0.48	0.07
Genotypes		Plant height (cm)	Leaf area (dm^2 $plant^{-1}$)	Number of Branches (branch $plant^{-1}$)	Chlorophyll Content (mg 100 g^{-1})	Total yield (g $plant^{-1}$)
Local		73.05	1639.72	8.96	1.68	1.02
Ramshorn		64.26	1656.48	10.95	2.01	1.54
Black Crowder		68.71	1730.84	13.13	2.42	2.04
LSD $_{0.05}$		7.23	69.12	1.09	0.43	0.06
Irrigation Levels	Genotypes	Plant height (cm)	Leaf area (dm^2 $plant^{-1}$)	Number of Branches (branch $plant^{-1}$)	Chlorophyll Content (mg 100 g^{-1})	Total yield (g $plant^{-1}$)
WA 50%	Local	92.30	2311.50	11.20	2.28	1.40
	Ramshorn	81.06	2189.42	13.00	2.70	2.23
	Black Crowder	87.64	2219.33	16.00	3.17	2.87
WA 65%	Local	75.90	1713.40	9.11	1.76	0.94
	Ramshorn	67.50	1800.67	11.73	2.08	1.52
	Black Crowder	72.38	1823.00	14.39	2.31	2.07
WA 80%	Local	50.95	894.26	6.59	1.02	0.73
	Ramshorn	44.23	979.35	8.14	1.27	0.89
	Black Crowder	46.11	1150.21	9.00	1.80	1.20
LSD $_{0.05}$		10.66	123.09	1.97	0.71	0.09

defense mechanisms, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), which help mitigate oxidative stress but can also divert energy from growth processes (Abdul Mohsin and Farhood, 2023). In addition, water stress triggers hormonal responses, particularly an increase in abscisic acid (ABA) levels, which further limits stomatal aperture and reduces transpiration. While this water conservation mechanism is crucial for survival, it also restricts carbon assimilation and affects overall plant growth. This results in decreased leaf area, smaller root systems, and reduced biomass production. In severe cases, as seen in the 80% irrigation treatment, plants may enter a state of dormancy or even experience irreversible damage, which severely limits productivity. Furthermore, the reduced turgor pressure in cells leads to wilting, and the osmotic pressure within the cells is compromised, further exacerbating stress-induced damage (Kang *et al*., 2021).

With an average plant height of 68.71 cm, leaf area of 1730.84 dm^2 $plant^{-1}$, 13.13 branches $plant^{-1}$, chlorophyll content of 2.42 mg 100 g^{-1} FW, and total yield of 2.04 g $plant^{-1}$, the Black Crowder genotype clearly demonstrated a superiority in all respects. This advantage is due to the genotype's resource efficiency and ability to maintain vital activities under pressure. The Ramshorn genotype performed well but less efficiently than Black Crower, with an average plant height of 64.26 cm, leaf area of 1656.48 dm^2 $plant^{-1}$, 10.95 branches $plant^{-1}$, 2.01 mg 100 g^{-1} chlorophyll content, and 1.54 g $plant^{-1}$ total yield. The local genotype had the lowest values for all variables with an average plant height of 73.05 cm, leaf area of 1639.72 dm^2 $plant^{-1}$, 8.96 branches $plant^{-1}$, chlorophyll content of 1.68 mg 100 g^{-1} FW, and total yield of 1.02 g $plant^{-1}$, showing weak water stress tolerance. Examining the relationship between genotypes and water stress levels revealed that the Black Crowder variant responded better at all irrigation levels. This genotype showed the highest values at the 50% irrigation level: plant height of 87.64 cm, leaf area of 2219.33 cm^2 $plant^{-1}$, 16.00 branches $plant^{-1}$, chlorophyll content of 3.17 mg 100 g^{-1} FW, and total yield of 2.87 g $plant^{-1}$. Black Crowder kept its advantage with a plant height of 72.38 cm, leaf area of 1823.00 cm^2 $plant^{-1}$, 14.39 branches $plant^{-1}$, chlorophyll content of 2.31 mg 100 g^{-1} FW, and total yield of 2.07 g $plant^{-1}$ when stress at the 65% watering level increased. Black Crowder recorded the highest values compared to Ramshorn and the local genotype at the 80% irrigation level, with a plant height of 46.11 cm, leaf area of 1150.21 cm^2 $plant^{-1}$, 9.00 branches $plant^{-1}$, chlorophyll content of 1.80 mg 100 g^{-1} FW, and total yield of 1.20 g $plant^{-1}$ despite the overall decline in values. Since these results reveal that the Black Crowder genotype can endure water stress and maintain required physiological functions even under stressful conditions, it is the most successful kind in drought resistance. This superiority is explained by its ability to raise antioxidant enzyme activity, preserve chlorophyll content, and reduce water loss by physiological and structural changes. The Ramshorn genotype showed a decent response although with less efficiency, whereas the native genotype was significantly impacted by inadequate tolerance to water stress, therefore reducing growth and yield.

Correlation Coefficient Analysis between the Studied Variables

Reflecting the response of the plant to water stress and adaptation mechanisms, the results of the correlation analysis between gene expression, enzyme activity, and physiological and growth parameters revealed several relations among these factors (Table 4). As well as the activity of antioxidant enzymes including SOD (0.930 and 0.945*, respectively), CAT (0.974* and 0.945*, respectively), and GR (0.942* and 0.958*, respectively), the gene expression of *VUNCED1* and *P5CS* revealed a clear positive connection. These interactions highlight the crucial roles these genes play in enhancing antioxidant defenses against water stress and in helping reactive oxygen species (ROS) accumulating under drought conditions to be eliminated. Conversely, a negative correlation between these genes and growth traits including plant height (-0.817* and -0.922*, respectively) and leaf area (-0.747* and -0.838*, respectively) suggests that activating stress defense pathways occurs at the expense of resource allocation for development (Ribeiro *et al*., 2023). With a substantial positive correlation between SOD and CAT (0.975), antioxidant enzyme activity revealed integrated internal interactions indicating their complementary roles in scavenging ROS: CAT eliminates hydrogen peroxide; SOD turns superoxide into hydrogen peroxide. Conversely, APX and SOD (-0.697) showed a negative connection as well as CAT (-0.718*). This shows the adaptability of the plant's defense system since a drop in APX activity under extreme stress circumstances could be offset by higher activity of other enzymes such as SOD and CAT (Barros *et al*., 2021). Leaf area and plant height (0.963) showed a substantial positive association for physiological and growth features, therefore indicating the integration of vertical and horizontal plant

Table 4. Correlation coefficient between gene expression, antioxidant enzyme activity, and physiological traits in cowpea genotypes under irrigation levels.

	VUNCED1 Gene Expression	P5CS Gene Expression	SOD Activity	APX Activity	CAT Activity	GR Activity	Plant height (cm)	Leaf area (dm^2 $plant^{-1}$)	Number of Branches (branch $plant^{-1}$)	Chlorophyll Content (mg 100 g^{-1})
VUNCED1 Gene Expression	----									
P5CS Gene Expression	0.878*	----								
SOD Activity	0.930*	0.930*	----							
APX Activity	-0.684*	-0.770*	-0.697*	----						
CAT Activity	0.974*	0.945*	0.975*	-0.718*	----					
GR Activity	0.942*	0.958*	0.943*	-0.664^{ns}	0.977*	----				
Plant height (cm)	-0.817*	-0.922*	-0.874*	0.893*	-0.889*	-0.878*	----			
Leaf area (dm^2 $plant^{-1}$)	-0.747*	-0.838*	-0.791*	0.956*	-0.802*	-0.771*	0.963*	----		
Number of Branches (branch $plant^{-1}$)	-0.419^{ns}	-0.537^{ns}	-0.386^{ns}	0.909*	-0.442^{ns}	-0.424^{ns}	0.714*	0.809*	----	
Chlorophyll Content (mg 100 g^{-1})	-0.552^{ns}	-0.678*	-0.561^{ns}	0.977*	-0.582^{ns}	-0.532^{ns}	0.796*	0.887*	0.944*	----
Total yield (g $plant^{-1}$)	-0.435^{ns}	-0.547^{ns}	-0.396^{ns}	0.887*	-0.442^{ns}	-0.414^{ns}	0.645^{ns}	0.739*	0.963*	0.946*

development. Furthermore, showing the significance of the plant's photosynthetic efficiency in raising production, particularly under stress, was a significant association between chlorophyll content and total plant yield (0.946) as well as branch number (0.944*). Further stressing its importance in enhancing photosynthetic efficiency, chlorophyll concentration also demonstrated a significant connection with leaf area (0.887*). Total yield has a strong positive correlation with branch number (0.963), indicating that optimal resource allocation improves plant physiological development and production. Gene expression and enzyme activity rarely correlated with yield, suggesting that stress defense should be prioritized before output (Omirou *et al*., 2019). The findings suggest plants must balance production, development, and antioxidant defenses. Although higher photosynthetic efficiency and resource allocation help plants adapt to difficult environments, *VUNCED1* and *P5CS* expression is essential for water stress resistance. This affects plant development and yield. Genes, enzymes, and physiological features must work together to improve a plant's water stress tolerance and material production in tough conditions (Khan *et al*., 2015).

CONCLUSION

The results revealed that SOD and CAT are activated by the *VuNCED1* and *P5CS* genes, therefore enhancing water stress resistance. Better than the native were the Black Crowder and Ramshorn genotypes. Under drought conditions, photosynthetic efficiency—especially chlorophyll content—improved output, therefore enhancing the genotype for the arid environment.

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Conflict of Interest. The authors declare that they have no competing interests to disclose.

Compliance with Ethical Standards. This study does not involve human or animal subjects and therefore does not require authorization by an ethical or bioethical committee. The research was conducted in compliance with all relevant institutional and national guidelines for research integrity and ethical conduct.

Data Availability. The data that support the findings of this study are available from the corresponding author, A.N. Farhood (ali.nazem@uokerbala.edu.iq), upon reasonable request.

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