Electronic Journal of Plant Breeding, 10 (4): 1461-1470 (Dec 2019) DOI: 10.5958/0975-928X.2019.00188.1
ISSN 0975-928X

Research Article

Physiological and biochemical response of cowpea (*Vigna unguiculata* L.) landraces of Kashmir valley under water stress

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(Received: 05 May 2019; Revised: 19 Dec 2019; Accepted: 23 Dec 2019)

Abstract
The present study is based on the hypothesis that natural variation for physiological and biochemical parameters can be effectively harnessed to improve water stress resilience. Under irrigated conditions, CTD decreased progressively. However, under drought stress, it decreased sharply. Under drought relative water content (RWC) had a mean value of 65.49% with the highest and lowest value recorded for C25 and C32. The largest and smallest reduction in RWC under drought was recorded in case of C32 and C9. There was wide variation in chlorophyll a and b content. The highest and lowest values for chlorophyll stability index (CSI) were recorded for C13 and C29 respectively. The DAB assay clearly differentiated the lines on the basis of darker staining of leaves. The lines showing greater per cent reductions in yield parameters such as C1, C2, C3, C7, C12, C14, C22 and C29 showed greater staining in leaves in DAB assay.

Key words
Cowpea, canopy temperature depression, relative water content, DAB assay

Introduction

Grain legumes form a major group food crops across the world due to its short duration, and high protein content. Among the important grain legumes grown in India, cowpea (*Vigna unguiculata* L.) plays a significant role in nutritional security. Globally cowpea is grown over an area of 12.61 million hectares, with a production of 5.59 million tones, of which India accounts for 15.06 and 8.45 per cent of area and production respectively (Singh, 2014). Cowpea is generally grown in the drier areas of the world with little or no irrigation facilities. Deficit rainfall early in the season has adverse effects on the growth of the crop. Cowpea is highly sensitive to drought stress during flowering stage (Lobato et al., 2008), leading to significant reductions in grain yield. It is anticipated that the occurrence of drought stress in the major grain legume-producing regions will increase in response to changing and variable climate (Semenov and Shewry 2011). In general, breeding efforts to improve crop yields under drought stress are focused on aboveground plant parts (Wachsman et al. 2015), and the knowledge about genotypic differences among cowpea genotypes in root architecture and shoot related traits influencing drought tolerance is limited.

Among above ground traits, earlier workers have reported no osmotic adjustment in cowpea under water stress, suggesting that differences at the maximum water deficit were not originated by the accumulation of osmotically active solutes (Souza et al., 2004). Therefore, stomatal behaviour in cowpea plants is important to preserve shoot water status under moisture stress conditions. Various other physiological, biochemical, and anatomical mechanisms such as CTD, RWC, Chlorophyll content and stability as well as oxidative damage have also been reported (Santos *et al.*, 2011). Cowpea is well known for its tight stomatal control with no difference in gas exchange between drought tolerant and susceptible genotypes (Verbree, 2012). CTD has also been implicated as an important trait, with some drought-tolerant cultivars, in particular, observed to have hotter canopy temperature (negative CTD), possibly on account of their ability to conserve moisture by closing its stomata whereas other drought-tolerant cultivars have the coolest canopy temperature (positive CTD). Similar results have also been reported in common bean (Khalid, 2017). Thus, it appears that no single method of phenotyping for drought tolerance can be broadly applied across all genotypes due to contrasting mechanisms of drought-tolerance and environmental differences. An understanding of the relationship of root traits to the shoot traits as well as physiological parameters that contribute to grain yield is essential to achieve improvements in productivity under water stress conditions. The present study is based on the hypothesis that natural variation in cowpea germplasm for root architecture, biomass partitioning and physiological parameters can be effectively harnessed to improve water stress resilience. The lines can be used in breeding programmes to develop stress resilient genotypes that could be used to improve productivity of cowpea.

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Materials and Methods

Plant materials: The experiment was carried out at the greenhouse facility and research field of Division of Genetics and Plant Breeding, Faculty of Agriculture Wadura, SKUAST-K, Sopore. A set of 20 genotypes of cowpea including 19 landraces collected from different areas of the Jammu-Kashmir valley and one released variety *viz.*, Shalimar Cowpea-1 as check were used in this study. These genotypes were evaluated under laboratory, green house and field conditions for root architecture (depth, biomass, volume, diameter, biomass density, tissue mass density, biomass at top, biomass at bottom and root-shoot ratio), shoot traits (height, biomass, number of leaves, leaf area), physiological (canopy temperature depression, relative water content), biochemical (DAB assay) as well as yield parameters and biomass partitioning.

Greenhouse experiment: The experiment was conducted during March-April, 2017, during which the air temperature ranged from 30 to 33 °C. The experimental design was factorial completely randomised design with three replications. The factor 1 was genotypes and factor 2 being the irrigation level. The plants were grown in Poly Vinyl Chloride (PVC) columns of dimensions 1.3-m height and 20 cm internal diameter. The growth medium comprising of soil and sand was chosen to simulate field screening. Using sand alone results in long roots (offering less friction) while using soil alone greatly impedes root growth especially under drought treatment on account of formation of hard pan. Initially four seeds of each genotype were sown at a depth of 3 cm after surface sterilisation with 10% NaOCl for 5 min and subsequent rinsing with distilled water. After the plants reached the first trifoliate leaf stage, only two competitive plants per column were maintained. The rooting medium was fertilized with Osmocote, a slow-release fertilizer with 19:6:12 ratio of $N:P_2O_5:K_2O$, respectively, at 4 g per column before sowing by mixing with top soil. A systemic insecticide, Vermitech 1% G (a.i.: Imidacloprid: 1–[(6–Chloro–3–pyridinyl) methyl]–N–nitro–2–imidazolidinimine) was applied at 1 g per column before sowing to control sucking pests.

Drought stress imposition: From sowing to harvest, the control plants were maintained at 100% field capacity by irrigating on daily basis. For the drought treatment, plants were maintained under 100% field capacity from sowing to the trifoliate stage. Drought stress was imposed by withholding water from trifoliate stage till pod development stage. The duration of drought stress was 41 days. The moisture content of medium at the end of the drought treatment was 30%, which was quantified on weight basis (Black 1965).

Canopy temperature depression: Canopy temperature was measured after 14, 21, and 28 days after drought stress imposition between 10:00 and 14:00 hrs using a hand held infrared thermometer (Fluke 68 Max, Fluke Corporation, WA, USA). Five readings per replication were recorded. Canopy temperature depression was calculated as difference between air temperature and canopy temperature.

Leaf relative water content: Leaf relative water content (RWC) was quantified 21 days after drought stress imposition from top most fully expanded leaves. Relative water content was measured between 11:00 and 13:00 hrs by adopting the procedure of Barr and Weatherley (1962).

Chlorophyll content (Chl. a and b): It was estimated by Ethanol extraction method following spectrophotometric absorbance at 649 and 664 nm (Koleyoreos, 1958). The quantification of Chlorophyll-a, Chlorophyll-b, by different 95 % Ethanol solvents using the spectral absorbance for Chlorophyll-a and Chlorophyll-b was done using the equation:

$$\text{Chl-a} = 13.36\ A664 - 5.19\ A649$$

$$\text{Chl-b} = 27.43\ A649 - 8.12\ A664$$

Chlorophyll stability index (CSI): CSI in the leaf was measured using a spectrometer following the method of Koleyoreas (1958). CSI was calculated as the difference in light transmission percentage between treated and untreated leaf samples by the formula:

$$CSI = \frac{\text{OD at 649 nm (stress)}}{\text{OD at 649 nm (control)}}$$

DAB assay for oxidative damage : DAB assay was done as per Daudi and O'Brien (2012). In this protocol, the *in situ* detection of hydrogen peroxide (one of several reactive oxygen species) is done by staining with 3,3'-diaminobenzidine (DAB). DAB is oxidized by hydrogen peroxide in the presence of some haem-containing proteins, such as peroxidases, to generate a dark brown precipitate. This precipitate is exploited as a stain to detect the presence and distribution of hydrogen peroxide in plant cells. Leaves are directly visualized for DAB staining. Photographs were taken under uniform lighting.

Results and Discussion

Canopy temperature depression: In the present study canopy temperature depression was measured at three stages namely second, third and fourth week after stress. Under irrigated conditions, CTD had mean value of 2.76, 1.87 and 0.27 at second, third and fourth week of stress impositions respectively (Table 1). At stage 1 highest value was recorded for C3 (4.85) while the lowest value was recorded for

C22 (0.74). At stage 2 highest value was recorded for C14 (3.40) followed by C11 (3.04) while lowest value was recorded for C4 (0.66). At stage 3 the largest value was recorded for C1 (1.83) while the lowest value was recorded for C7 (-2.01).

Wide variation was noticed in genotypic response to irrigated and water stress treatments in terms of CTD. Under irrigated treatment, across genotypes, CTD remained mostly positive but decreased progressively, possibly on account of increased evaporative demand due to canopy expansion as the growth progressed, indicating that plants tend to keep themselves cooler when water is available. However, under drought stress, the CTD had sharp decrease and was mostly negative at the third stage of measurement, possibly on account of lack of moisture in the column. Under irrigated conditions CTD averaged across three stages was positive for all the genotypes with the highest value recorded for C11 (3.10 ^{o} C), while the lowest value was observed in 22 (0.49 ^{o}C). Under drought stress, however 10 genotypes exhibited negative values with highest negative value recorded for C8 (-1.41^{o}C) followed by C7 (-1.02 ^{o} C) and C13 (-0.78 ^{o} C). Positive CTD values under drought were recorded for many genotypes with highest value recorded for C11 (2.25 ^{o}C) followed by C1 (1.87 ^{o}C) and C12 (1.7 ^{o}C). When correlated with seed yield under well irrigated and water stressed conditions, we observed that genotypes (C6, C25, C7, C4, C11, and C10) with higher positive CTD had higher yields under field condition. Ndiso *et al.* (2016) reported similar results in cowpea with an increase in canopy temperatures under water stress both at vegetative as well as flowering stage. Under greenhouse conditions, genotypes with a cooler canopy temperature (higher CTD) under drought stress use more available soil moisture to cool the canopy by transpiration to avoid excessive dehydration (Reynolds *et al.,* 2009). Canopy temperatures under well-watered conditions also indicate potential yield performance during drought and could effectively be used as a technique to assess genotypic response to drought (Mohammadi *et al.,* 2012).

Under water stress conditions, an interesting observation was recorded. Genotypes with higher yields (Data not shown) were identified on both sides of the CTD extremes (+ and -). Genotypes could be classified into water spenders (+ CTD) and water savers (-CTD). Blum (2015) proposed ideotypes of crop plants based on CTD for use in plant breeding as per the drought types such as the isohydric ("water saving") model and the anisohydric ("water spending") model. The water saving model has a distinct advantage in the harsher environments, whereas the water spending model is expected to perform relatively better under more moderate/mild drought situations. Polania *et al.* (2016) proposed that the water spender genotypes can be used for cultivation in areas exposed to intermittent drought stress with soils that can store greater amount of available water deep in the soil profile. However, water savers would be more suitable in semiarid to dry environments dominated by the terminal drought stress. The water savers or isohydric genotypes are characterized by a shallow root system with intermediate root growth and penetration ability and thin roots. Such genotypes are early and have high water use efficiency, reduced transpiration and limited leaf area and canopy biomass development, reduced sink strength and superior photosynthate remobilization to pod and grain formation. Contrary to this, water spenders or anisohydric genotypes have a vigorous and deep rooting system with rapid root growth rate and penetration ability, and a thicker root system. Such genotypes are early and have high water use efficiency, moderate transpiration and fast leaf area and canopy biomass development, moderate sink strength and superior photosynthate remobilization to pod and grain formation.

Canopy temperature can be related to the genetic potential of root's capacity to explore soil moisture (Pinto and Reynolds 2015) and as such can be used as effective surrogate trait for the analysis of root development and biomass partitioning under drought stress (Bhandari, 2016). Cool canopies (+ CTD) are reported to be associated with enhanced plant access to water by virtue of deeper roots (Lopes and Reynolds 2010) and the genotypes with cooler canopies have been reported to yield 30% more, with a concomitant increase of 40% in root dry weight. CTD has been reported to be correlated with yield under both drought stress (Purushothaman *et al.,* 2017) and hot irrigated conditions (Pinto and Reynolds, 2015). Drought susceptible genotypes which suffer relatively greater yield loss under drought stress tend to have warmer canopies at midday. Our studies have revealed that CTD can be a reliable indicator of crop performance under both irrigated and drought stress conditions. Under irrigated conditions there was a linear trend of higher yield with CTD, however, under drought stress, both negative CTD and positive CTD could be identified, and in both classes, high yielding genotypes were identified. The water savers probably could sense drought stress in early phases of growth and could trigger conservative water use that could be used in later stages of growth (Khalid, 2017).

Relative water content: Under irrigated conditions RWC had a mean value of 73.63 (Table 2). The highest value was recorded for C6 (89.83) followed by C12 (88.72), while as lowest value was recorded for C4 (62.57). Similarly under drought conditions RWC had a mean value of 65.49 with highest value was recorded for C25 (77.30) followed by C13 (74.23, while as lowest value was recorded for C32 (47.49). The largest reduction in RWC under drought conditions was recorded in case of C32 (37.789 %) followed by C12 (23.794%) and C2 (20.332%), while the lowest value was recorded for C9 (1.343%).

Leaf relative water content (RWC) is a reliable indicator of water status in plants and reflects the balance between water supply to the leaf tissue and transpiration rate (Lugojan and Ciulca 2011). It provides a reliable basis for building a relationship between leaf water status and plant metabolism under drought stress, an easily measured, robust indicator of water status for comparison of tissues and species, which 'normalizes' water content by expressing it relative to the fully turgid (hydrated) state (Lawlor and Cornic 2002). Lugojan and Ciulca (2011) proposed that RWC is a relatively better indicator of water status than water potential. Under drought stress, leaf RWC plays an important role in the identification of tolerance of plants to stress by inducing osmotic adjustment due to the accumulation of osmoprotectants (Ritchie *et al.* 1990). The genotype having higher RWC would possibly maintain protoplast hydration for a longer duration under drought conditions than susceptible genotype, which is critical for optimum physiological functions and growth processes. Variation in drought response among genotypes may be associated with dehydration avoidance through lower stomatal conductance leading to higher transpiration efficiency (Khan et al. 2007). Identification of drought tolerant lines through RWC is a rapid method by which a large germplasm pool can be quickly and efficiently narrowed to a manageable number of candidate germplasm for use in a more focused way.

Relative water content is a semi-high throughput method for identification of drought tolerant lines (Knepper and Mou 2015). Studies on faba bean and common bean have shown that maintenance of a relatively high RWC during mild drought is indicative of drought tolerance (Swapna and Shylaraj 2017). Omae et al. (2005) reported that genotypic differences in leaf water status of French bean correlated with grain yield under drought conditions. RWC has been used as an integrative indicator of internal plant water status under drought conditions to identify drought-resistant cultivars in common bean (Choudhury *et* al. 2011).

Chlororophyll content and stability: Under irrigated conditions chlorophyll a had a mean value of 11.15 (Table 3) with the highest value recorded for C14 (12.90) and the lowest value was recorded for SCP-1(8.90), Under drought conditions it had a mean value of 9.01 with the highest value recorded for C9 (14.96) and the lowest value was recorded for C5 (2.57). Similarly, the highest value for chlorophyll b under irrigated conditions was recorded for C2 (37.89) and the lowest value was recorded for C3(16.68) while as under drought conditions the highest value recorded for C9(35.83) and the lowest value was recorded for C5(12.57). Under irrigated conditions chlorophyll a/b ratio had a mean value of 0.46 with the highest value recorded for C6 (0.62) and the lowest value was recorded for C2(0.82) while as under drought conditions it had a mean value of 0.48 with the highest value recorded for C9(0.89) and the lowest value was recorded for C5(0.21).

The chlorophyll stability index (CSI) had a mean value of 0.61 with the highest value recorded for C13 (0.99) and the lowest value was recorded for C5 (0.47). The chlorophyll stability index (CSI) is an indication of the stress tolerance capacity of plants. A high CSI value means that the stress did not have much effect on chlorophyll content of plants. A higher CSI helps plants to with stand stress through better availability of chlorophyll. This leads to increased photosynthetic rate, more dry matter production, and higher productivity. CSI indicates how well chlorophyll can perform under stress (Mohan *et al*, 2000). The reduced values of CSI can be attributed to reduced synthesis and increased breakdown of chlorophyll as induced by water stress. Therefore, the highest values of CSI presented by certain genotypes indicate better maintenance of leaf chlorophyll and hence active photosynthesis thus contributing to their yield stability.

DAB (1, 3-Diaminobenzidine) Assay: The DAB staining of 20 cowpea genotypes (Fig. 1) was carried out under drought conditions to elucidate role of oxidative damage under stressful conditions. In our study, the DAB assay clearly differentiated the lines on the basis of darker staining of leaves under drought. The lines showing greater per cent reductions in yield parameters such as C1, C2, C3, C7, C12, C14, C22 and C29 showed greater staining in DAB assay underlining the reliability of using this assay as a reliable supplement to phenotyping protocols for characterizing large germplasm sets. However, certain genotypes such as C4 having no staining were low yielding and genotypes such as C7 with darker staining recorded better yields under stress. This is possibly due to the fact that antioxidant systems are not significantly implicated in stress response in cowpea (Cavalcanti *et al.*, 2004). However in other crops H_2O_2 has been reported to initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity, actions that result in senescence (Omae *et al.*, 2005).

Physiological parameters such as CTD, RWC and the biochemical parameters such as chlorophyll content and stability can be used as reliable indicators of plant response under stress in crops including cowpea especially in the initial screening of large germplasm sets in view of their high throughput nature and ease of screening.

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Electronic Journal of Plant Breeding, 10 (4): 1461-1470 (Dec 2019) DOI: 10.5958/0975-928X.2019.00188.1
ISSN 0975-928X

Table 1. Mean performance of cowpea genotypes (*Vigna ungiculata*) for canopy temperature depression

Genotype	Irrigated			Drought			Mean (irrigated)	Mean (drought)	Change under drought
	2 weeks	3 weeks	4 weeks	2 weeks	3 weeks	4 weeks			
C1	4.40	2.58	1.83	3.35	1.86	0.39	2.94	1.87	1.07
C2	4.57	1.00	1.31	1.97	0.50	0.03	2.29	0.83	1.46
C3	4.85	1.44	1.56	2.32	0.85	-0.23	2.62	0.98	1.64
C4	3.62	0.66	1.36	1.67	0.88	-0.18	1.88	0.79	1.09
C5	3.90	1.45	1.21	2.29	-0.41	-0.11	2.18	0.59	1.59
C6	2.47	2.30	-0.78	2.20	-1.20	-3.30	1.33	-0.77	2.10
C7	2.44	2.34	-2.01	0.79	-0.40	-3.44	0.92	-1.02	1.94
C8	1.86	2.75	0.66	1.06	-0.85	-4.45	1.76	-1.41	3.17
C9	1.52	2.10	-1.38	1.85	-0.85	-2.15	0.75	-0.38	1.13
C10	2.90	2.60	-0.82	2.22	-0.33	-2.65	1.56	-0.25	1.81
C11	4.85	3.04	1.41	4.87	1.88	0.01	3.10	2.25	0.85
C12	3.87	2.20	-1.00	4.15	0.63	0.33	1.69	1.70	-0.01
C13	3.69	1.03	-0.15	3.35	-1.45	-4.25	1.52	-0.78	2.30
C14	2.30	3.40	0.30	2.05	-0.23	-1.64	2.00	0.06	1.94
C22	0.74	1.53	-0.78	1.65	0.50	-4.40	0.49	-0.75	1.24
C24	1.88	1.71	1.07	1.39	-0.16	-0.60	1.55	0.21	1.34
C25	1.42	1.15	0.27	0.87	-0.80	-0.33	0.95	-0.09	1.04
C29	1.11	1.51	-0.21	0.84	-0.53	-0.93	0.80	-0.21	1.01
C32	1.45	0.90	1.24	1.35	-0.33	-0.73	1.19	0.09	1.10
SCP-1	1.40	1.75	0.26	1.20	0.17	-1.66	1.14	-0.09	1.23
Mean	**2.76**	**1.87**	**0.27**	**2.07**	**-0.01**	**-1.51**	**1.63**	**0.18**	**0.45**
CD (<0.05)	G= 0.58	W= 0.46	S= 0.66		G x W= 0.44				
	G x S = 0.72	W x S= 0.92 G x	W x S= 0.55						
P value	G= <0.001	W= <0.001	S= <0.001			G x W= <0.001			
	G x S = <0.001	W x S= <0.001	G x W x S= <0.001						

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Table 2. Mean performance of cowpea (*Vigna ungiculata*) genotypes for relative water content

Genotype	Irrigated	Drought	Percent reduction
C1	74.385	68.999	7.241
C2	87.460	69.677	20.332
C3	72.009	65.033	9.687
C4	62.575	59.178	5.428
C5	82.200	66.292	19.352
C6	89.836	71.590	20.310
C7	69.548	61.362	11.770
C8	69.284	67.549	2.504
C9	64.759	63.889	1.343
C10	74.223	65.156	12.215
C11	64.128	59.884	6.618
C12	88.726	67.614	23.794
C13	80.269	74.232	7.521
C14	72.185	66.982	7.207
C22	67.427	58.432	13.340
C24	69.838	64.659	7.415
C25	86.752	77.304	10.890
C29	65.115	53.037	18.548
C32	76.338	47.490	37.789
SCP-1	75.820	61.205	19.275
Mean	74.643	64.478	13.129
CD (<0.05)	Genotype= 3.29 Water= 1.04 Genotype × Water= 4.65		
P value	Genotype= <0.0001 Water= <0.0001 Genotype × Water= <0.0001		

Table 3. Mean performance of cowpea (*Vigna Ungiculata*) genotypes for various chlorophyll a and b and chlorophyll stability index.

Genotype	Chl a		Chl b		Chl a/b		CSI
	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	
C1	11.57	6.97	26.93	16.26	0.45	0.43	0.61
C2	10.43	12.05	37.89	25.61	0.28	0.45	0.78
C3	11.89	11.57	16.68	27.92	0.44	0.70	0.71
C4	10.07	6.33	23.96	18.30	0.43	0.35	0.73
C5	9.67	2.57	22.43	12.57	0.44	0.21	0.47
C6	11.52	6.17	18.82	13.84	0.62	0.45	0.66
C7	9.27	10.69	20.74	27.50	0.45	0.39	0.79
C8	12.43	13.82	28.21	27.75	0.45	0.50	0.98
C9	11.27	14.96	16.91	35.83	0.32	0.89	0.68
C10	12.26	9.42	21.53	22.05	0.57	0.43	0.94
C11	14.13	9.78	25.52	21.78	0.56	0.45	0.79
C12	11.78	9.93	27.04	22.48	0.44	0.45	0.84
C13	10.01	11.32	21.69	24.33	0.43	0.53	0.99
C14	12.90	8.01	29.35	19.57	0.44	0.41	0.66
C22	12.06	6.19	25.33	13.73	0.48	0.45	0.54
C24	9.78	7.92	20.85	16.01	0.47	0.49	0.78
C25	11.78	7.41	24.72	15.12	0.48	0.49	0.62
C29	11.46	6.74	23.81	13.58	0.49	0.50	0.58
C32	9.80	9.54	21.50	19.47	0.46	0.49	0.93
SCP-1	8.90	8.70	18.44	17.90	0.48	0.49	0.98
Mean	11.15	9.01	23.12	20.58	0.46	0.48	0.61
CD	**Genotype=** N/A **Water regime=1.255** **Interaction=** N/A		**Genotype=** N/A **Water regime=** N/A **Interaction=12.346**		**Genotype=**0.008 **Water regime=**0.003 **Interaction=**0.012		**Genotype=** 0.008

Electronic Journal of Plant Breeding, 10 (4): 1461-1470 (Dec 2019) DOI: 10.5958/0975-928X.2019.00188.1
ISSN 0975-928X

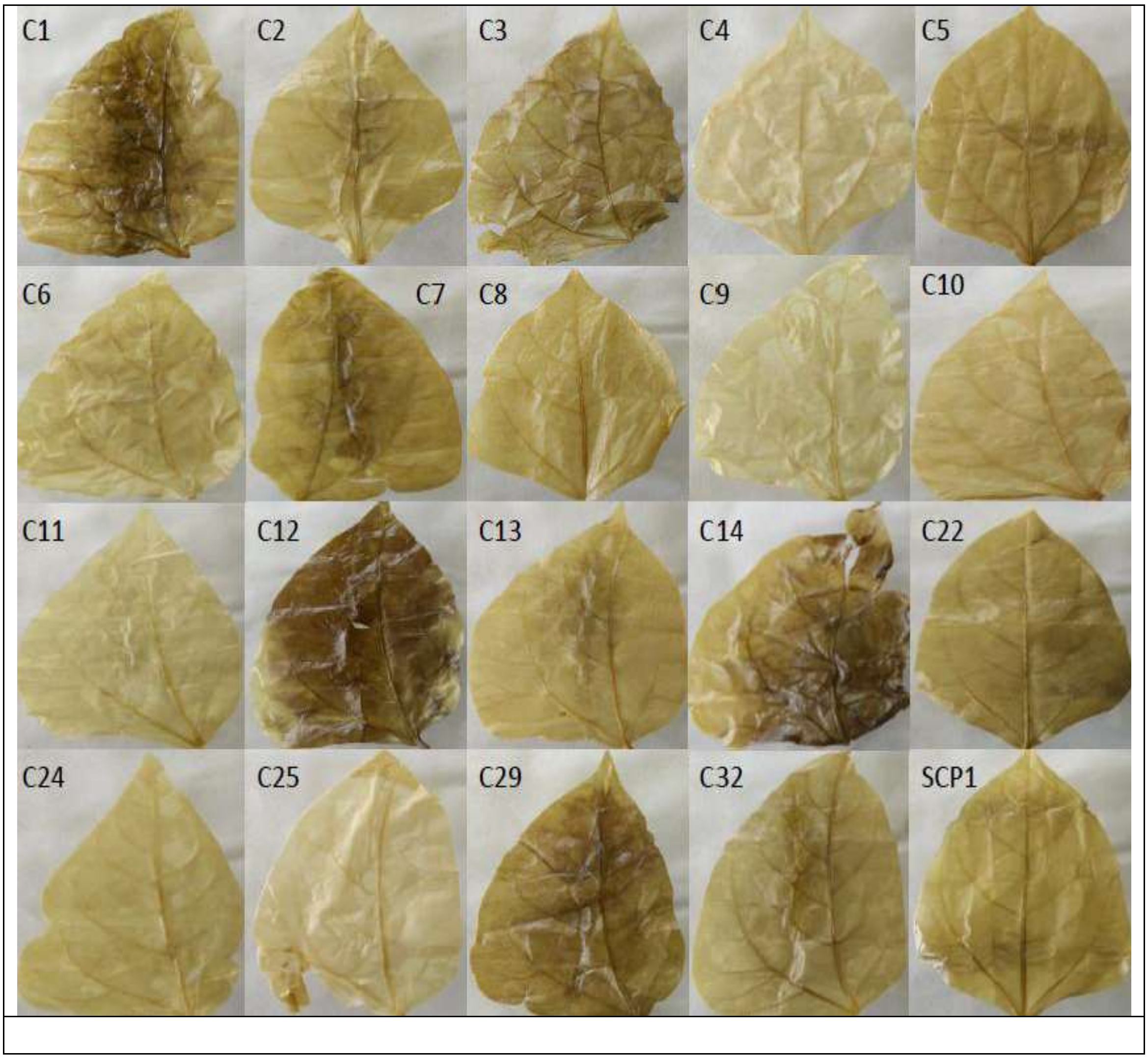

Fig. 1. DAB staining assay of 20 cowpea genotypes under water stress