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Physiological, Molecular and Anatomical Studies on Drought Tolerance in Cowpea

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COWPEA is a high-protein legume. Water stress is the most significant obstacle that hinders agriculture development in regions with limited water resources. Drought stress affects cowpea production, i.e., growth, yield and quality. An experiment was conducted to evaluate some cowpea accessions for drought tolerance at reproductive stage and studying the physiological, molecular and anatomical basis of tolerance. Six accessions were selected from a previous investigation to study the nature of drought tolerance at reproductive stage. Accessions TVU-14997, TVU-15304 and TVU-15306 exhibited high drought tolerance based on their high levels of each of antioxidant capacity, total phenols, catalase, peroxidase and abscisic acid during reproductive stage. The molecular study using specific primers confirmed the agronomical findings. The identification of potential genetic markers associated with drought tolerance in this study is a promising avenue for future research. The development of molecular markers for drought tolerance can facilitate marker-assisted selection in breeding programs, accelerating the development of drought-tolerant cowpea varieties. Regarding anatomical structure, under drought condition, accession TVU-15306 had the highest value for thickness of the midvein, spongy tissue, dimension of vascular bundle and mean vessels diameter. Water stress was found to negatively affect growth of studied cowpea accessions. Results showed that accessions TVU-14997, TVU-15304 and TVU-15306 were drought tolerant as evidenced by high levels of each of antioxidant capacity, total phenols, catalase, peroxidase and abscisic acid. They can be used as sources of tolerance to drought stress in breeding programs.

Keywords: *Vigna unguiculata*, deficit irrigation, Relative water content, reproductive stage, Antioxidant enzymes, Chemical antioxidants.

Introduction

Cowpea (*Vigna unguiculata* L. Walp.) belongs to the *Fabaceae* (syn. *Leguminosae*) family, which has a remarkable ability to fix atmospheric nitrogen in soil (Talukdar, 2013). As a significant crop in semi-arid tropical regions, cowpea is mostly grown in Africa, which accounts for 96.9% of global production. Egypt's production of cowpea in 2022 was 7272.23 tons, area cultivated was 1939 ha (≈ 4615 feddan) (FAO, 2022). Cowpea is mostly consumed as dry grains or fresh vegetable. The grains have significant levels of proteins, carbohydrates, vitamins, and fibers (Hall., 2012). Its low-fat content is also beneficial in preventing a variety of metabolic and cardiovascular disorders (Gonçalves *et al*., 2016).

Agriculture growth is severely hampered by climate change, especially in underdeveloped countries (Elsherpiny, 2023). In arid and semiarid regions worldwide, the scarcity of freshwater presents a significant challenge to the irrigation of crops and overall food production (Mohammed *et al*., 2023; Abd ElAty *et al*., 2023).One of the keys limiting factors to cowpea growth and production is drought, which is intensified by global climate change (Ajayi *et al.*, 2018). The impact of drought stress on crops, morphological, physiological, molecular, and biochemical features has been widely documented (Carvalho *et al*., 2019). Drought stress can negatively affect

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the amount of chlorophyll in leaves, the rate of photosynthesis, and the rate at which roots absorb nutrients. This can impede plant growth and development and leads to significant decreases in crop yield (Ghahremani *et al*., 2021; Namaki *et al*., 2022; Moustafa *et al*., 2024).

In many plant species, stomata are important regulators of gas exchange between internal leaf components and the outside environment. Under drought stress, stomata are reported to be smaller and fewer in number, with positive correlations between stomatal conductance, net CO_2 assimilation rate, and water use efficiency (WUE). Reproductive stage can be significantly reduced by drought; therefore, decreasing cowpea's productivity (Hamidou *et al*., 2007). One of the earliest ways that plants respond to water stress is by accumulating proline to decrease cell damage (Anjum *et al*., 2011). Proline concentration is a good indicator of drought tolerance. It has been detected in stress-tolerant plants when compared to susceptible ones (Toscano *et al*., 2016). One of the major challenges to cowpea production and yield is drought. Therefore, improving cowpea cultivars with increased drought tolerance is urgently needed in order to avoid the negative effects of water scarcity on crops. It is challenging to improve superior cowpea genotypes for growing in soils with moisture deficits using a selection strategy that just considers grain production since genotypic variation for yield is minimal under these soil moisture-deficient circumstances. However, it is possible to efficiently and effectively breed improved, tolerant, and high yielding genotypes of cowpea if characteristics driving good yield under moisture deficiency are identified in advance and used as selection standards in breeding programs (Kumar *et al*., 2008). Cowpea has a genome size of 613-640.6 Mb with diploidy (2n = 22) (Lonardi *et al*., 2019). The gene expression and QTLs in plants were identified using the start codon targeted marker (SCoT), Simple Sequence Repeats (SSR), and Amplified fragment length (AFLP) (Khaled *et al*., 2022).

It is possible to select genotypes that are tolerant to drought using morphophysiological parameters. Therefore, the objectives of this study were: 1) selection of cowpea accessions with contrasting morphophysiological parameters and 2) studying the nature of drought tolerance in the selected accessions molecularly.

2. Materials and methods

2.1. Plant Materials and evaluation for drought tolerance during reproductive stage

Six cowpea accessions (Table 1) were selected out of 70 cowpea accessions that were provided by The International Institute of Tropical Agriculture (IITA) and evaluated to their drought tolerance in previous study. The selected six accessions were divided into two groups as tolerant and sensitive ones, then evaluated in pots during the reproductive stage. All genotypes were evaluated for drought tolerance in three replicates. In each replicate, six 30-cm pots were used for each genotype. Pots were filled with a mixture of sand, peat moss and soil at 1:1:1. The experiment was carried out during 2021 (Sept. 18) and 2022 (Sept.1) growing seasons at the farm of Department of Vegetable Crops, Faculty of Agriculture, Cairo University, Giza, Egypt (30°01'02.1"N 31°12'37.5"E). Seeds were sown in pots in three replicates. Drought treatments levels: 1.0 ETc (full irrigation) and 0.50 ETc (severe drought) started 5 weeks after plant emergence.

2.2. Crop evapotranspiration

The reference evapotranspiration (ET_o) was calculated using Penman-Monteith method (Allen, 2000).

2.3. Physical characters

Eighteen cowpea plants (3 plants from each genotype per replicate and each watering level) were selected after 45 days of the drought treatments for measuring various characters during the remaining period till senescence. Leaf greenness (SPAD), leaf area using a portable leaf area meter (YMJ-A), shoot dry weight, root dry weight, days to first flowering, number of pods per plant, seed yield per plant and 100- seed weight were measured.

2.4. Physiological parameters

Stomata conductance and transpiration rate in the 5 th mature leaf from meristem tip of the plant were determined using a portable steady-state porometer (LI-1600M, LI-COR, Nebraska, USA) according to Surendar *et al*. (2013). Photosynthesis efficiency was determined according to Kumar *et al*. (2022) using the following formula: Photosynthesis II efficiency = (F_v/F_m), where F_v indicates the variable chlorophyll fluorescence ratio and F_m represents the maximal chlorophyll fluorescence ratio. A modulated chlorophyll fluorometer was utilized to estimate in a dark environment for 30 minutes. The relative water content (RWC) was estimated according to Medeiros *et al*. 2012.

Table 1. List of evaluated cowpea accessions (*Vigna* accessions) provided by The International Institute of Tropical Agriculture (IITA).

ID	Accession name	Accession number (IITA)	Cultivar name (IITA)	Country of origin
1	TVU-3812	3812	KR157	Nigeria
2	TVU-9908	9908	CHEWEATAN NO.1	Malawi
3	TVU-11619	11619	EX TVU 8247	Mali
4	TVU-14997	14997	AO87N-207	Niger
5	TVU-15304	15304	PS87CH-399	Chad
6	TVU-15306	15306	PS87CH-405	Chad

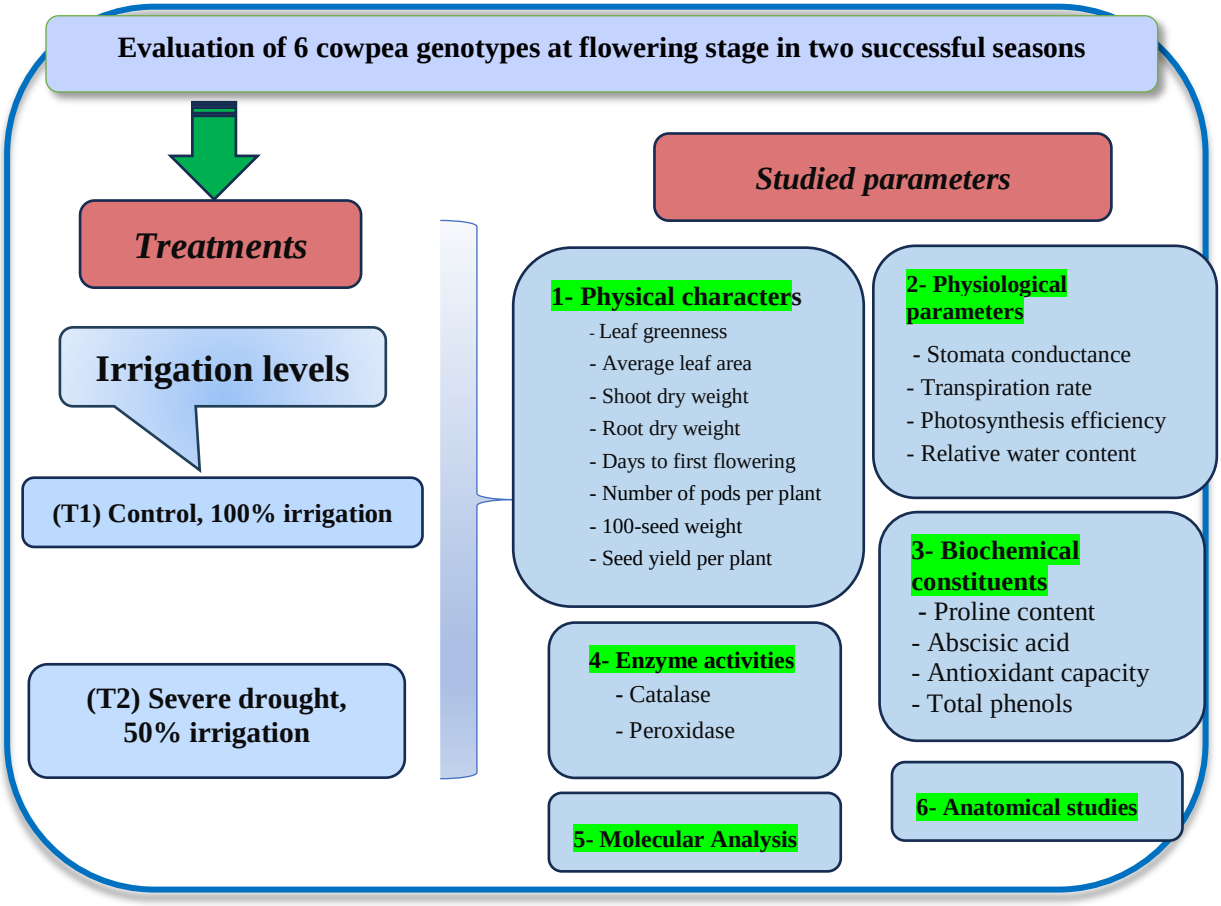

Fig. 1. An overview on the main treatments and measurements during the study.

2.5. Biochemical constituents

Biochemical constituents were measured in the 5 th mature leaf from meristem tip of the plant after 45 days of drought treatments. Free proline content was determined using a spectrophotometer (UNICO S2100, Cole Parmer Instruments, Vernon Hills, IL, USA) at 520 nm according to Bates *et al*. (1973). Abscisic acid (ABA)

was extracted and quantified in accordance with the guidelines provided by the AOAC (1990). The ABA concentration was determined using a technique outlined by Fales *et al*. (1973). The antioxidant activity was determined by measurement of DPPH radical scavenging activity according to Sanchez-Moreno (2002). The radical scavenging activity was calculated as a percentage of DPPH discoloration using the following equation:
DPPH radical scavenging % = $[(A_0 – A_1)/A_0] \times 100$
Where A_0 is the absorbance of the DPPH solution and A_1 is the absorbance of the sample. Total phenols were determined using Folin-Ciocalteu reagent by measuring the absorbance spectrophotometrically at 765 according to Singleton *et al*. (1999).

2.6. Enzyme activities

To measure peroxidase (POD) activity, 0.5 g of frozen leaf sample was crushed in 10 ml of extraction buffer (50 mM phosphate buffer, pH 7, containing 0.5 mM EDTA and 2% PVPP (w/v)). The mixture was then centrifuged at 3930 rpm for 20 minutes. The POD activity was measured according to Aebi (1984). The activity of catalase (CAT) was assessed using the Aebi (1983) procedure. Ten µl of enzymatic extract was combined with 120 µl of potassium phosphate buffer (KH2PO4, 50 mM, pH 7.0) and allowed to stabilize for 5 minutes at 25°C. Then, 70 µl of 0.2% (v/v) H_2O_2 was added, and the decomposition of H_2O_2 was recorded at 240 nm. The enzyme activity was calculated using H_2O_2's molar extinction coefficient of 0.0394 mM−1cm−1. One CAT unit is defined as the amount of enzyme required to break down one µmol of H_2O_2 per minute at 25°C.

2.7. Molecular Analysis

The fresh young leaves were the source for isolating genomic DNA, utilizing the method described by Khaled et al.,2015. To confirm the DNA's integrity, 1% agarose gel electrophoresis (AGE) in TBE buffer was employed. Subsequently, the DNA was adjusted to a working concentration of approximately 10 ng/µL using TE buffer. The PCR assay was executed in a reaction mix of 25 µL, incorporating specific primers to evaluate expression patterns. Primers were constructed according to literature and genes in GenBank detailed in Table 2. The amplification process and gel electrophoresis were performed using an MJ 200CT Thermal Cycler and a BioRad submarine gel, adhering to the methods described by AL-Taweel *et al*., (2019). The band patterns produced were documented with the JSC Gel Documentation system. CLIQS software, developed by Total Lab Ltd, was used to decipher the expression band patterns.

Table 2. Details of specific primers used to show expression patterns of cowpea under drought tolerance.

Primer code	Primer sequence	GC%	Tm
dT1-F	ATGGAGAATAATAAAGGAAATGCTG	32	59.2
dT1-R	TTTGCAAAACTTAAAGAGAATGAAA	24	56
dT2-F	ATGGAGAATAATAAAGGAAATGCTG	32	59.2
dT2-R	TTATATTTTCTTTTGGGATCTAAGGA	27	58.4

2.8. Anatomical studies

Samples used in anatomical studies were taken from the terminal leaflet of the fifth mature blade of cowpea plants during reproductive stage in the 2022 growing season. Samples represented six genotypes (3 most tolerant and 3 least tolerant), each subjected to 2 irrigation treatments. Approximately 1.0 cm from the specimens were killed and fixed in FAA solution (5 mL glacial acetic, 10 mL formalin, 35 mL water, and 50 mL ethyl alcohol 70%) for at least 48 h. The selected materials were washed in 30% ethyl alcohol, dehydrated in a normal ethanol and butyl alcohol series, embedded in paraffin wax with a melting point of 56°C, sectioned to a thickness of 15 µm stained with crystal violet-erythrosin, cleared in xylene and mounted in canada balsam in accordance with Mohammed and Guma (2015). Transverse sections were done with a Leica Microtome RM 2125, and then micrographed and measured using a Leica Light Image Analysis System DM 750 at the Faculty of Agriculture, Cairo University-Research Park (CURP). The following parameters were recorded: thickness of the midvein (µm), lamina (µm), palisade tissue (µm), and spongy tissue (µm), main bundle dimension (length –width) (µm), and mean vessels diameter (µm).

2.9. Statistical analyses

Analysis of variance and mean comparisons were conducted using MSTATC followed by Duncan´s multiple range seasons ($P \leq 0.05$) to determine significant differences (Snedecor and Cochran, 1989).

3. Results

3.1. Evaluation of 6 selected cowpea genotypes for drought tolerance during the reproductive stage

Six genotypes were selected and evaluated for drought tolerance during the reproductive stage. The average leaf area and leaf greenness of studied traits was influenced by irrigation, genotype and interaction in two consecutive seasons (Table 3). Significant differences in leaf area and leaf greenness were found among 6 cowpea accessions under both well-watered and drought conditions (Fig. 2 and 3). TVU-14997 had the largest leaf area under well-watered conditions, while TVU-3812 had the smallest. Under drought, TVU-15306 and TVU-14997 maintained the largest leaf area, while TVU-3812 was again the smallest.

For leaf greenness, TVU-3812 scored highest under well-watered conditions, while TVU-15304 and TVU-11619 scored lowest. Under drought, TVU-15304 and TVU-3812 remained highest, while TVU-9908 and TVU-11619 were lowest. Shoot dry weight was influenced by irrigation, genotype, and their interaction (Table 3), while root dry weight was affected by genotype and interaction (Table 3). Under well-watered conditions, TVU-15304 had the highest shoot dry weight in the first season (Fig. 4), while TVU-9908 and TVU-14997 were highest in the second. TVU-3812 and TVU-11619 were consistently lowest. Under drought, TVU-15306 had the highest shoot dry weight in the first season, while in the second season, it shared the highest values with TVU-14997 and TVU-15304. TVU-3812 and TVU-11619 were again lowest. For root dry weight (Fig. 5), TVU-9908 and TVU-14997 were highest under well-watered conditions in the first season, while TVU-14997, TVU-15304, and TVU-15306 were highest in the second. TVU-3812 and TVU-11619 were consistently lowest. Under drought, TVU-15304 had the highest root dry weight in both seasons, sharing the highest value with TVU-14997 in the second season. TVU-3812 and TVU-9908 were consistently lowest. The days to first flowering were affected by genotype and interaction, but not irrigation (Table 3). The number of pods per plant was influenced by irrigation and genotype (Table 3). TVU-15304 and TVU-15306 flowered earliest under well-watered conditions, while TVU-11619 and TVU-14997 flowered latest (Table. 4). Under drought, TVU-9908 flowered earliest, while TVU-11619, TVU-3812, and TVU-14997 flowered latest. TVU-14997 and TVU-11619 were least affected by drought in flowering time, while TVU-11619 and TVU-15304 were most affected. Under well-watered conditions, TVU-15304 had the highest number of pods per plant in the second season. TVU-3812, TVU-11619, and TVU-14997 were consistently lowest. When 6 cowpea accessions were evaluated for drought tolerance, accessions TVU-15304 and TVU-14997 showed the least negative impact in terms of number of pods per plant under drought (Table 4).

Seed yield per plant and 100-seed weight were significantly affected by irrigation, genotype, and their interaction (Table 3). Under sufficient irrigation, there were no significant differences in the first season, but in the second season, TVU-15306 had the highest 100-seed weight (Table 5). Under drought, TVU-15304 and TVU-14997 maintained higher 100-seed weights compared to other accessions (Table 5). Accessions TVU-14997, TVU-15304, TVU-9908, and TVU-15306 had the highest seed yield per plant under sufficient irrigation, while TVU-9908, TVU-3812, and TVU-11619 had the lowest (Table 5). Under drought, TVU-14997, TVU-15304, and TVU-306 performed best, while TVU-9908 and TVU-11619 performed worst (Table 5). TVU-15306 and TVU-15304 were the least affected by drought in terms of seed yield per plant. Significant differences in relative water content (RWC) and photosynthesis efficiency (Fv/Fm) were found among 6 cowpea accessions under sufficient irrigation and drought (Fig. 6 and 7).

Table 3. Analysis of variance (mean squares) for drought tolerance in 6 selected cowpea genotypes concerning the studied traits during the reproductive stage in two consecutive seasons.

Source of variation		Irrigation (I)	Genotype (G)	I×G
d.f.		1	5	5
Average leaf area (cm^2)	**1st season**	7639.634**	3325.167**	383.337**
	2nd season	10819.467 **	2717.695**	429.214**
Leaf greenness (SPAD)	**1st season**	1110.000**	267.079**	182.625**
	2nd season	366.723**	209.424**	143.662**
Shoot dry weight (g)	**1st season**	116.892 **	34.819 **	4.790 **
	2nd season	72.818 **	9.955**	4.184**
Root dry weight (g)	**1st season**	0.344 ns	9.006 **	4.274 **
	2nd season	0.267 ns	45.741**	7.909**
Days to first flowering	**1st season**	0.694 ns	179.917 **	96.228 **
	2nd season	0.444 ns	331.333 **	90.978 **
Number of pods per plant	**1st season**	40.111 **	12.667 **	1.578 ns
	2nd season	64.000 **	14.267 **	2.933 *
100-seed weight (g)	**1st season**	129.050 **	11.297 **	0.757 ns
	2nd season	290.702**	12.944 **	3.955 **
Seed yield per plant (g)	**1st season**	31.622**	3.544 **	0.664 **
	2nd season	51.361**	3.076 **	1.186 **
Relative water content (%)	**1st season**	3502.075 **	257.105 **	460.394**
	2nd season	4747.210 **	465.789 **	749.150 **
Photosynthesis efficiency (F_v/F_m)	**1st season**	1.356 **	0.011 **	0.012 **
	2nd season	1.171**	0.038 **	0.035 **
Stomata conductance (cm/s)	**1st season**	0.009 **	0.004 **	0.001 **
	2nd season	0.009 **	0.002 **	0.001 **
Transpiration rate (µg H_2O/ cm^2.s)	**1st season**	1.660 **	1.970 **	0.500 **
	2nd season	6.829**	0.560 **	0.111 **
Proline content (m mole/100g FW)	**1st season**	5584.573 **	643.957 **	660.333 **
	2nd season	1709.823 **	489.514 **	347.612 **
Abscisic acid (µg/g FW)	**1st season**	151.992 **	7.427 **	7.635 **
	2nd season	168.783 **	6.524 **	6.752 **
Total phenols (mg/100g FW)	**1st season**	648696.023 **	78716.913 **	22072.880 **
	2nd season	775074.788 **	73525.783 **	21323.701**
Antioxidant capacity (%)	**1st season**	623.334 **	1024.932 **	692.807 **
	2nd season	701.190 **	546.461 **	544.694 **
Catalase (units mg^{-1} protein)	**1st season**	152.300 **	2.556 **	5.773 **
	2nd season	156.876 **	4.917 **	10.059 **
Peroxidase (units mg^{-1} protein)	**1st season**	83.509**	5.334**	5.135 **
	2nd season	89.114 **	4.533 **	4.924 **

*, **, ns significant at P= 0.05 and P= 0.01 levels and not significant, respectively.

Under drought, TVU-14997 and TVU-15306 showed the highest RWC, while TVU-9908 was the most affected. For F_v/F_m, TVU-15306 and TVU-15304 were the highest, while TVU-11619 and TVU-9908 were most affected by drought. Stomata conductance and transpiration rate were also affected by irrigation, genotype, and their interaction (Table 3). Significant differences in stomata conductance and transpiration rate were found among 6 cowpea accessions under sufficient irrigation and drought (Table 6). Under drought, TVU-3812 had the highest stomata conductance, while TVU-14997, TVU-15304, and TVU-15306 had the lowest. TVU-9908 and TVU-11619 maintained the highest transpiration rate under drought. Proline content and abscisic acid were also affected by irrigation, genotype, and their interaction (Table 3). Significant differences in proline and abscisic

acid content were found among 6 cowpea accessions under sufficient irrigation and drought (Fig. 8 and 9). Under drought, TVU-15304 and TVU-15306 showed the highest proline content, while TVU-9908 and TVU-11619 were the lowest. TVU-15304 and TVU-15306 also had the highest abscisic acid content, while TVU-9908 and TVU-11619 were the lowest. Total phenols and antioxidant capacity were also affected by irrigation, genotype, and their interaction (Table 3). Significant differences in total phenols and antioxidant capacity were found among 6 cowpea accessions under sufficient irrigation and drought (Table 7). Under drought, TVU-15304, TVU-15306, and TVU-14997 had the highest total phenols, while TVU-9908 and TVU-11619 were the lowest. TVU-15304, TVU-15306, and TVU-14997 also had the highest antioxidant capacity under drought, while TVU-9908 and TVU-11619 were the lowest. TVU-15306 and TVU-14997 were the least affected by drought in antioxidant capacity. Catalase and peroxidase were also affected by irrigation, genotype, and their interaction (Table 3). Significant differences in catalase and peroxidase activity were found among 6 cowpea accessions under sufficient irrigation and drought (Fig. 10 and 11). Under drought, TVU-15304 and TVU-15306 had the highest catalase activity, while TVU-9908 and TVU-11619 had the least. For peroxidase activity, TVU-15306 had the highest activity under drought, while TVU-9908 and TVU-11619 had the least.

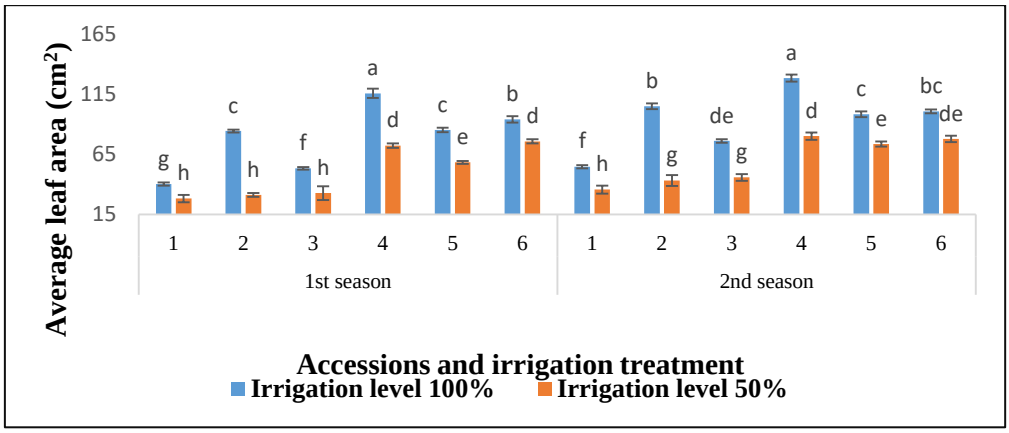

Fig. 2. Effect of drought stress (full irrigation 100% (control), severe drought 50%) on average leaf area of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306.

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05

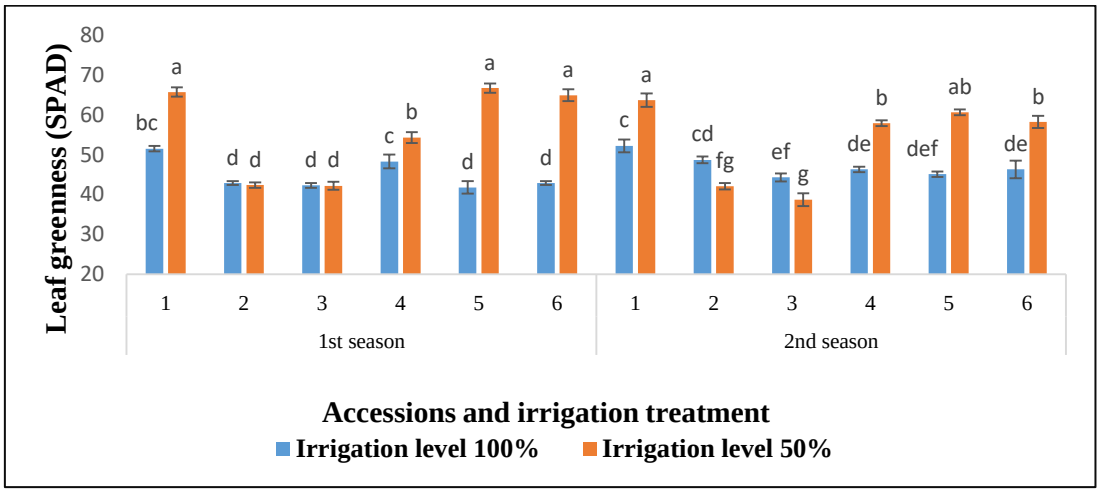

Fig. 3.Effect of drought stress (full irrigation 100% (control), severe drought 50%) on leaf greenness of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306.

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05

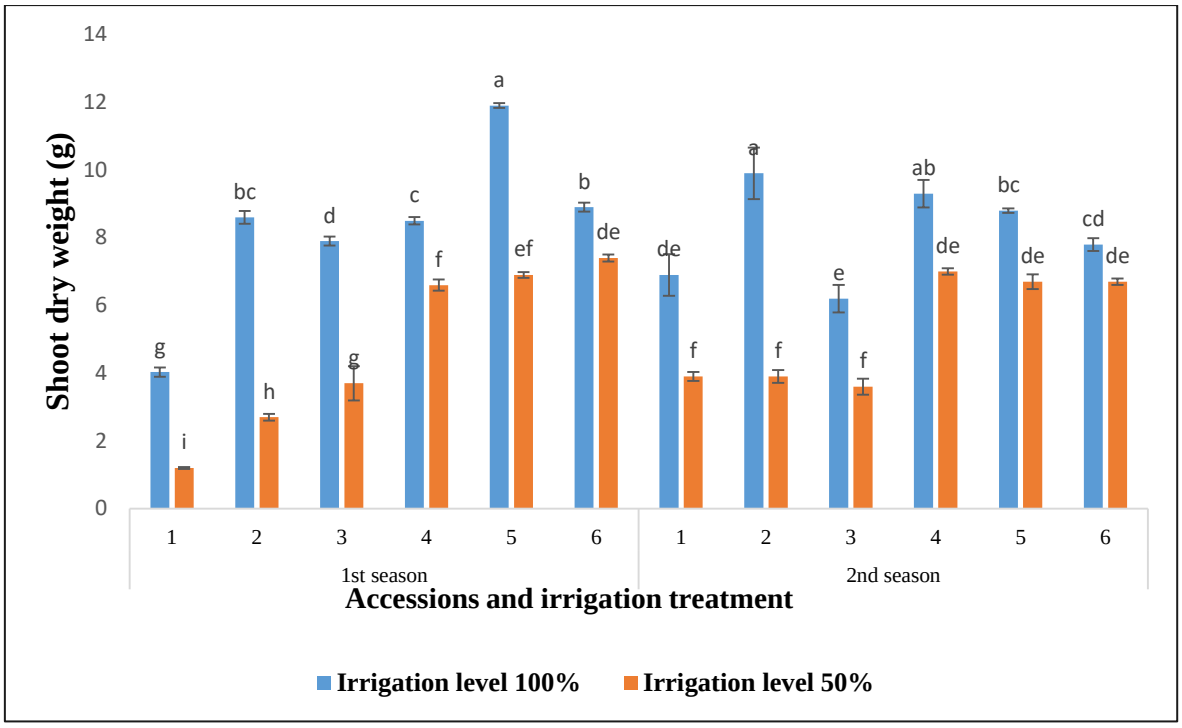

Fig. 4. Effect of drought stress (full irrigation 100% (control), severe drought 50%) on shoot dry weight of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306.

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05

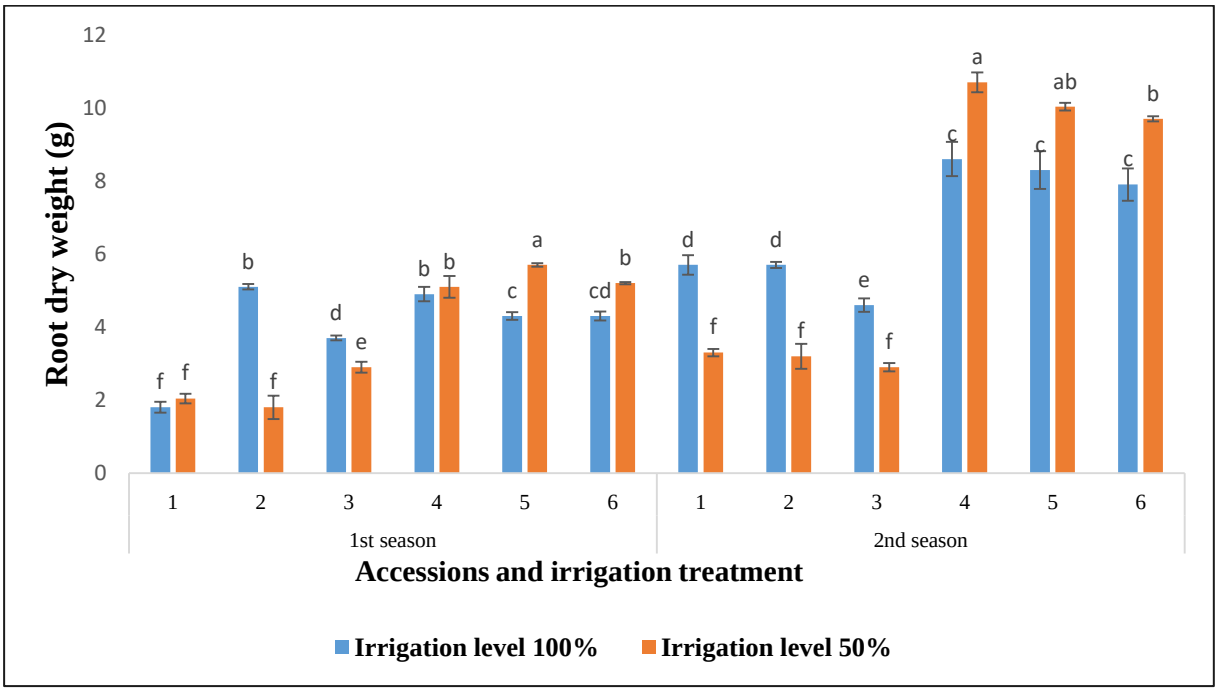

Fig. 5. Effect of drought stress (full irrigation 100%(control), severe drought 50%) on root dry weight of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306.

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

Table 4. Effect of drought stress (full irrigation 100%(control), severe drought 50%) on days to first flowering and number of pods per plant of 6 selected cowpea genotypes during the reproductive stage in two consecutive seasons.

Genotypes	Days to first flowering						Number of pods per plant					
	1st season			2nd season			1st season			2nd season		
	Irrigation		% Change	Irrigation		% Change	Irrigation		% Change	Irrigation		% Change
	100%	50%		100%	50%		100 %	50%		100 %	50%	
TVU-3812	62.0 de	65.6 bc	+5.8	68.3 bc	74.6 ab	+9.2	4.3	2.6	-39.5	4.6 b	2.3 c	-50
TVU-9908	65.0 cd	56.7 fg	-12.7	63.0 cde	55.6 f	-11.7	6.6	2.6	-60.6	7.3 a	2.3 c	-68.4
TVU-11619	66.3 bc	76.3 a	+15.08	77.0 a	65.3 cd	-15.1	4.3	2.0	-53.4	4.6 b	1.6 c	-65.2
TVU-14997	69.0 b	58.3 f	-15.5	74.0 ab	76.3 a	+3.1	6.3	5.3	-15.8	5.3 b	4.6 b	-13.2
TVU-15304	53.7 g	57.3 f	+6.7	55.0 f	61.0 def	+10.9	7.0	5.3	-24.2	8.3 a	5.6 b	-32.5
TVU-15306	56.7 fg	60.0 ef	+5.08	58.0 ef	63.7 cde	+9.8	7.6	5.6	-26.3	7.6 a	5.3 b	-30.2
F-test	**			**			ns			*		

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

Table 5. Effect of drought stress (full irrigation 100%(control), severe drought 50%) on 100-seed weight and seed yield per plant of 6 selected cowpea genotypes during the reproductive stage in two consecutive seasons.

Genotypes	100-seed weight (g)						Seed yield per plant (g)					
	1st season			2nd season			1st season			2nd season		
	Irrigation		% Change	Irrigation		% Change	Irrigation		% Change	Irrigation		% Change
	100%	50%		100%	50%		100%	50%		100%	50%	
TVU-3812	12.16	8.57	-29.5	11.23 b	4.63 e	-58.7	4.26 b	2.43 f	-42.9	5.30 b	3.33 e	-37.1
TVU-9908	8.86	5.66	-36.1	7.83 c	3.33 f	-57.4	3.41 d	2.11 g	-38.1	6.33 a	2.30 f	-63.6
TVU-11619	11.86	7.01	-40.8	12.13 ab	3.63 f	-70.07	4.38 b	2.21 fg	-49.5	5.17 b	2.60 f	-49.7
TVU-14997	12.87	8.46	-34.2	11.27 b	6.93 cd	-38.5	5.77 a	3.71 c	-35.7	6.30 a	3.90 d	-38.09
TVU-15304	12.52	9.50	-24.1	11.57 ab	6.97 cd	-39.7	5.99 a	3.10 e	-48.2	6.23 a	4.70 c	-24.5
TVU-15306	12.18	8.53	-29.9	12.33 a	6.77 d	-45.09	4.22 b	3.21 de	-23.9	6.57 a	4.73 c	-28
F-test	ns			**			**			**		

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

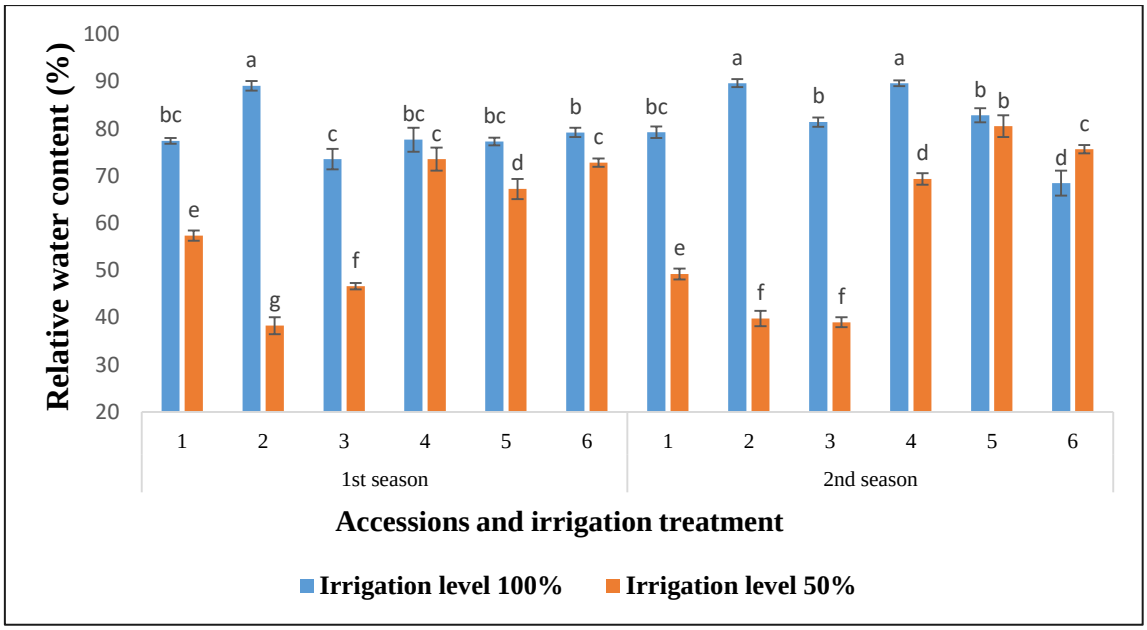

Fig. 6. Effect of drought stress (full irrigation 100% (control), severe drought 50%) on relative water content of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

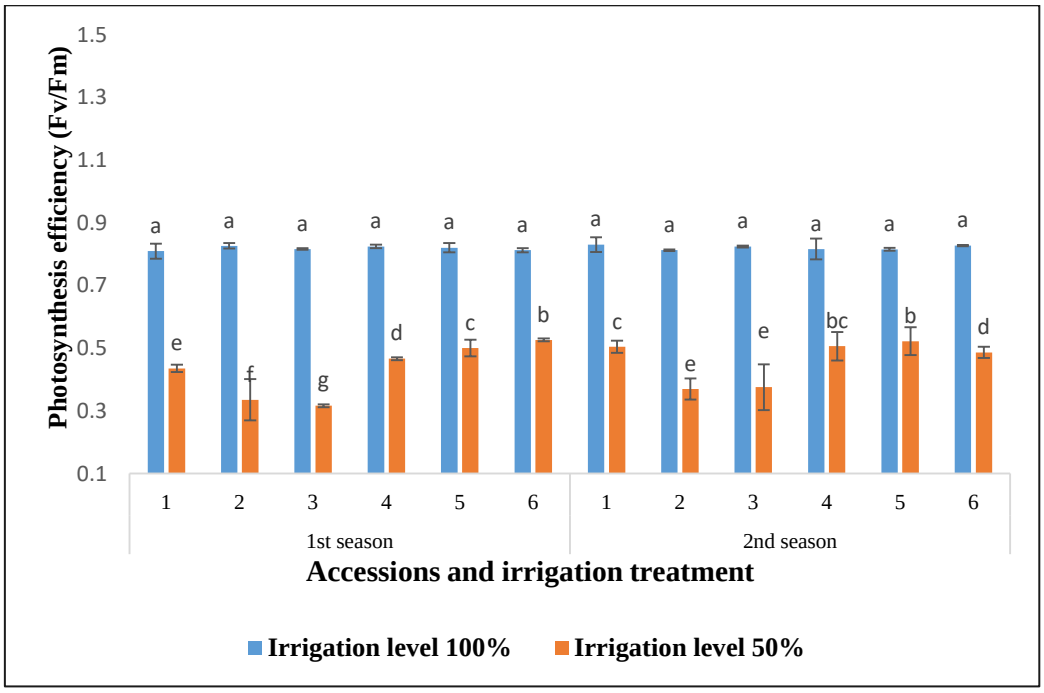

Fig. 7. Effect of drought stress (full irrigation 100% (control), severe drought 50%) on photosynthesis efficiency of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306.

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

Table 6. Effect of drought stress (full irrigation 100%(control), severe drought 50%) on stomata conductance and transpiration rate of 6 selected cowpea genotypes during the reproductive stage in two consecutive seasons.

genotypes	Stomata conductance (cm/s)						Transpiration rate (µg H_2O/ cm^2.s)					
	1st season			2nd season			1st season			2nd season		
	Irrigation		% Change	Irrigation		% Change	Irrigation		% Change	Irrigation		% Change
	100%	50%		100%	50%		100%	50%		100%	50%	
TVU-3812	0.227 bc	0.237 b	+4.4	0.214 bc	0.209 bc	-2.3	5.09 b	4.62 cd	-9.2	5.29 a	4.48 c	-15.3
TVU-9908	0.280 a	0.217 c	-22.5	0.239 a	0.220 b	-7.9	5.75 a	5.87 a	+2.08	5.35 a	4.93 b	-7.8
TVU-11619	0.217 c	0.211 c	-2.7	0.216 bc	0.209 bc	-3.2	5.77 a	5.63 a	-2.4	5.52 a	4.78 b	-13.4
TVU-14997	0.217 c	0.172 de	-20.7	0.207 bc	0.156 d	-24.6	4.53 cd	4.68 c	+3.3	5.24 a	4.21 d	-19.6
TVU-15304	0.242 b	0.179 de	-26.03	0.223 ab	0.159 d	-28.6	5.10 b	3.88 e	-23.9	5.26 a	4.18 d	-20.5
TVU-15306	0.188 d	0.170 e	-9.5	0.199 c	0.156 d	-21.6	5.25 b	4.24 de	-19.2	4.91 b	3.77 e	-23.1
F-test	**			**			**			**		

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

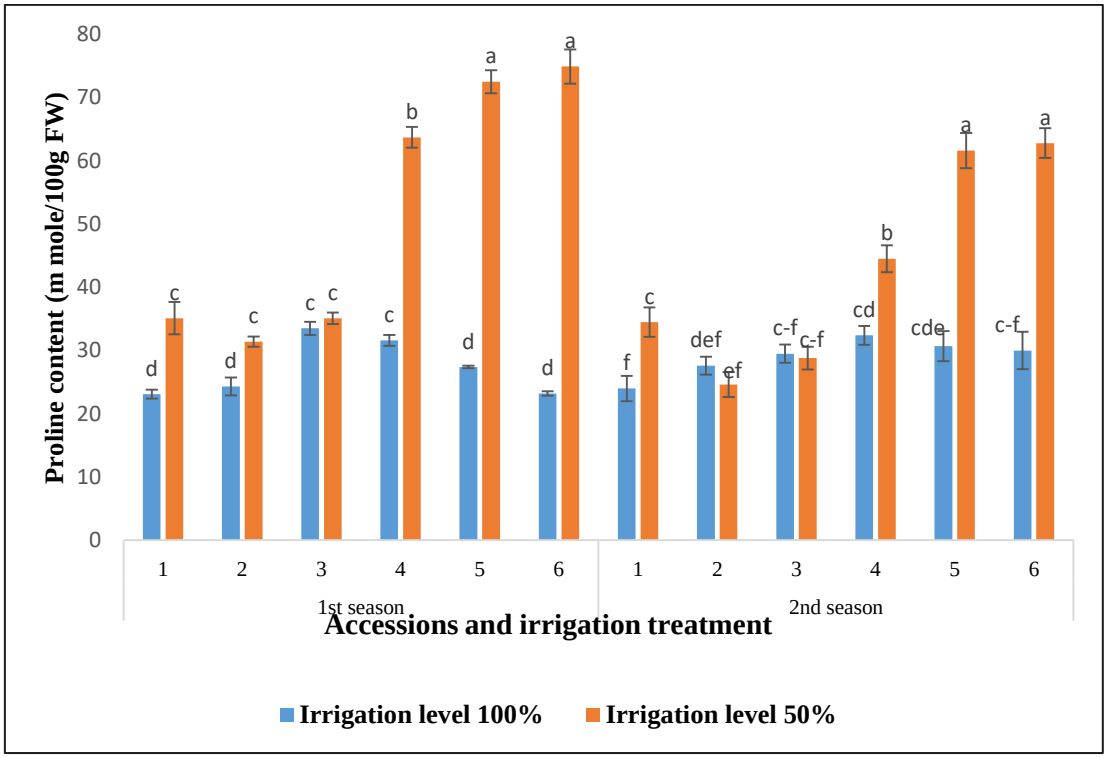

Fig. 8. Effect of drought stress (full irrigation 100% (control), severe drought 50%) on proline content of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306.

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

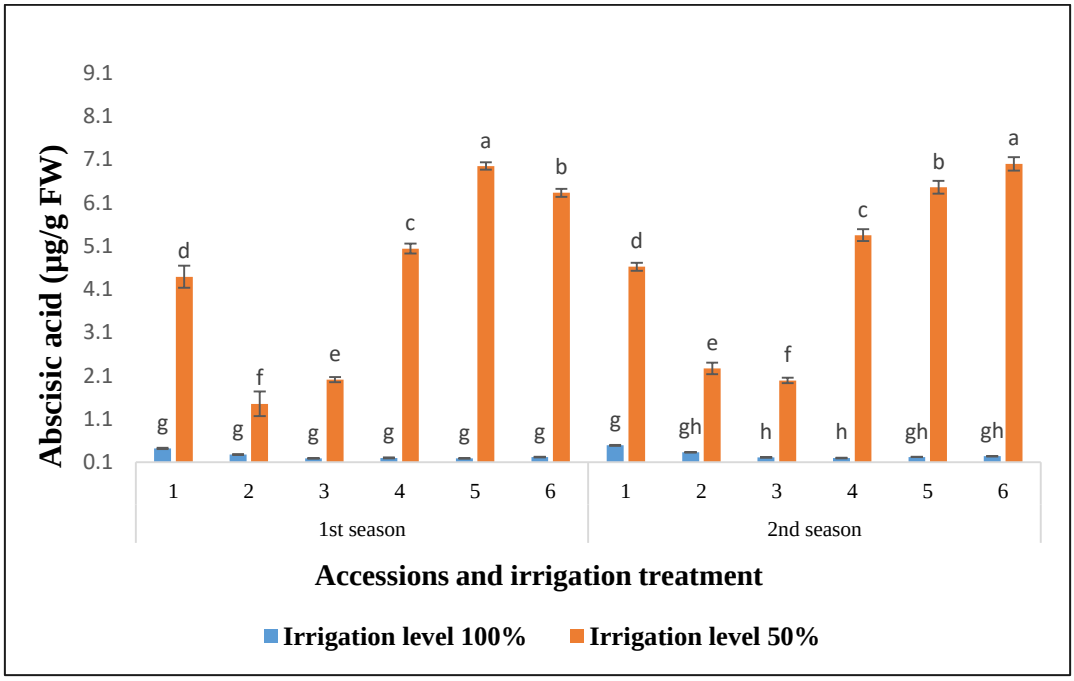

Fig. 9. Effect of drought stress (full irrigation 100% (control), severe drought 50%) on abscisic acid of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306. Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

Table 7. Effect of drought stress (full irrigation 100%(control), severe drought 50%) on total phenols and antioxidant capacity of 6 selected cowpea genotypes during the reproductive stage in two consecutive seasons.

Genotypes	Total phenols (mg/100g FW)						Antioxidant capacity (%)					
	1st season			2nd season			1st season			2nd season		
	Irrigation		% Change	Irrigation		% Change	Irrigation		% Change	Irrigation		% Change
	100%	50%		100%	50%		100%	50%		100%	50%	
TVU-3812	310.1 g	536.6 c	+73.04	295.4 e	549.8 b	+86.1	36.9 g	44.6 e	+20.8	40.8 e	59.9 b	+46.8
TVU-9908	241.1 h	385.3 de	+59.8	247.7 f	415.1 c	+67.5	51.4 d	26.2 h	-49.02	52.4 c	31.1 g	-40.6
TVU-11619	258.5 h	414.0 d	+60.1	260.9 f	409.2 c	+56.8	37.9 fg	29.4 h	-22.4	37.2 f	30.6 g	-17.7
TVU-14997	340.0 fg	593.1 b	+74.4	356.3 d	758.0 a	+112	37.6 fg	59.0 c	+56.9	35.0 f	64.4 a	+84
TVU-15304	368.6 ef	779.5 a	+111	349.8 d	760.0 a	+117	55.3 c	84.5 a	+52.8	54.6 c	66.9 a	+22.5
TVU-15306	372.3 ef	792.8 a	+112	367.0 d	745.7 a	+103	41.1 ef	66.5 b	+61.8	44.9 d	65.0 a	+44.7
F-test	**			**			**			**		

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

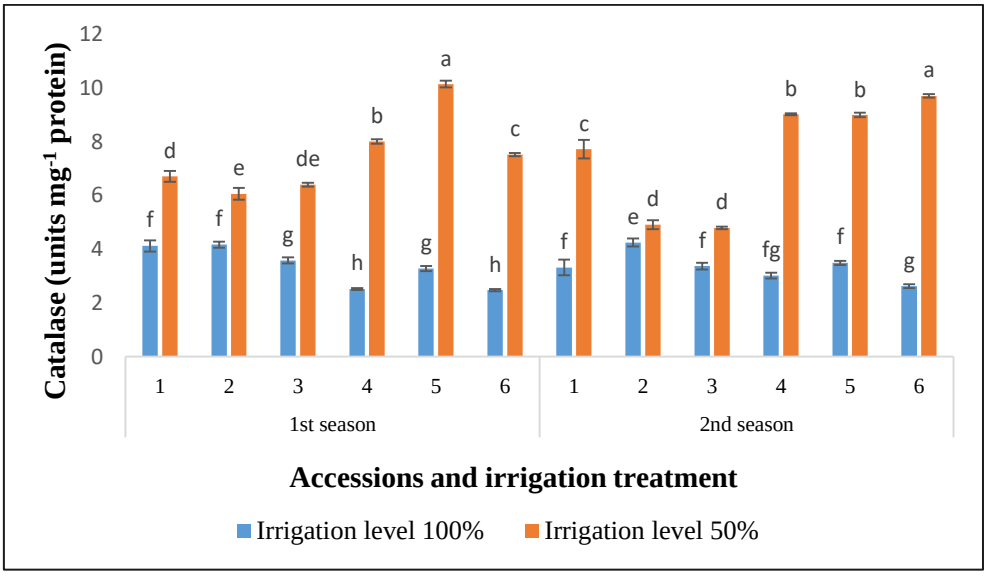

Fig. 10. Effect of drought stress (full irrigation 100% (control), severe drought 50%) on catalase of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306. Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

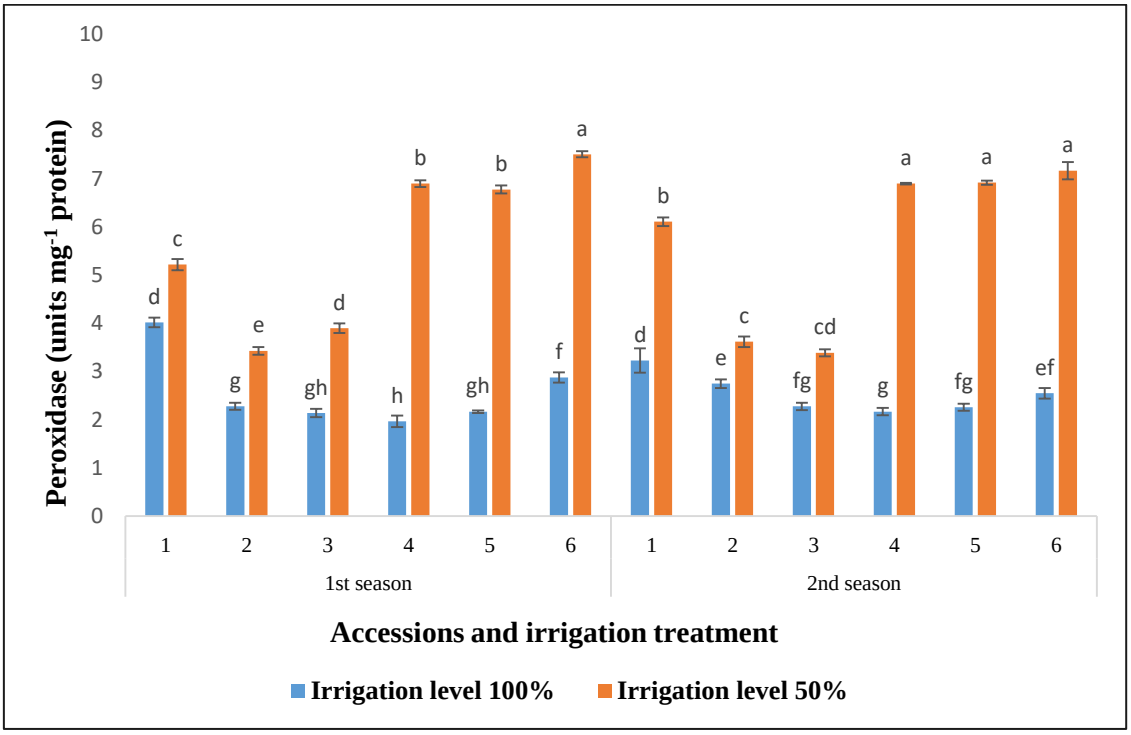

Fig. 11. Effect of drought stress (full irrigation 100% (control), severe drought 50%) on peroxidase of 6 selected cowpea accessions during the reproductive stage in two consecutive seasons. 1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306. Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

3.2. Molecular basis of drought tolerance

The molecular results corresponding to the genetic analysis of the cowpea genotypes show DNA fragments amplified using two different primers under drought conditions. These primers target specific genes or regions of the cowpea genome that are thought to be associated with drought tolerance. The presence or absence of specific bands in the gel could indicate genetic variations between the cowpea genotypes. Primer 1 has a distinct band with 245 bp which is present in genotypes TVU-3812, TVU-9908, TVU-11619 and TVU-15304, while these bands are absent in genotypes TVU-14997 and TVU-15306 (Figure 12). This suggests that the cowpea genotypes TVU-3812, Tvu-9908, TVU-11619 and TVU-15304 share a genetic variant that is not present in genotypes Tvu-14997 and Tvu-15306. This variant could be related to a gene or regulatory region involved in drought tolerance. This primer appears to target a gene that is downregulated in response to drought in most of the cowpea genotypes. This is evident by the fainter bands in the drought-treated genotypes (TVU-3812, TVU-9908, TVU-11619, TVU-15304, TVU-15306). This finding is consistent with the results in (Fig. 4 and 5), which shows that drought significantly reduced shoot dry weight and root dry weight in most of these genotypes.

The downregulation of genes involved in root development could explain this reduction in root biomass. However, TVU-14997 is an exception, suggesting that the expression of this gene might not be affected by drought in this genotype. This aligns with the finding in (Fig. 5) that TVU-14997 was one of the least affected genotypes in terms of root dry weight reduction under drought. Similarly, in Primer 2 (Figure 13), there are two distinct bands (320 and 350 bp), the band of 350 bp appeared in genotypes TVU-3812, TVU-9908, TVU-11619, TVU-14997 and TVU-15304, with genotype TVU-15306 appearing to have an additional band (320 bp). This could indicate that the cowpea genotype TVU-15306 possesses an additional genetic variant that might be associated with drought tolerance. This primer seems to target a gene that is upregulated under drought conditions in most genotypes. The bands in the drought-treated genotypes (TVU-3812, TVU-9908, TVU-11619, TVU-14997, TVU-15304) are more intense.

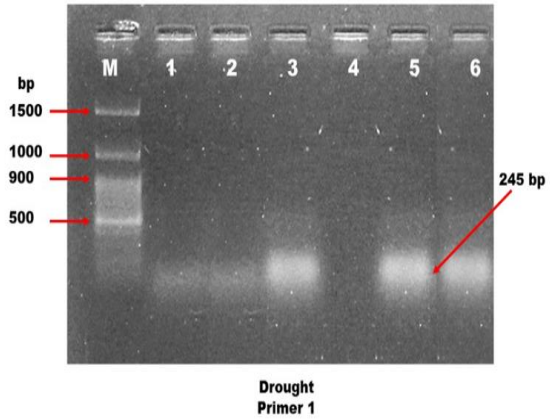

Fig. 12. Patterns of primer 1 against six cowpea genotypes revealing the drought tolerance markers. M= DNA ladder, **1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306.**

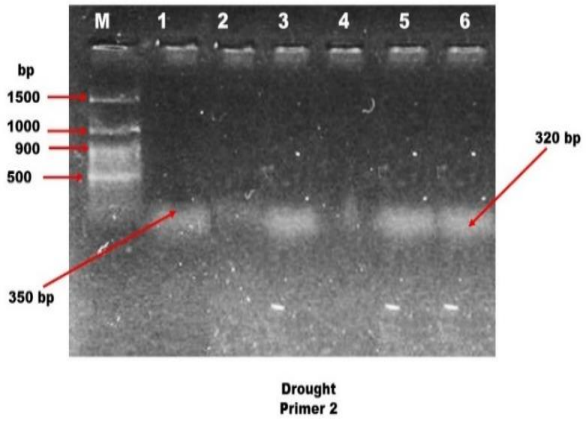

Fig. 13. Patterns of primer 2 against six cowpea genotypes revealing the drought tolerance markers. M= DNA ladder, **1= TVU-3812, 2= TVU-9908, 3= TVU-11619, 4 = TVU-14997, 5= TVU-15304 and 6= TVU-15306.**

3.3 Anatomical studies

Anatomical characteristics of cowpea leaves of various genotypes growing under drought stress during the reproductive stage are shown in Table 8 and Figure 14, 15. Referring to full irrigation, the TVU-14997 accession exhibited the highest values in terms of thickness of midvein and dimension of vascular bundle

(2158.16 µm and 785.11µm respectively, figure D). While the Tvu-15306 accession excelled in the thickness of spongy tissues and mean vessels diameter (235.43 µm and 23.16 µm, respectively, figure F). Meanwhile, TVU-9908 accession showed the least thickness of the midvein, lamina, palisade tissue, spongy tissue, dimension of vascular bundle and mean vessels diameter (1500.64 µm, 248.78 µm, 119.14 µm, 682.53 µm and 27.98 µm, respectively, figure A). Under drought condition, accession TVU-15306 had the highest value for thickness of the midvein, spongy tissue, dimension of vascular bundle and mean vessels diameter (1716.15 µm, 256.16 µm, 750.96 µm and 32.16 µm, respectively, figure F). whilst, TVU-15304 accession gave the highest value for the thickness of lamina and palisade tissue (383.64 µm and 214.20 µm, respectively, figure E). The least affected accession by drought stress in leaf anatomical characteristics was TVU-15306 for the thickness of the midvein, dimension of vascular bundle and mean vessels diameter by 8.27%, 3.78% and 0.27%, respectively, less than the control (TVU-15306, 100% irrigation, respectively, figure F and F1), while the thickness of lamina, palisade tissue and spongy tissue increased by 9.94%, 12.92% and 8.80%, respectively, when compared to the control (TVU-15306, 100% irrigation, figure F and F1).

Table 8. Effect of drought stress treatment on the terminal leaflet anatomical parameters of cowpea genotypes in the reproductive during the 2022 growing season. Measurements are in mm.

Water regime (WR)	**Sufficient irrigation**	**Drought stress**	**Sufficient irrigation**	**Drought stress**	**Sufficient irrigation**	**Drought stress**
genotypes	Thickness of midvein		Thickness of lamina		Thickness of palisade tissue	
TVU-3812	1515.21 e	1126.03 h	422.05 a	366.34 e	190.72 b	157.23 d
TVU-9908	1500.64 f	1114.65 i	248.78 j	228.90 k	129.64 f	112.10 g
TVU-11619	1618.48 d	1475.20 g	414.29 b	338.18 h	192.66 b	150.40 e
TVU-14997	2158.61 a	1708.53 c	350.98 g	380.49 d	159.26 d	174.40 c
TVU-15304	1866.25 b	1710.62 c	352.31 g	383.64 c	148.55 e	214.20 a
TVU-15306	1870.90 b	1716.15 c	325.81 i	358.22 f	90.38 i	102.06 h
F-test	**		**		**	
Treatments	Thickness of spongy tissue		Mean diameter of xylem vessels		Dimensions of main vascular bundle	
TVU-3812	231.33 c	209.11 e	30.82 f	30.75 g	689.00 e	542.47 h
TVU-9908	119.14 i	116.80 i	27.98 i	25.79 j	682.53 f	500.92 i
TVU-11619	221.63 d	187.75 g	30.90 e	30.85 ef	689.12 e	551.17 g
TVU-14997	190.72 g	206.09 ef	32.11 c	32.00 d	785.11 a	710.67 d
TVU-15304	203.76 f	169.44 h	32.96 a	30.00 h	770.47 b	711.28 d
TVU-15306	235.43 b	256.16 a	32.19 b	32.10 c	780.51 a	750.96 c
F-test	**		**		**	

Values followed by a letter in common are not significantly different from each other according to Duncan´s multiple range season at P =0.05.

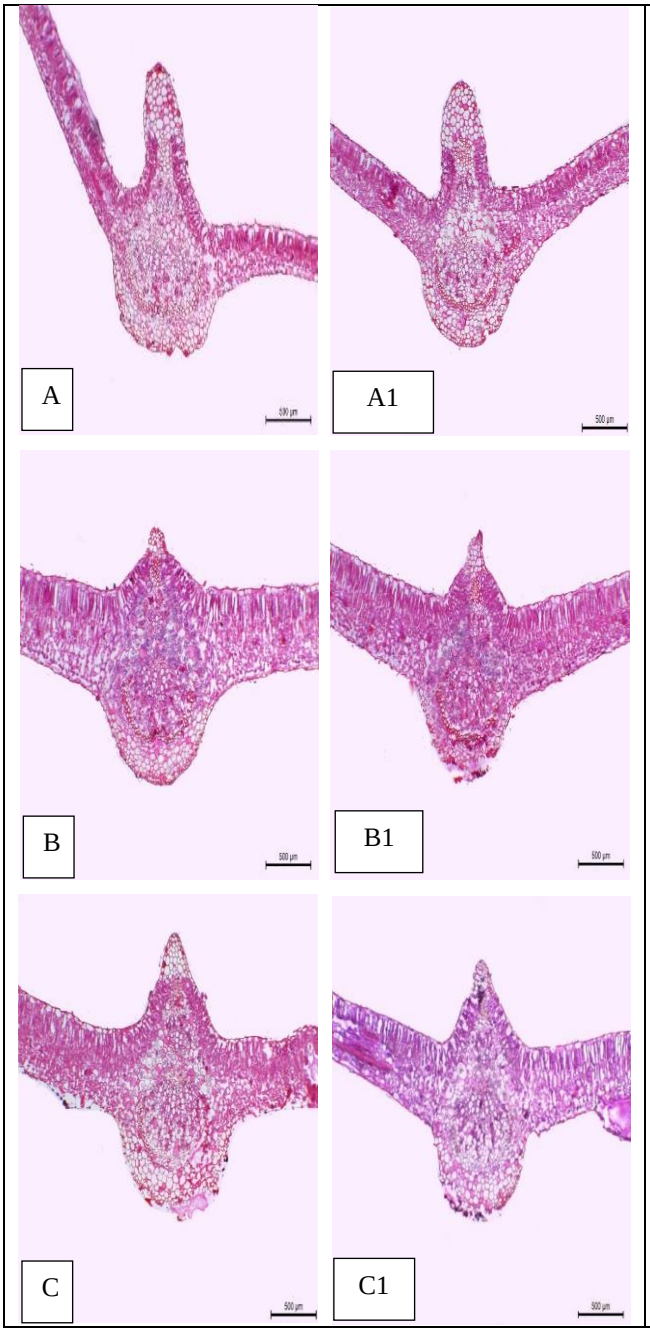

Fig. 14. Microphotographs of cross sections through the terminal leaflet of cowpea plants of genotypes affected by drought stress treatment during reproductive stage in the 2022 growing season. Scale bars = 500 µm. A- TVU-9908, B- TVU-3812, C- TVU-11619 (full irrigation), A1- -TVU-9908, B1- TVU-3812, C1- TVU-11619 (severe drought).

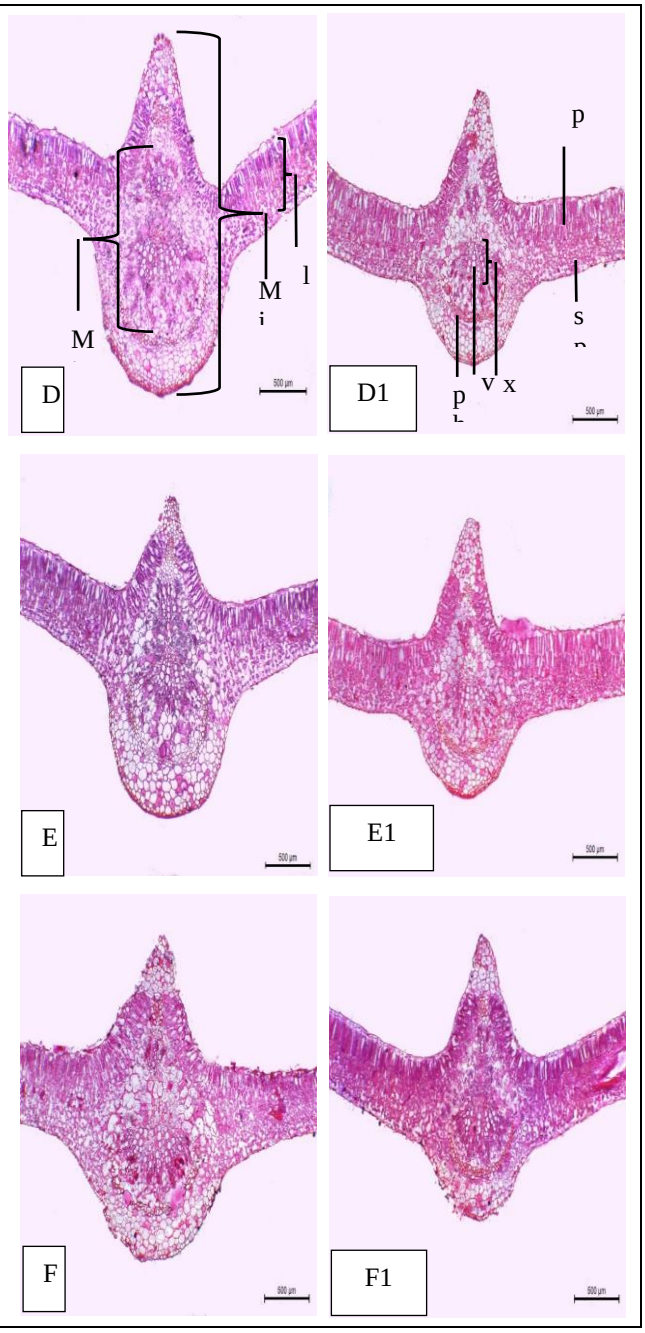

Fig. 15. Microphotographs of cross sections through the terminal leaflet of cowpea plants of genotypes affected by drought stress treatment during reproductive stage in the 2022 growing season. Scale bars = 500 µm. D- TVU-14997, E- TVU-15304, F- TVU-15306 (full irrigation), D1- TVU-14997, E1- TVU-15304, F1- TVU-15306 (severe drought).

abbreviations: mid b, midvein bundle; mid r, midvein region; ph, phloem; v, vessel; x, xylem; spo, spongy tissue; p, palisade tissue and l, lamina.

4. Discussions

Flowering and pod-filling are critical for cowpea grain yield (Hamidou *et al*., 2007). Our study indicates decreases in leaf area and shoot dry weight in all accessions under drought stress (Fig.2 and Fig. 4), these results agree with those of Rivas *et al*. (2016), Goufo *et al*. (2017) and Zegaoui *et al*. (2017). Constable and Hearn, (1978) indicated that reduced leaf area can be attributed to the acceleration of leaf senescence and abscission. Decreases in crop yield due to water stress was primarily caused by a reduction in leaf area leading to reduced photosynthesis (Correia *et al*., 2001). Reduced shoot biomass helps maintain hydration (Iseki *et al*., 2018). The decrease in growth caused by water stress can be linked to a reduction in cellular expansion caused by a reduction in plant water content (Kramer and Boyer, 1995).

Under the stress condition of drought, it has been noticed significant increases in Photosynthetic efficiency (Fig. 7) as a result of increasing chlorophyll levels in leaves (Fig. 3) and leaf area (Fig. 2) stress resulted also in reductions in midvein and vascular tissue along with increased lamina and palisade/spongy tissue, resulting in enhancing yield (Table 5) in accessions TVU-14997, TVU-15304 and TVU-15306. Genotypes under water stress may experience an increase in root biomass because cowpeas have the capability to redirect nutrients to promote root growth, allowing them to access deeper soil water (Turk and Hall, 1980). Drought stress also affects flowering time (Lalsaga *et al*., 2016) and reduces grain yield due to production of fewer pods in cowpea (Bastos *et al*., 2011) and reduces development and productivity in rice (Abd EL-Aty *et al*. 2023). This is attributed to decreased photoassimilate synthesis for seed filling (Lalsaga *et al*., 2016). Accessions TVU-14997, TVU-15304, and TVU-15306 showed the least impact on seed weight and yield (Table 5). Relative water content (RWC), an indicator of plant water status, was significantly affected by drought (Fig. 6), consistent with Lobato *et al*. (2009). Higher RWC in drought-tolerant cultivars helps maintain leaf health (Siddique *et al*., 2000). Bousba *et al*. (2009) reported that decrease in chlorophyll content in stressed plants may be due to the fact that chlorophylls are broken down more than they are produced in these plants, whereas in unstressed plants.

The decrease in Chl levels is recognized as a drought reaction to reduce light absorption by chloroplasts (Pastenes *et al*., 2005). Photosynthetic efficiency decreased under drought, negatively impacting accessions TVU-11619, TVU-9908, and TVU-3812 (Fig. 7). That was in support of Al-Khatib and Paulsen (1984) who reported reduced maximum fluorescence (F_m) and Fv/Fm under drought; thus, reducing photosynthesis. Drought also affects stomatal conductance, crucial for regulating transpiration (Martínez-Vilalta and Garcia-Forner, 2017). Stomatal limitation is often caused by increased ABA in levels under reduced soil water (Wilkinson and Davies, 2010). All genotypes showed reduced stomatal conductance under drought, confirming cowpea's stomatal closure mechanism in response to leaf water potential changes (Martínez-Vilalta and Garcia-Forner, 2017). Cowpea varieties close their stomata as a drought avoidance strategy to prevent and reduce water loss, as was found in previous research (Souza et al.2004). Genotypes TVU-15306 and TVU-15304 maintained greater stomatal conductance despite reduced transpiration (Table 6). Belko *et al*. (2013) found that genotypes sensitive to drought exhibited greater transpiration rates compared to drought-tolerant genotypes under both well-watered and severe drought conditions. In a Accessions TVU-3812, TVU-9908 and TVU-11619 it significant decreases in RWC were noticed (Fig. 6) as a result of transpiration increase (Table 6), and showed deficiencies in anatomical characteristics like midvein, lamina, and palisade/spongy tissue thickness, resulting in yield decline (Table 5) under drought stress.

Anya and Herzog (2004) reported that cowpea plants use the reduction of transpiration surfaces as a strategy to avoid drought. Leaf proline content increased significantly under stress (Fig. 8), consistent with other studies (Merwad *et al*., 2018). Ahmed *et al*. (2009) reported that proline is recognized as an osmolyte that builds up in the leaves of various species experiencing water stress, aiding in maintaining cell and tissue function in a water-stress. Proline accumulation, through increased synthesis or reduced degradation, enhances drought tolerance (Gill and Tuteja 2010). Plant phenolics, common in legumes, act as antioxidants protecting against ROS damage (Sombié *et al*., 2018; Fariaszewska *et al*., 2017). Significant differences were observed in antioxidant activity and total phenols among genotypes with/without drought stress (Table 7), agreeing with Sombié *et al*. (2018). Enzymatic antioxidants like peroxidase (POD) and catalase play crucial roles in ROS defense under drought (Naveed *et al*., 2014). Salama *et al*., (2024) showed that catalase, peroxidase and proline were increased under drought stress compare control. Accessions TVU-15304 and TVU-15306 showed higher POD and catalase activity under drought, suggesting greater drought tolerance (Fig. 10 and 11). These findings align with observations of increased peroxide activity in drought-tolerant soybean cultivars under stress (Iqbal *et al*., 2019).

Molecular data confirmed gene expression changes observed in field trials. Down regulation of a gene potentially involved in root development (Primer 1) could explain reduced root biomass, while up regulation of a gene potentially involved in stress response (Primer 2) suggests a drought tolerance mechanism. Exceptions in TVU-14997 (Primer 1) and TVU-15306 (Primer 2) highlight genetic diversity in drought responses. Similar gene expression changes under drought have been reported in other crops like wheat (Khaled *et al*., 2022), rice (Todaka *et al*., 2015), and maize (Zheng *et al*., 2010). Drought's impact on photosynthesis can lead to reduced leaf size (Nunes et al., 2022), consistent with findings in cowpea cv. Tepa (Arnaout *et al*., 2019) and broad bean (Abdelaal, 2015).

5. Conclusions and Future Prospective

The comprehensive evaluation of six cowpea genotypes under drought conditions during the reproductive stage has elucidated significant physiological, molecular, and anatomical traits linked to drought tolerance. Accessions such as TVU-14997 and TVU-15304 consistently demonstrated resilience, maintaining leaf area, shoot and root dry weights, and seed yield in the face of water stress. Molecular analyses further revealed genetic variations that may confer drought resistance, with specific bands indicating the presence of beneficial genetic traits in selected genotypes. Anatomically, TVU-15306 showcased superior adaptations, largely retaining key structural attributes under drought while enhancing others, reinforcing its potential as a drought-tolerant variety. This study underscores the importance of genetic diversity in developing resilient cowpea cultivars that can withstand the challenges posed by climate change. Future research should aim at further exploring these promising genotypes, with a focus on their genetic mechanisms and potential for breeding programs aimed at enhancing drought tolerance in cowpea crops, ultimately contributing to food security in vulnerable regions.

Ethics approval and consent to participate. Not applicable

Consent for publication. Not applicable

Declarations. Ethics clearance is not required.

Availability of data and material. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authors' contributions AAH and KEAA with KAMAK contributed conceptualization, TAAI collected, prepared, and drafted data. TAAI, KAMK and AAH contributed writing and approval of the contents. KEAA, SNA and KAMK did the work of paraphrasing and measuring the plagiarism ratio. AAH, KEAA and KAMK generated the numbers and did the final revision. AAH, KEAA, SNA and KAMK supervised all stages of manuscript preparation. AAH, KEAA and KAMK have read, reviewed, approved, and approved the content of the final version of this review.

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