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Regulation of photosynthesis, fluorescence, stomatal conductance and water-use efficiency of cowpea (*Vigna unguiculata* [L.] Walp.) under drought

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ABSTRACT

Drought is the major abiotic stress factor that causes extensive losses to agriculture production worldwide. The objective of this study was to evaluate the dynamics of photosynthesis and water-use efficiency parameters in 15 cowpea genotypes under well-watered and drought condition. Photosynthesis (A) and chlorophyll fluorescence (Fv'/Fm') declined linearly with decreasing soil water content whereas intrinsic water-use efficiency (WUE) increased under drought stress, suggesting stomatal regulation was a major limitation to photosynthesis. However, under increasing drought conditions, increase in ratio of intercellular CO_2 to ambient CO_2 concentrations along with reduced WUE showed the role of non-stomatal limitation of photosynthesis. The resistant nature of Fv'/Fm' and electron transport rate under drought appeared to be important mechanisms for photoinhibition protection under drought stress. Oxidative stress was apparent due to drought-induced reduction in total chlorophyll and carotenoid which was accompanied with increased leaf wax contents. The accumulation of proline appeared to be in response of drought injury rather than a drought tolerance mechanism. A clear separation based on the genotypes site of origin among the genotypes for drought tolerance could not be established when analyzed using principal component analysis. The identified genotypes and physiological traits from this study may be useful for genetic engineering and breeding programs integrating drought adaptation in cowpea.

1. Introduction

Drought is a major abiotic stress limiting plant productivity worldwide, especially in the arid and semi-arid agro-ecosystems [1]. The predicted changes in climate may lead to precipitation extremes and drought intensities on regional scale. However, decrease in precipitation will be widespread in subtropical region associated with higher temperature and increased evapotranspiration [2]. In general, drought stress induces an array of morphological, physiological, biochemical, and molecular responses, in which photosynthesis being one of the primary physiological target [3]. Understanding the detrimental effects of drought on plant processes and mechanisms of drought tolerance in crop species, particularly those adapted to dry conditions will help to improve their agronomic performance by incorporating the superior traits into new species or cultivars [4].

Stomatal regulated reduction in transpiration is a common response of plants to drought stress which also provides an opportunity to increase plant water-use efficiency [5]. Under moderate drought stress conditions, reduced stomatal conductance (g_s) is the primary cause of photosynthetic inhibition from reduced supply of CO_2 to the intercellular space [6]. In general, atmospheric CO_2 diffuses through stomata to the intercellular space (i.e. stomatal limitation) then across the mesophyll (mesophyll limitations) at the carboxylation site. Therefore, mesophyll conductance (g_m) and biochemical limitation (b_L) (often termed as non-stomatal limitations) to photosynthesis mainly under severe water stress has also gained importance in the recent years and their relative importance to photosynthesis limitation has been subjected to long-standing debate [7–11].

The finite role of g_m has been accepted and the concentration of CO_2 in to the inter-cellular spaces (C_i) has been estimated to differ from CO_2 concentration in the chloroplast (C_c) which varies within species due to their sensitivity to a range of internal and external factors including, leaf development stage, leaf structure and anatomy, radiation, CO_2, temperature, water, and nutrient condition [7 and references therein, 8,12]. Severe water stress can also lead to metabolic impairments including limitations to phosphorylation [13], RuBp (ribulose 1,5-bisphosphate) regeneration [14], and Rubisco activity [15] thus indicating, biochemical limitations to photosynthesis. Although, the role of non-stomatal limitations to photosynthesis is apparent, controversies still exit due to the assumptions and errors in the estimation of g_m and b_L under drought [16,17]. Photosystem II (PSII) is highly sensitive to light and down regulation of photosynthesis under drought stress

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causes an energy imbalance in the PSII reaction center leading to photoinhibition [18]. Mechanisms have evolved in the plant to protect from photoinhibition, such as non-photochemical quenching, electron (e^-) transport to molecules other than CO_2, most importantly to oxygen, which leads to photorespiration and/or Mehler reaction [11,19], non-radiative energy dissipation mechanisms [20,21], and chlorophyll concentration changes [18]. However, these processes ultimately lead to the lower quantum yield of PSII [19].

Cowpea is an important legume crop mostly grown in the arid and sub-arid zones of the world where the production mostly depends upon rain as a sole source of water supply [22,23]. Among all legumes, cowpea has the maximum diversity for plant type, growth habit, maturity, seed type and adapted to a wide range of environments which may serve as a model legume crop [22,24]. Cowpea exhibits broad adaptation mechanisms to drought, such as drought escape, drought avoidance by decreasing leaf area, dehydration avoidance, and vegetative stage drought tolerance by delayed leaf senescence [24–27]. Cowpea plants have shown dehydration avoidance by maintaining high leaf water status without osmotic adjustment which has indicated a common response pattern of photosynthetic processes in relation to soil water content (SWC) or drought induced changes in g_s independent to leaf osmotic potential [25–27].

Crop adaptation to rain-fed conditions can be achieved by improved water-use efficiency (WUE) or by increasing water supply to the plant through improved root system [24]. Intrinsic water-use efficiency estimated as a ratio of A/g_s has been recognized as a measure of carbon gain per unit of water loss and found to be inversely proportional to the ratio of intercellular and ambient CO_2 concentrations (C_i/C_a) [28,29]. Large variability in WUE has been reported among several species as well as cultivars within a species including cowpea [28,30,31]. Because higher rates of leaf photosynthesis are often associated with faster crop growth rates, a combination of higher photosynthesis and improved WUE may play a vital role for yield enhancement of crops under drought stress conditions [5,31].

Although, studies have shown that cowpea photosynthetic performance can recover considerably once the drought stress is relieved, transient photoinhibition or residual impairment of photosystems at very low g_s have also been observed [21,27]. The protective mechanisms for maintaining the photosynthetic apparatus under drought stress condition in cowpea are not well understood [32]. In drought stress, g_s has been shown to relate well and exhibit a specific pattern over almost all the important photosynthetic parameters similarly [11,16,33] which may not essentially reflect the cause and effect relationship. However, it will be helpful to evaluate the relative importance of different processes limiting photosynthesis at a wide range of g_s caused by drought stress and may shed light on the current debate regarding to stomatal *vs.* non-stomatal limitations. The extent of stomatal limitation to various photosynthetic parameters can be assessed by simultaneous measurement of leaf gas exchange, fluorescence, and WUE parameters under drought stress conditions [5,16]. The genotypic diversity in cowpea for the physiological responses to drought will help to comprehend how one or a combination of physiological processes interacts with each other to manage drought stress. The objectives of the study were to determine the relative regulation of various photosynthetic parameters to drought induced stomatal conductance and to evaluate the relative responses of different photosynthetic processes among cowpea genotypes under drought stress and determine whether the genotypes representing diverse sites of origin would group based on their relative physiological tolerance to drought.

2. Material and methods

2.1. Plant material and experimental conditions

An outdoor container experiment was conducted in 2006 at the R.R. Foil Plant Science Research Center, Mississippi State University, Mississippi State (33°28′N, 88°47′W) MS, USA. Fifteen cowpea genotypes representing diverse sites of origin (Table 1) were seeded in 12-L pots, filled with fine sand on 2 August 2006. The pots were 0.65 m in height and 0.15 m in diameter with a small hole at the bottom to drain excess water. The study was comprised of 600 pots with 40 pots per genotype in two complete sets (20 control and 20 stressed pots for each genotype). The pots were arranged randomly in 30 rows, oriented in an east to west direction with 1-m spacing between rows. Seedlings were thinned to two per pot 7 days after emergence. All plants were irrigated with full-strength Hoagland nutrient solution three times a day from emergence to 30 days after sowing (DAS). Thereafter, the control plants continued to receive full irrigation and the other set (drought stressed) received 70%, 50%, 40%, and 0% irrigation compared to control for next 34, 36, 40, and 50 DAS in a manner to create water stress progressively overtime in order to generate a range of water regimes. The pots in the drought-stressed treatments were covered with plastic sheeting at the base of the plants to prevent evapotranspiration, and rain water entering into the pots. The air temperature (mean = 28.7 ± 1.4 °C), relative humidity (mean = 65.5 ± 5.5%), solar radiation (mean = 19.6 ± 2.8 MJ m^{-2} d^{-1}), and precipitation (total = 4.33 cm) were recorded during experimental period from an onsite weather station.

2.2. Leaf and soil water content measurements

From 30 to 50 DAS, photosynthetic processes, leaf relative water content (LRWC), and soil water content (SWC) were measured daily. Immediately after the photosynthetic measurements, the same leaves were detached to measure the leaf fresh, turgid and dry weights and the leaf relative water content was determined as follows: LRWC = (fresh weight − dry weight)/turgid weight − dry weight). The turgid weight of the leaves was determined after 24 h keeping submerged in distilled water in dark. Also, immediately after the photosynthetic measurements, SWC of the upper 6-10 cm of soil was measured with a soil moisture probe (Type ML2X attached to HH2 moisture meter, Delta-T Devices, Burwell, UK). The value for SWC derived from this moisture probe ranged from 0.002 to 0.06 m^3 m^{-3} with obvious difference between drought stressed and irrigated (till excess water drained off the pot) plant. However, these values appear to be very low for plant growth in real world situation [34,35]. Therefore the reported values for SWC in the pots appear to be underestimated and here mainly used for comparison stand point.

2.3. Gas exchange and fluorescence measurements

Gas exchange and chlorophyll fluorescence parameters were measured simultaneously using a Li-Cor 6400 Photosynthesis system (Li-Cor Inc., Lincoln, Nebraska, USA) with an integrated fluorescence chamber (Li-Cor 6400-40 mounted with Leaf Chamber Fluorometer; LCF). These measurements were made on the 3rd or 4th fully expanded leaves from the stem apex between 10:00 and 13:00 h over 2-cm^{-2} leaf area in each genotype. The measurements were taken, when a steady-state (around 3–5 min) was obtained, at 1500 μmol photon m^{-2} s^{-1} photosynthetically active radiation, cuvette temperature set to 30 °C, 360 μmol mol^{-1} CO_2, and 50 ± 5% relative humidity. The quantum efficiency by oxidized

Table 1
Characteristics of the regression equations describing relationship of soil water content (SWC) with photosynthesis (A), fluorescence (Fv′/Fm′), stomatal conductance (g_s), intrinsic water-use efficiency (WUE) and estimated maximum WUE (WUE_{max}) of fifteen cowpea genotypes. $P \leqslant 0.01$ and n varied from 30 to 36.

Genotype	Origin	A Coefficient[▽]		R^2	Fv′/Fm′ Coefficient		R^2	g_s Coefficient			R^2	WUE Coefficient			R^2	WUE_{max}
		a	b		a	b		$y0$	a	b		$y0$	a	b		
Black Crowder (BC)[†]	USA	4.04	624	0.66	0.43	2.91	0.43	−0.87	0.85	21.10	0.45	−26.5	115.4	15.8	0.45	89
CB-5[‡HS]	USA	−2.36	1006	0.87	0.37	5.06	0.72	−3.26	2.97	13.54	0.63	4.7	111.7	42.2	0.66	116
CB-27[‡ HT]	USA	4.29	617	0.67	0.46	2.09	0.56	−1.30	1.24	16.29	0.77	−47.5	128.6	11.7	0.58	81
CB-46[‡ HS]	USA	6.79	617	0.70	0.44	2.81	0.56	−0.72	0.71	28.49	0.69	18.9	103.1	65.4	0.60	122
Magnolia Blackeye (MBE)[†]	USA	3.29	589	0.71	0.43	2.76	0.68	−0.10	0.10	51.33	0.83	53.2	141.9	166.4	0.61	195
Melakh[*]	Senegal	5.09	768	0.70	0.45	3.10	0.62	−0.71	0.67	28.73	0.80	−3.5	118.8	35.9	0.51	115
Mississippi Pinkeye (MPE)[†]	USA	1.80	753	0.82	0.43	3.01	0.73	−1.78	1.62	15.67	0.77	−31.9	138.4	20.9	0.75	107
Mississippi Shipper (MS)[†]	USA	3.05	673	0.71	0.39	3.77	0.65	−0.27	0.25	45.78	0.61	−16.3	130.4	29.3	0.69	114
Mississippi Purple (MP)[†]	USA	2.99	715	0.71	0.40	3.29	0.61	−0.68	0.65	20.15	0.70	33.2	93.4	73.6	0.65	127
Prima[* HT]	Nigeria	2.66	766	0.73	0.42	3.10	0.64	−0.91	0.84	23.88	0.70	24.1	100.5	62.2	0.62	125
Tennessee White Crowder (TWC)[†]	USA	4.04	690	0.78	0.41	3.24	0.63	−0.65	0.56	32.22	0.59	−7.4	124.0	32.1	0.72	117
Top Pick Pinkeye (TPP)[†]	USA	1.89	735	0.75	0.46	2.63	0.63	−0.27	0.25	45.26	0.58	23.3	101.8	48.6	0.66	125
TVu-4552 (TVu)[*]	Senegal	3.55	732	0.71	0.44	2.21	0.61	−0.26	0.25	41.00	0.69	19.5	98.5	49.6	0.55	118
UCR-193 (UCR)[* HT]	India	0.90	808	0.87	0.45	2.51	0.63	−0.24	0.20	49.79	0.82	25.0	161.4	70.0	0.77	186
Zipper cream (ZC)[†]	USA	4.51	614	0.76	0.43	2.34	0.60	−0.64	0.55	30.37	0.73	4.5	131.3	38.6	0.58	136

"*" = genotypes already known to be adapted in dry and hot environment. "†" these genotypes mostly grown in the southern part of USA. "‡" developed at the University of California Davis (CB-5 and CB-27) and Riverside (CB-27). The superscripts "HS" and "HT" represent known heat sensitive and heat tolerant nature of these genotypes.
"▽" The regression equations used: A and Fv′/Fm′ [$Y = a + (b \times x)$]; g_s [$Y = y0 + (a \times \exp.^{bx})$]; WUE [$Y = y0 + (a \times \exp.^{-bx})$].

(open) PSII reaction center in light was calculated as (Fv′/Fm′) = (Fm′-Fo′)/Fm′ [36], where Fm′ = maximal fluorescence of light adapted leaves, Fo′ = minimal fluorescence of a light adapted leaf that has momentarily been darkened. The actual flux of photons driving photosystem II (PSII), i.e. electron transport rate (ETR), was computed according to the equation $[(Fm' - Fs)/Fm'] \times f I \alpha_{leaf}$, where, Fs = steady state fluorescence, f = the fraction of absorbed quanta that is used by PSII, typically, 0.5 for C_3 plants (in this study), I = incident photon flux density ($\mu mole\ m^{-2}\ s^{-1}$), and α_{leaf} = leaf absorptance set to 0.85 in this study (adopted from LI-6400 Instruction Manual, version 5 (LI-COR) and references therein). Intrinsic WUE was estimated as the ratio of A/g_s [28]. ETR/A was taken as the relative measure of electron transport to oxygen molecules [11].

2.4. Pigments, proline, and wax measurements

Total chlorophyll and carotenoid concentrations, proline and wax contents were measured from the 3rd or 4th leaf from the stem apex at 45 DAS in the control and drought-stressed plants when averaged SWCs were 0.06 and 0.01 $m^3\ m^{-3}$, respectively. The pigments were extracted by placing five 0.38 cm^{-2} leaf disks for each replication in a vial containing 5 mL of dimethyl sulfoxide and incubated in dark for 24 h. Thereafter, the absorbance of the supernatant was measured at 664, 648, and 470 nm by using a Bio-Rad UV/VIS spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The chlorophyll a, chlorophyll b, and carotenoids have absorption maxima at 664 nm, 648 nm, and 470 nm, respectively [37]. The total chlorophyll and carotenoids were estimated by using the equation of Lichtenthaler [37] as described by Chappelle et al. [38] and expressed on leaf area basis ($\mu g\ cm^{-2}$).

For proline extraction, one leaf from each replication were collected at noon and 0.5 g of leaf tissue was immediately placed in a vial containing 10 mL of 3% aqueous sulfosalicylic acid and stored at −20 °C. For analysis, mixture was homogenized after bringing to room temperature and the homogenate was filtered through Whatman No. 2 filter paper. Two milliliters of filtrate was combined with 2 mL each of acid-ninhydrin reagent and glacial acetic acid in a test tube, and heated on a water bath maintained at 100 °C for 1 h and the reaction was terminated in an ice bath [39]. The reaction mixture was extracted with 4 mL of toluene, mixed vigorously and chromophore containing toluene was aspirated from the aqueous phase and the absorbance was read at 520 nm by using a Bio-Rad UV/VIS spectrophotometer. The free proline was determined as outlined by Bates et al. [39] and expressed as $\mu mol\ g^{-1}$ using a proline standard (L-Proline, Sigma–Aldrich, Inc., MO, USA).

The extraction and quantitative analysis of leaf epicuticular waxes were carried out as per the method of Ebercon et al. [40] with minor modifications. Ten leaf discs constituting an area of 35.36 cm^{-2} from 3rd or 4th leaf from the stem apex were cut from each genotype from five plants in each replication. Leaf waxes were removed by stirring the leaf disks in 15 mL of chloroform (Sigma–Aldrich, Inc., MO, USA) in a test tube for 20 s. The wax extract was evaporated on a water bath maintained at 80 °C, cooled to room temperature; 5 mL of dichromate reagent was added and further heated on a water-bath maintained at 80 °C for 30 min. The reagent was prepared by dissolving 20 g $K_2Cr_2O_7$ in 40 mL of de-ionized water and the resulting slurry was mixed with 1 L of H_2SO_4 and heated below boiling point until clear solution was obtained. The samples were removed from the water bath and cooled, and then 12 mL of de-ionized water was added, allowed to stand for 15 min, and the intensity of the color was measured at 590 nm using a Bio-Rad UV/VIS spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The wax content was expressed on a leaf area basis ($\mu g\ cm^{-2}$) by using a standard curve developed from the wax obtained from the same species.

2.5. Statistical analysis

Cowpea response to drought was assessed by combining the data for all genotypes and also within a genotype. The relationships among the SWC, LRWC, and different gas exchange and water-use efficiency parameters were tested for linear, exponential, and logarithmic functions and the best fit regressions were selected. The relationship between g_s and SWC was analyzed by exponential three parameter regression equation [$Y = y0 + (a \times \exp.^{bx})$] where b represents the rate of stomatal conductance in response to decreasing SWC. An exponential decay function [$Y = y0 + (a \times \exp.^{-bx})$] was used to describe the relationship between WUE and SWC, where ($y0 + a$) is the maximum WUE (WUE_{max}). The exponential rise to maximum function [$Y = y0 + a \times (1 - \exp.^{-bx})$] was used to obtain the relationships between g_s and A, Fv′/Fm′, C_i/C_a, ETR and E, where ($y0 + a$) provided the maximum photosynthesis (A_{max}) and fluorescence (Fv'/Fm'_{max}). The relationship between g_s and C_i/C_a ratio was fit under the conditions in which g_s was the primary factor

controlling the observed decrease in photosynthesis as described by Brodribb [29]. The minimum C_i/C_a ratio (C_i/C_amin) and the corresponding g_s values were obtained from these functions. To determine the co-regulation of these parameters (A, Fv′/Fm′, C_i/C_a, ETR, and E) as a function of g_s, all parameters were normalized to the g_s value of three mol m^{-2} s^{-1}, representative of the plants grown under saturated SWC (0.06 m^3 m^{-3}). Important to note that some of the g_s values were unexpectedly higher (out of 512 values about 12, 24 and 33 data points were above 2.5, 2.0 and 1.8 mol H_2O m^{-2} s^{-1}, respectively) in current experiment. Taking into consideration such a large data points from 15 genotypes, perhaps one could argue and may discard these higher values from the analysis. However, based on the norm for instrumentation and data collection, it seemed to be very appropriate to document the original findings. Such large values have also been reported in other studies [33,41,42].

The regression analyses were carried out using SigmaPlot version10 (Systat Software Inc, 2006). The random nature of individual measurements or data points (among the pots for each genotype) varied among the pots each day due to instantaneous measurement and generated a range in the measured parameters. Since, all the data points (30–36) for each genotype were used to study the relationships, the differences between genotypes for their response of photosynthetic parameters to either SWC or stomatal conductance could not be compared statistically. However, the analyses of variance (ANOVA) were performed to assess the genotypes × treatment effect on chlorophyll, carotenoid, proline, and wax contents using PROC MIXED of SAS at $\alpha = 0.05$ level of significance [43]. The mean values of genotypes are reported and differences between control and treated means were separated by least square means procedure of Tukey–Kramer method. Replication nested within the treatments was considered as random effect.

Principal component analysis (PCA) is a statistical technique for multivariate data analysis and is quite useful in separating experimental units into subgroups [44,45]. PCA was performed on the correlation matrix of fifteen genotypes and six response variables, i.e. regression slopes of A and Fv′/Fm′ response to SWC, A_{max}, Fv′/Fm'_{max}, WUE_{max}, and C_i/C_amin^{-1} using the PROC PRINCOMP procedure [43]. The A and Fv′/Fm′ values were normalized to obtain the slopes in response to SWC, the A and Fv′/Fm′ values at saturated SWC was used as a denominator for each genotype so that the derived values range between a relative scales of 0–1. Then, the A and Fv′/Fm′ were regressed against SWC to estimate the slopes. This analysis was necessary to measure differences among the cowpea genotypes. PCA produced loadings for these response variables termed as eigenvectors, principal component (PC) scores for each genotypes and eigenvalues for each PC. A superimposed biplot with the PC scores and the corresponding eigenvectors was developed with the same scale units along the abscissa and ordinates having the same physical length as illustrated by ter Braak [46]. The eigenvectors derived from the PC analysis were used to identify the variables that tend to have a strong relationship (i.e. having elements larger in absolute value than the other elements in the same eigenvector) with a particular PC. This criterion was used to describe and group cowpea genotypes for their drought stress tolerance.

3. Results

3.1. Gas exchange, photosynthetic and water-use efficiency parameters under drought

The combined analysis of all cowpea genotypes showed no relationship between LRWC and SWC (Fig. 1A). However, photosynthesis (A) showed a linear relationship with SWC (Fig. 1B). The g_s exhibited an exponential relationship with SWC and decreased to zero under severe drought conditions (Fig. 1C). Similar to the A, the transpiration rate (E) also exhibited a linear relationship with SWC (Fig. 1D).

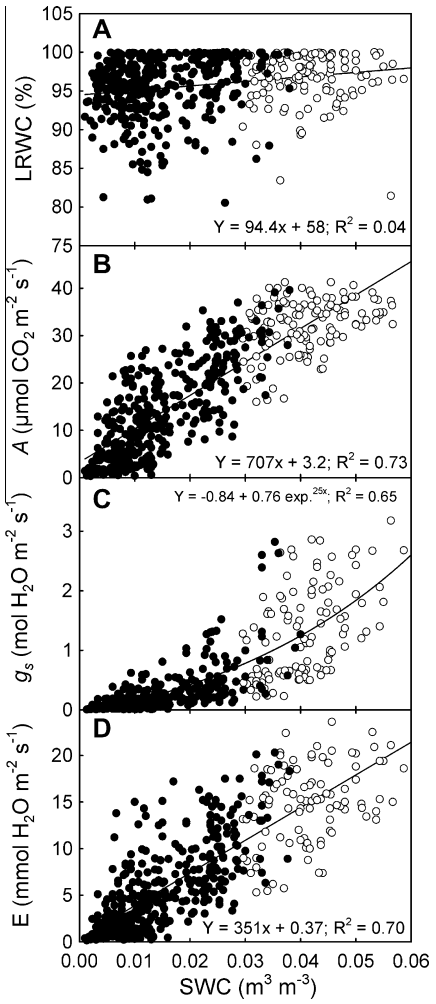

Fig. 1. Relationships between soil water content (SWC) and (A) leaf relative water content (LRWC), (B) photosynthesis (A), (C) stomatal conductance (g_s) and (D) transpiration rate (E). Data is from 15 cowpea genotypes (P = >0.05 (LRWC), <0.001 (A and g_s) and n = 512), Open and closed circles represent data from watered and drought treated plants, respectively. The regression equations used for figure A, B and D was [$Y = a + (b \times x)$]; figure C was [$Y = y0 + (a \times \exp.^{bx})$].

Photosynthesis rate declined linearly as drought stress-induced C_i decreased to a minimum value of 95 µmol mol^{-1} (Fig. 2A, symbol circle). However, at severe water stressed condition an increased in C_i was observed, i.e. closed 'square' symbols in Fig. 2A, whereas A remained low. In order to obtain a minimum C_i value, a linear regression was performed using the C_i at g_s values above 0.04 mol m^{-2} s^{-1} because C_i in most of the genotypes increased when g_s decreased further. Based on the regression analysis, it is estimated that A reached zero at C_i value of approximately 180 µmol mol^{-1}. The WUE, on the other hand, showed increasing trend as g_s decreased and peaked roughly around the g_s value of 0.04 mol m^{-2} s^{-1} (Fig. 2B).

Fig. 3 shows measured photosynthetic and fluorescence parameters in response to g_s (Fig. 3A–F). All photosynthetic parameters exhibited an exponential relationship with g_s, except ETR/A. The C_i/C_a decreased until a minimum value of C_i/C_a (predicted C_i/C_a-min = 0.41 and g_s = 0.002 mol m^{-2} s^{-1}) was realized (Fig. 3C). However, an increase in C_i/C_a was also observed near 0.04 mol m^{-2} s^{-1} g_s value. In contrast to A, ETR was maintained until very low values of g_s were reached; whereas, Fv′/Fm′ started to decrease earlier

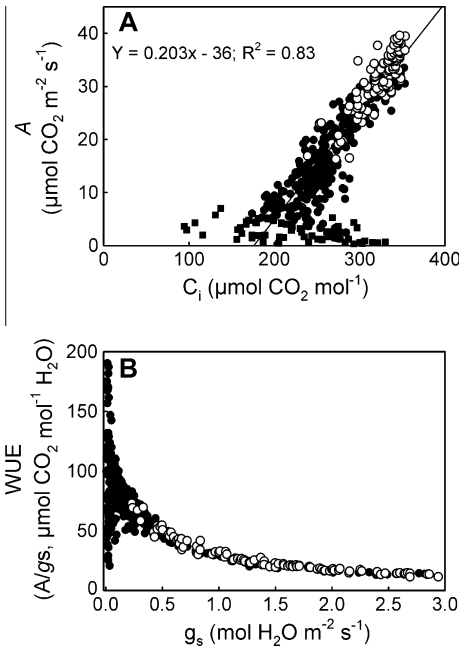

Fig. 2. Relationships between (A) drought induced changes in C_i and A and (B) g_s and WUE in cowpea. Data is from fifteen cowpea genotypes (P = <0.001 and n = 512). The linear regression [$Y = a + (b \times x)$] in figure (A) was only extended to the C_i value obtained above 0.04 mol m^{-2} s^{-1} g_s (circles) thus, only 438 data was included. Open and closed circles/squares represent data from watered and drought treated plants, respectively.

than ETR and remained comparatively higher at low g_s (Fig. 3D and E).

To understand the relative regulation of g_s, the data on gas exchange, photosynthetic and water-use efficiency parameters were normalized (Fig. 4). Based on the profound changes in the different photosynthesis parameters, four well-defined stomatal controlled regions ($g_s > 1.8$, $0.4 < g_s < 1.8$, $0.04 < g_s < 0.4$ and $g_s < 0.04$ mol H_2O m^{-2} s^{-1}) exhibiting the co-regulation of these parameters were realized and illustrated in Fig. 4. For instance, the first region ($g_s > 1.8$) was set where all parameter attained a saturation except transpiration which continued to increase. Also, g_s value above 1.8 mol m^{-2} s^{-1} has no effect on A, ETR, Fv′/Fm′, C_i/C_a, and ETR/A (ETR/A as in the Fig. 3F). E continued to increase and was accompanied with reduction in A/g_s (A/g_s as in the Fig. 2B). The second region ($0.4 < g_s < 1.8$ mol m^{-2} s^{-1}) was distinguished because in this region all parameters started to decline but ETR remained constant. When g_s decreased by 77% (from 1.8 to 0.4 mol); A (36%), E (58%), Fv′/Fm′ (13%), and C_i/C_a (14%) were decreased continuously without any change in ETR. In contrast, at this g_s range, ETR/A showed about 22% increase. This is also the region when A/g_s showed an increasing trend (Fig. 2B).

The third region was apparent when g_s declined from 0.4 to 0.04 mol m^{-2} s^{-1} ($0.04 < g_s < 0.4$) and identified by a sharp decrease in ETR. Also, until this region of g_s, the percent reduction in g_s was always higher than the reduction of any other parameters. The reductions accounted by different photosynthetic parameters due to decline in g_s until 0.04 mol m^{-2} s^{-1} were 85% (A),>90% (E), 46% (C_i/C_a), 48% (ETR), and 23% (Fv′/Fm′); however, the ETR/A increased by >200% (Fig. 3F). The A/g_s also continued to increase

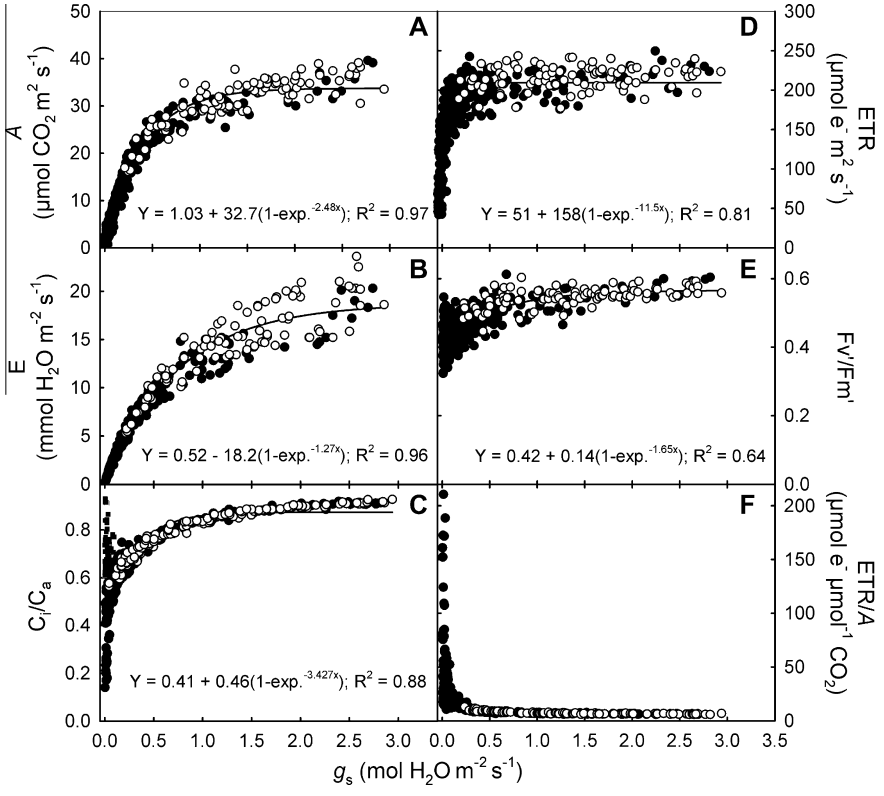

Fig. 3. Relationships between stomatal conductance (g_s) and (A) photosynthesis (A), (B) transpiration rate (E), (C) C_i/C_a ratio, (D) electron transport rate (ETR), (E) fluorescence (Fv′/Fm′) and (F) ETR/A ratio for fifteen cowpea genotypes. P = <0.001 and n = 512 for all except C_i/C_a ratio in which n = 438 and remaining 74 values were not included in the regression fit. The line in Fig. 3C, represents the relationship between the g_s and C_i/C_a ratio under condition in which g_s was the primary factor controlling decrease in photosynthesis (following Brodribb, 1996, Plant Physiology vol. 111, pp. 179–185).These line has been extended only to the g_s value at which C_i/C_a was minimal. The regression equation shown in above relationship was [$Y = y0 + a \times (1 - \exp.^{-bx})$]. Open and closed circles represent data from watered and drought treated plants, respectively. The parameter A/g_s was estimated from Fig. 2B.

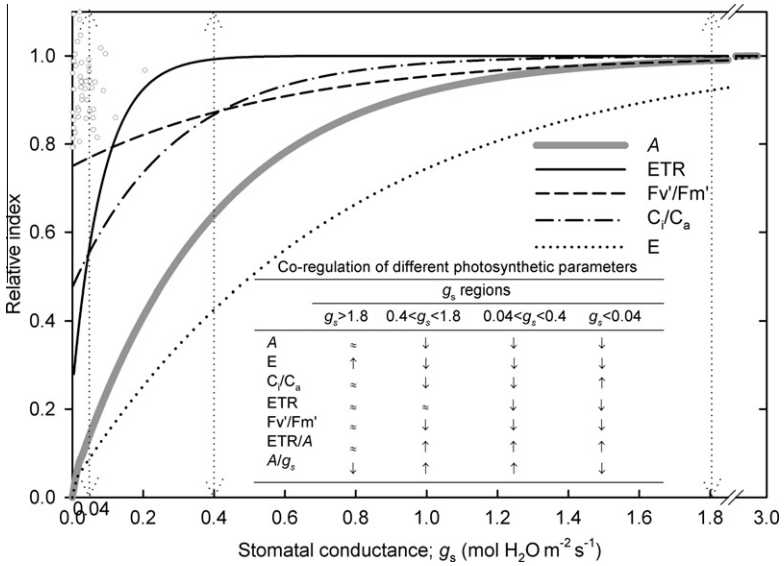

Fig. 4. Analysis of the extent of the stomatal co-regulation to the different photosynthetic parameters in cowpea, using drought induced decrease in stomatal conductance (g_s) as a reference parameter. The normalized data of fifteen cowpea genotypes from Fig. 3 were used. The circular symbols at the top left corner of the figure indicate increase in C_i/C_a ratio. The four stomatal conductance (g_s) regions are distinguished. The symbols ≈, ↑ and ↓ indicate no change, increasing and decreasing trend of the parameters in a specified region of g_s, respectively. The parameter A/g_s was estimated from Fig. 2B.

in this region. Further decrease in g_s, signified the fourth region (g_s < 0.04 mol m^{-2} s^{-1}) where *A* and *E* approached almost zero; whereas, ETR and Fv′/Fm′ were decreased by about 75% (51 μmol e^- m^{-2} s^{-1}) and 25% (0.42) of the maximum, respectively. In contrast to the previous region, a sudden drop in A/g_s was also observed (Fig. 2B). The C_i/C_a also increased in some genotypes as much as its maximum value along with a continuous increase in ETR/*A*.

3.2. Pattern of cowpea response to photosynthesis, fluorescence and WUE

3.2.1. Effect of the soil water content

The analysis showed that photosynthesis and Fv′/Fm′ declined linearly with decreasing SWC (Table 1, and Fig. 5A and B). The slope showing the genotypic response to SWC ranged from 614 in ZC to 1006 in CB-5 for *A* and from 2 in CB-27 to 5 in CB-5 for Fv′/Fm′ (Table 1). Changes in *A* in response to SWC were much greater than the changes in Fv′/Fm′; former approached to zero while the latter remained higher across all genotypes under severe drought stress conditions (Fig. 5A and B). The g_s exhibited an exponential decrease in response to decrease in SWC (Fig. 5C). The rate of stomatal conductance expressed as slope of the relationship between g_s and SWC varied from 13.54 in CB-5 to 51.3 in MBE (Table 1). In contrast to g_s, WUE increased exponentially as SWC decreased (Fig. 5D). The WUE_{max} varied from 81 in CB-27 to 186 in UCR-193 among the 15 genotypes (Table 1).

3.2.2. Effect of stomatal conductance

The coefficient of determination (R^2) observed for the relationship between *A* and g_s ranged from 0.95 to 0.99 among genotypes (Table 2). At low g_s, the response of *A* was comparable, whereas at higher g_s values, a similar increase in g_s yielded greater increase in *A* for many genotypes (Fig. 6A). A similar response was also observed between g_s and Fv′/Fm′, however it occurred at higher g_s compared to the relationship between *A* and g_s (Fig. 6B). The C_i/C_a ratio exhibited biphasic response pattern over decreasing g_s, an initial stomatal regulated reduction followed by an increase, roughly below the g_s level of 0.048 (CB-5) and 0.002 mol m^{-2} s^{-1} (UCR-193) reflecting the onset of a non-stomatal limitation to photosynthesis (Fig. 6C). Around this g_s level, the Fv′/Fm′ also decreased sharply. Among cowpea genotypes, the A_{max} ranged from 30.7 (MS) to 36.6 μmol m^{-2} s^{-1} (UCR-193) and the Fv'/Fm'_{max} ranged from 0.545 (MP) to 0.630 (TPP) (Table 2). The $C_i/C_a min$ varied from 0.323 (UCR-193) to 0.592 (CB-27) with a corresponding g_s level of 0.002 and 0.048 mol m^{-2} s^{-1}, respectively (Table 2).

3.3. Leaf pigments, proline and wax content

Table 3 shows changes in leaf pigments, proline, and leaf epicuticular wax contents in irrigated and drought-stressed plants. Except wax content, other parameters showed a significant genotype × treatment interaction. Based on a conservative mean comparison test (Tukey Kramer), many genotypes exhibited significant difference between irrigated and drought stressed values. Drought stress caused reduced total chlorophyll and carotenoids concentrations with MPE (53%) showing the maximum decrease for both the pigments. Proline and wax content increased by 332% in PMP and 46% in MPE, respectively, under drought stress conditions compared to irrigated plants.

3.4. Principal component analysis of drought tolerance

The differences and similarities in the response of cowpea genotypes to drought were assessed using PCA. The first two PC's, based on the scree plot, explained 67% total variations among cowpea genotypes for the six selected parameters. The eigenvectors for PC1 had high positive scores for A_{max}, Fv'/Fm'_{max}, WUE, and $C_i/C_a min^{-1}$; whereas, the eigenvector for PC2 had high positive scores for A_{slope} and Fv'/Fm'_{slope} (Fig. 7). These slopes (A_{slope} and Fv'/Fm'_{slope}) were also referred as drought sensitivity as higher the slope the more sensitive indices to drought because of the steep drop in A and Fv′/Fm′ due to decrease in SWC. Therefore, genotypes with high PC scores should have higher values for these parameters. For instance, in the biplot of PC1 and PC2 (Fig. 7), the genotype UCR-193 had the highest value for A_{max}, Fv'/Fm'_{max}, WUE, and $C_i/C_a min^{-1}$ with lower scores of A_{slope} and Fv'/Fm'_{slope} and was determined as tolerant to drought. Similarly, genotypes with relatively high scores for PC1 and low scores for PC2 were classified as drought tolerant (UCR-193, TPP, and MBE). Genotypes near the

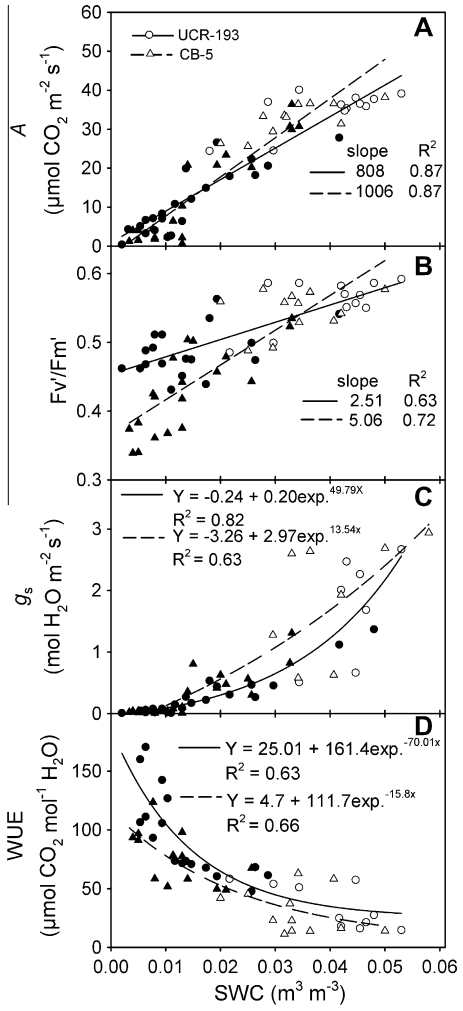

Fig. 5. Relationships between soil water content (SWC) and (A) photosynthesis (A), (B) fluorescence (Fv′/Fm′), (C) stomatal conductance (g_s) and (D) intrinsic water-use efficiency (WUE) in cowpea. Only two genotypes with their regression fits are shown. P = <0.001 for all curves, n = 32 (UCR-193) and 30 (for CB-5). Open and closed circles/triangles represent data from watered and drought treated plants, respectively. The regression equations used for figure A and B was [$Y = a + (b \times x)$]; figure C was [$Y = y0 + (a \times \exp.^{bx})$] and figure D was [$Y = y0 + (a \times \exp.^{-bx})$].

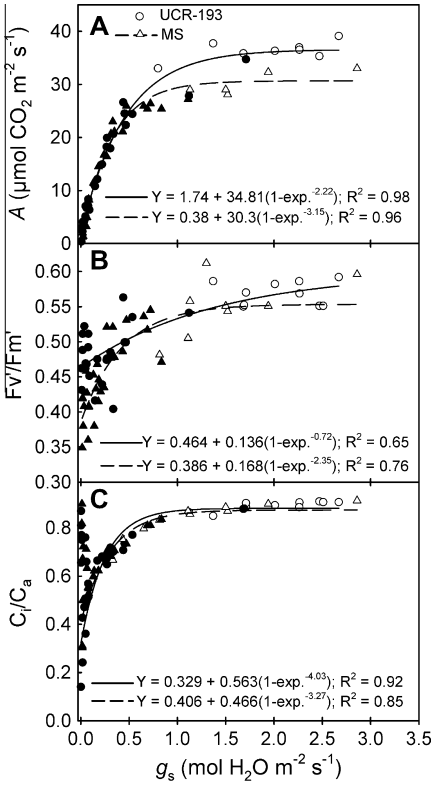

Fig. 6. Relationships between stomatal conductance (g_s) and (A) photosynthesis (A), (B) fluorescence (Fv′/Fm′) and (C) C_i/C_a ratio in cowpea. Only two genotypes with their regression fits are shown. P = <0.001, n = 32 (UCR-193) and 36 (MS), except the fit for C_i/C_a with g_s in which n = 27 (UCR-193) and 30 (MS) and the remaining values were not included in the regression fit. The regression equations shown in above relationship was [$Y = y0 + a \times (1 - \exp.^{-bx})$]. Open and closed circles/triangles represent data from watered and drought treated plants, respectively.

center of the plot have medium PC scores, reflecting their intermediate photosynthetic performance and medium drought sensitivity. These genotypes included Melakh, MPE, TVu, ZC, TWC, and Prima. Due to high negative values for both PC scores, genotypes, BC, CB-46, and CB-27, were less drought sensitive with low A_{max}, Fv'/Fm'_{max}, WUE and $C_i/C_a min^{-1}$; and were therefore classified as

Table 2
Characteristics of the regression equations describing relationship of stomatal conductance (g_s) with photosynthesis (A), fluorescence (Fv′/Fm′), and ratio of intercellular CO_2 (C_i) to the ambient CO_2 (C_a) concentration (C_i/C_a) for fifteen cowpea genotypes. The estimated maximum A (A_{max}), maximum Fv′/Fm′ (Fv'/Fm'_{max}) and minimum C_i/C_a ($C_i/C_a min$) are also presented. The values in the parenthesis for $C_i/C_a min$ column represent stomatal conductance at which the $C_i/C_a min$ was realized. $P \leqslant 0.001$ and n varied from 30 to 36.

Genotype	A Coefficient♀			R^2	A_{max} (μmol CO_2 m^{-2} s^{-1})	Fv′/Fm′ Coefficient			R^2	Fv'/Fm'_{max}	C_i/C_a Coefficient			R^2	$C_i/C_a min$
	$y0$	a	b			$y0$	a	b			$y0$	a	b		
BC	0.70	31.9	2.37	0.97	32.5	0.420	0.169	1.17	0.55	0.589	0.506	0.346	3.38	0.95	0.550 (0.041)
CB-5	0.52	33.3	2.48	0.98	33.8	0.381	0.178	1.92	0.84	0.559	0.464	0.443	2.24	0.90	0.477 (0.013)
CB-27	0.49	32.6	2.40	0.98	33.1	0.434	0.128	1.85	0.72	0.562	0.562	0.333	1.94	0.94	0.592 (0.048)
CB-46	0.11	31.5	2.91	0.95	31.6	0.414	0.144	2.31	0.71	0.558	0.370	0.508	3.63	0.89	0.388 (0.010)
MBE	0.35	33.0	2.75	0.98	33.4	0.419	0.159	2.28	0.71	0.578	0.266	0.540	6.83	0.91	0.302 (0.010)
Melakh	1.86	34.3	2.10	0.97	36.1	0.426	0.159	2.32	0.85	0.584	0.389	0.503	3.30	0.90	0.424 (0.022)
MPE	0.96	34.2	2.48	0.98	35.1	0.401	0.175	3.09	0.85	0.576	0.442	0.453	3.04	0.85	0.459 (0.013)
MS	0.38	30.3	3.15	0.96	30.7	0.386	0.168	2.35	0.76	0.553	0.408	0.466	3.27	0.85	0.430 (0.014)
MP	0.43	31.5	2.87	0.98	31.9	0.385	0.160	2.38	0.83	0.545	0.405	0.431	4.24	0.89	0.426 (0.012)
Prima	0.91	32.1	2.60	0.96	33.0	0.420	0.150	1.28	0.76	0.571	0.408	0.463	3.44	0.81	0.428 (0.013)
TWC	2.29	31.8	2.23	0.97	34.1	0.417	0.161	1.11	0.70	0.578	0.458	0.424	2.80	0.96	0.484 (0.023)
TPP	0.98	35.2	2.34	0.97	36.2	0.477	0.153	0.66	0.50	0.630	0.447	0.423	3.06	0.84	0.469 (0.018)
TVu	0.81	32.6	2.64	0.99	33.4	0.427	0.147	0.93	0.80	0.574	0.371	0.503	3.45	0.94	0.376 (0.003)
UCR	1.74	34.8	2.22	0.98	36.6	0.464	0.136	0.72	0.65	0.599	0.319	0.563	4.03	0.92	0.323 (0.002)
ZC	1.49	32.9	2.45	0.98	34.4	0.420	0.139	1.67	0.76	0.560	0.464	0.415	2.78	0.96	0.471 (0.007)

"♀" The regression equations used for all above relationship was [$Y = y0 + a \times (1 - \exp.^{-bx})$].

Table 3
The summary of analysis of variance (ANOVA) for genotypes (*G*) and treatments and average total chlorophyll, carotenoids, proline, and leaf epicuticular wax contents of cowpea genotypes under irrigated and drought stress (SWC = 0.01 m^3 water m^{-3} soil) conditions. Percent changes (%) form irrigated to drought-stressed plants are also shown.

Genotype	Total chlorophyll (μg cm^{-2})			Carotenoids (μg cm^{-2})			Proline (μmol g^{-1})			Wax (μg cm^{-2})		
	Irrigated	Drought	%	Irrigated	Drought	%	Irrigated	Drought	%	Irrigated	Drought	%
BC	47.5$^{a\Psi}$	33.6^{a}	−29	10.80^{a}	7.04^{a}	−35	1.51^{a}	3.54^{b}	135	7.97^{a}	10.80^{a}	26
CB-5	62.1^{a}	32.6^{b}	−48	13.41^{a}	7.70^{b}	−43	1.12^{a}	3.59^{b}	221	9.70^{a}	18.20^{b}	47
CB-27	61.2^{a}	33.5^{b}	−45	13.13^{a}	7.62^{b}	−42	1.02^{a}	2.80^{b}	175	9.93^{a}	17.26^{a}	42
CB-46	67.1^{a}	39.1^{b}	−42	14.36^{a}	8.63^{b}	−40	0.71^{a}	2.90^{b}	311	12.74^{a}	18.72^{a}	32
MBE	63.5^{a}	41.8^{b}	−34	13.92^{a}	8.76^{b}	−37	0.52^{a}	2.26^{b}	332	13.16^{a}	18.15^{a}	27
Melakh	53.7^{a}	33.1^{b}	−38	11.15^{a}	6.81^{a}	−39	0.95^{a}	3.90^{b}	310	11.07^{a}	17.26^{a}	36
MPE	62.9^{a}	29.7^{b}	−53	13.75^{a}	6.53^{b}	−53	1.56^{a}	3.07^{b}	96	10.17^{a}	18.67^{b}	46
MS	52.9^{a}	40.9^{a}	−23	11.07^{a}	8.69^{a}	−21	1.75^{a}	2.30^{a}	31	6.61^{a}	9.91^{a}	33
MP	49.1^{a}	32.4^{a}	−34	10.85^{a}	7.58^{a}	−30	1.11^{a}	3.61^{b}	225	7.92^{a}	13.85^{a}	43
Prima	53.0^{a}	29.6^{b}	−44	11.60^{a}	6.71^{b}	−42	0.79^{a}	1.74^{a}	119	9.34^{a}	13.43^{a}	30
TWC	54.6^{a}	33.4^{b}	−39	11.01^{a}	7.20^{a}	−35	0.90^{a}	1.43^{a}	59	10.59^{a}	17.05^{a}	38
TPP	57.7^{a}	39.6^{a}	−31	11.46^{a}	8.57^{a}	−25	0.44^{a}	0.77^{a}	73	9.70^{a}	13.53^{a}	28
TVu	54.2^{a}	36.0^{a}	−34	12.00^{a}	7.86^{a}	−34	0.68^{a}	0.94^{a}	39	10.23^{a}	13.27^{a}	23
UCR	44.2^{a}	37.1^{a}	−16	10.29^{a}	8.41^{a}	−18	0.80^{a}	1.10^{a}	37	13.43^{a}	15.47^{a}	13
ZC	42.0^{a}	30.0^{a}	−29	7.89^{a}	6.67^{a}	−15	0.77^{a}	1.31^{a}	70	8.02^{a}	8.86^{a}	9
ANOVA[#]												
G	0.0009***			0.0003***			<0.0001***			<0.0001***		
Treatment	0.0002***			0.0001***			0.0003***			0.0007***		
G * Treatment	0.038*			0.02*			<0.0001***			<0.227^{NS}		

$^{\Psi}$ Indicates that within each genotypes and between treatments for each measured variables, means followed by common letter do not differ significantly by the Tukey–Kramer method ($\alpha = 0.05$).

[#] Statistical significance (*P*-value) of ANOVA is given as: *** ($P \leqslant 0.001$), ** ($P \leqslant 0.01$), * ($P \leqslant 0.05$) and NS ($P \geqslant 0.05$).

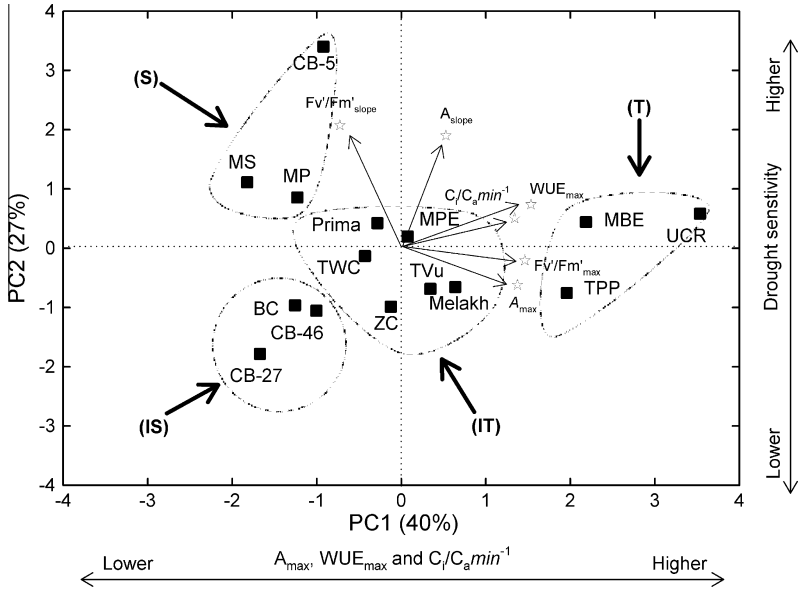

Fig. 7. The biplot of principal components (PC) scores of PC1 vs. PC2 related to the classification of fifteen cowpea genotypes (solid diamond symbols) for their drought sensitivity. The eigenvectors (PC1 and PC2) for the photosynthetic parameters (solid stars) are superimposed with the PC biplot scores at the similar scale reflecting their contribution in determination of drought sensitivity. The arrows radiating from the center indicate the direction (angle) and magnitude (length) for the parameters. The eigenvectors were multiplied by four in order to obtain clear and superimposed figure. The arrow along the right *y*-axis and the bottom *x*-axis indicate the interpretation of the PCs. The genotypes are distinguished for their relative sensitivity to drought in the circumscribed area as tolerant (T), intermediately tolerant (IT), intermediately sensitive (IS), and sensitive (S) to drought stress condition.

intermediately drought sensitive. Genotypes CB-5, MS, and MP showing high negative scores for PC1 and high positive scores for PC2 reflected their low photosynthesis and WUE and high sensitivity to drought.

Since, PC1 and PC2 represents the main components of drought responsiveness, they were correlated with the photosynthetic parameters to find the traits contributing to drought responsiveness (Table 4). The strong correlation of all the parameters with either PC1 or PC2 exhibited the importance of these parameters in determining drought sensitivity. A positive correlation between A_{slope} and Fv'/Fm'_{slope} ($r = 0.55$, $P < 0.05$), A_{max} and Fv'/Fm'_{max} ($r = 0.72$, $P < 0.01$), and WUE and $C_i/C_a min^{-1}$ ($r = 0.90$, $P < 0.001$) were also observed.

4. Discussion

4.1. Role of stomatal conductance under drought stressed conditions

Since, g_s is responsive to almost all external and internal factors related to drought, it represents a highly integrative basis for overall effect of drought on photosynthetic parameters [16]. Once this

Table 4
The Pearson's correlation (r) matrix showing the relationship between six photosynthetic parameters used in principal component analysis and their relationship with the first two principal component scores.

Variables	PC1	PC2	A_{slope}	A_{max}	Fv'/Fm'_{slope}	Fv'/Fm'_{max}	WUE_{max}
A_{slope}	0.27	0.79***					
A_{max}	0.76***	−0.08	0.35				
Fv'/Fm'_{slope}	−0.37	0.87***	0.55**	−0.29			
Fv'/Fm'_{max}	0.70****	−0.26	0.19	0.72**	−0.36		
WUE_{max}	0.79***	0.27	0.17	0.30	−0.11	0.21	
$C_i/C_a min^{-1}$	0.69***	0.21	0.05	0.13	−0.14	0.10	0.90***

Statistical significance of correlation are given as: **($P \leqslant 0.01$) and ***($P \leqslant 0.001$).

relationship is determined, the proportional changes in each process can then be estimated at any point of g_s, representing various degree of water stress. The important event observed in the first g_s region ($g_s > 1.8$) clearly indicates that cowpea transpired excessively without any gain in photosynthetic rate under well-watered conditions causing an indirect decrease in WUE (A/g_s). It has been suggested that stomata control E more than A, as A levels off at high g_s, E continues to increase linearly [31].

In the second g_s region ($0.4 < g_s < 1.8$), stomatal limitation to photosynthesis appeared to be the main cause of A inhibition as deduced from a parallel decrease in C_i/C_a ratio. A similar response pattern at the initial phase of stomatal closure was also observed in grape [11], and studies suggest a large portion of excess electrons at reduced A might be used for photorespiration [19] to protect leaves from photoinhibition. This was supported by the observed unchanged ETR while both A and C_i/C_a decreased with a concomitant increase in ETR/A in this region of g_s. The occurrence of other processes such as non-radiative energy dissipation in the form of heat might have also been involved, as inferred by a small decrease in Fv′/Fm′ in the current and other studies [20,21]. In the third region ($0.04 < g_s < 0.4$) a smaller reduction in ETR compared to A due to water stress indicates a relative increase in photorespiration [47] which was in accordance with the continuous increase in ETR/A observed in this region. The stomatal-limitation still appeared to account more of photosynthesis inhibition because the reduction in the C_i/C_a was still parallel to the reduction in A, and the Fv′/Fm′ was maintained relatively higher (73% of the maximum). It was also supported by the fact that until this region of g_s, the percent reduction in g_s was always higher than the reduction of any other photosynthetic parameters. However, the presence of non-stomatal limitation to A might be possible because accumulation of soluble sugars and decrease in capacity for RuBp regeneration have also been reported to occur under these conditions that could cause a minimal non-stomatal limitation to photosynthesis by feedback inhibition [16,21,28].

The appearance of non-stomatal limitation to photosynthesis was evident in the fourth region of g_s ($g_s < 0.04$ mol m^{-2} s^{-1}) as designated by increased C_i/C_a, drop in A/g_s, and greater percentage decreases in measured photosynthetic parameters compared to the percentage decline in g_s. A similar increase in C_i/C_a at very low g_s has also been observed in other species under severe water stressed conditions [28,29,33]. While ETR/A continued to increase, Fv′/Fm′ remained higher. Maintenance of high Fv′/Fm′ has been suggested as a protective mechanism of the photosystem from photo-inhibitory damage which may lead to the recovery of photosynthesis after water stress is released [48,49]. Moreover, the lowest estimated C_i value (≈180 μmol CO_2 mol^{-1}) corresponded to the g_s level of 0.04 mol m^{-2} s^{-1}, which was the inflexion point of C_i/C_a in response to g_s under drought stressed condition. This suggests that above this level of g_s (0.04 mol m^{-2} s^{-1}), photosynthesis was predominantly controlled by stomata and below this g_s level the non-stomatal limitation to photosynthesis was more evident. Although, mesophyll conductance (g_m) and biological limitations were not measured in this study, the drastic changes in the above mentioned processes clearly indicate the pre-dominance of non-stomatal limitation to photosynthesis in this region of g_s. The processes that govern CO_2 and water fluxes, essentially during photosynthesis, may also involve mesophyll conductance (lower C_c, than C_i) and biochemical limitations (reduced carboxylation efficiency and Rubisco activity, phosphorylation) to photosynthesis [7,13,16,17,28].

The non-uniform stomatal distribution (or patchy stomatal closure) in some species is known to cause an over estimation of C_i, particularly under severe drought conditions, which may lead to an erroneous conclusion of non-stomatal limitation to photosynthesis [29]. Recently, Sekiya and Yano [50] found that, in cowpea subjected to various environmental conditions, soil water content had no significant effect on stomatal index and exhibited uniform stomatal indices across environmental conditions including water stress. In this study, the uniformity of stomatal response to drought was assumed; however, precaution is recommended while using the results related to C_i.

4.2. Pattern of cowpea response to photosynthesis and WUE

Although, the differences between genotypes could not be tested statistically, the ranges in slopes of A and Fv′/Fm′ as a function of SWC showed an indirect measure of drought sensitivity among cowpea genotypes. Genotypes with steeper slopes (e.g., CB-5) would be more sensitive to drought and experience larger reduction in A per unit decrease in SWC, compared to genotypes (e.g., UCR-193) with lower slopes (For example, Fig. 5A). The functional relationship between A and g_s can be useful to identify genotypes with higher WUE which is more due to increase in A as compared to increase in g_s. Because the WUE is a first derivative of the curve (A/g_s), at a given g_s, moving vertically in the Fig. 6A, towards higher A, will also confer higher WUE. Drought-induced increases in intrinsic WUE have also been reported in other crops and found to represent water-use by plants under field conditions [31,33]. This aspect of genotypic variation has been described as an important goal for crop breeding programs in order to induce drought tolerance and yield enhancement in dry environments [5].

Water-use efficiency is a negative function of C_i/C_a ratio, hence $C_i/C_a min^{-1}$ represents the maximum WUE attainable during drought [29]. Under drought stress, g_s influences the supply of CO_2 to the leaf intercellular spaces; whereas, the capacity of A determines the demand of CO_2, therefore as shown in Fig. 6C and Table 2, lower $C_i/C_a min$ (e.g., UCR-193 compared to MS) obtained at similar or lower g_s values should increase WUE as a consequence of higher capacity for A at a specific range of g_s. This was also supported by a strong correlation between WUE_{max} and $C_i/C_a min^{-1}$ observed in this study.

4.3. Leaf pigments, proline and wax content

Drought-induced reduction in leaf pigments are considered to be a typical oxidative stress indicators which might be attributed to

pigment photo-oxidation, chlorophyll degradation and/or chlorophyll synthesis deficiency [51]. Reduction in chlorophyll concentration is identified as a drought response mechanism in order to minimize the light absorption by chloroplasts [18]. Proline is a known osmolyte that accumulates under water stressed leaves of several species and helps to sustain cell and tissue activity under water limited environment [21,35,39,51]. Similar to the present study, Souza et al. [21] also reported substantial increment in proline content in cowpea under extreme drought stressed conditions. Since no osmotic adjustment has been found in cowpea so far [26,27], despite the known role of proline in osmotic adjustment it has been considered a symptom of injury in some plants including cowpea [21]. The observed enhancement of leaf surface wax content under drought might contribute to reductions in cuticular transpiration [52]. However, no association between wax content and any of the photosynthetic parameters was observed in this study.

4.4. Classification of genotypes

All genotypes that have origins from tropical countries and adapted to dry and hot environments (Prima, TVu-4552, UCR-193, and Melakh: Table 1) [24] along with some genotypes grown in the southern region of USA (MPE, ZC, and TPP) [53,54] were classified as either tolerant or intermediately tolerant to drought stressed conditions. The genotype Melakh is adapted to the dry conditions of the West African countries and has shown high tolerance to drought during vegetative growth stages [24]. One of the genotypes, CB-5, classified as physiologically drought sensitive, is mostly grown in irrigated conditions in California of USA [23] and had poor performance under drought conditions in this study. However, an unambiguous separation based on their site of origins among the studied genotypes could not be found. The identified tolerant genotypes exhibited lower drought sensitivity with relatively higher photosynthesis and improved WUE. The higher rate of photosynthesis during initial stage of drought confers greater plant survival and more dry matter accumulation [5].

Higher g_s in response to drought stress and increased WUE in the identified tolerant genotypes compared to the sensitive genotypes delineate the differences in susceptibility to drought. In fact, there was higher or more stable *A*, as inferred from the lower slope of the linear relationship between SWC and *A* as drought intensity increased in the identified tolerant genotypes. Thus, under drought stressed conditions, the stomatal limitation to *A* seems to be very important in tolerant genotypes and higher WUE observed in theses genotypes could be due to better functioning of carboxylation mechanism [5,55]. This was also supported by a smaller $C_i/C_a min$ in the tolerant genotypes and a strong correlation between WUE and $C_i/C_a min^{-1}$ observed in this study. The significant correlation between A_{slope} and Fv'/Fm'_{slope}, and A_{max} and Fv'/Fm'_{max} indicates that genotypes showing comparatively more stable or higher *A* under drought stressed conditions are also reflecting less photoinhibition by maintaining higher Fv′/Fm′. Lizana et al. [55] demonstrated that bean cultivars showing large plasticity at the biochemical and cellular levels for g_s and *A* also exhibited resistance to photoinhibition.

5. Conclusions

The current study showed the drought avoidance behavior of cowpea by maintaining higher leaf water status. However, soil water status affected the g_s and photosynthetic parameters measured in leaves, exhibiting a pattern of gradual response of photosynthetic parameters to the distinguished four regions of stomatal conductance. Stomatal conductance is the major limitation to *A* under drought conditions in cowpea; however, a pronounced non-stomatal limitation can occur under severe drought stressed conditions that may also lead to impairment of photosynthetic activity. The less responsiveness of Fv′/Fm′ and maintenance of high electron transport as SWC declined, and accompanied with increased photorespiration under drought appeared to be an important protective mechanisms from photoinhibition. The drought-induced reduction in leaf pigments clearly exhibited the oxidative stress which was associated with an increased wax contents. Accumulation of osmolyte such as proline was not associated with any measured photosynthetic parameters indicating a response to drought injury rather than tolerance mechanisms. A lack of clear separation of the genotypes from the different regions was observed for their sensitivity to drought. Based on photosynthetic performance and water-use efficiency characteristics, the cowpea genotypes were classified as tolerant (UCR-193, MBE and TPP), intermediately tolerant (Prima, MPE, TWC, Melakh, ZC and TVu-4552), intermediately sensitive (BC, CB-46 and CB-27), and sensitive (CB-5, MS and MP) to drought stress. The identified genotypes and physiological parameters could be used by breeding programs and/or genetic engineering concerning drought adaptation of legumes.

6. Abbreviations

A	photosynthesis
C_c	CO_2 concentration in the chloroplast
C_i	intercellular CO_2 concentration
C_a	ambient CO_2 concentration
Fv′/Fm′	quantum efficiency by oxidized (open) PSII reaction center
g_s	stomatal conductance
g_m	mesophyll conductance
SWC	soil water content
WUE	water-use efficiency

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