

THE EFFECT OF OSMOTIC STRESS ON ANTI-OXIDATIVE CAPACITY OF BLACK GRAM (*VIGNA MUNGO* L.)

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SUMMARY

In order to assess the response of four popularly grown black gram (*Vigna mungo* L.) genotypes (T9, PU 19, USJD 113, KU 301) under osmotic stress, the present study was carried out by monitoring the changes in relative leaf water content (RLWC), carotenoid, total soluble protein (TSP), membrane lipid peroxidation (MDA), H₂O₂ scavenging capacity, and the activities of superoxide dismutase (SOD) and catalase (CAT). Osmotic stress was applied by withholding irrigation for 15 consecutive days at vegetative, flowering and pod filling stages. Under stress condition, several fold increase in the content of MDA, H₂O₂ scavenging activity and antioxidant enzyme activity was recorded while, RLWC, carotenoid and TSP were found to decrease. Flowering stage was found to be most sensitive in terms of economic yield. The genotype T9 experienced lowest reduction in yield (12.10–33.91%) with maximum value of drought tolerance index (DTI) (7.48) which can be attributed to its higher tolerance capacity to drought. On the other hand, USJD 113 had the highest yield loss (26.48–60.99%) and lowest DTI (6.07) value, indicating its susceptibility towards osmotic stress.

INTRODUCTION

Black gram is one of the most important highly priced pulse crops grown in a large scale in India and its subcontinents. It enhances soil fertility by fixing atmospheric nitrogen, carbon sequestration and soil amelioration. Among different pulse crops, black gram occupies first position in Assam in terms of area (37,412 ha) which covers about 32.5% of total pulse area. But, the production potential is much below the national level due to selection of marginal lands for its cultivation. As majority of black gram cultivation is done during Rabi or late Rabi season, therefore, the insufficient irrigation facility with erratic rainfall pattern leads to water deficit stress at various stages of crop growth (Belayet *et al.*, 2010).

Drought disturbs normal turgor pressure in cells altering the physiological and biochemical processes, disrupting cell membrane and ultrastructure of subcellular organelles (Yordanov *et al.*, 2003). RLWC is a measure to express the amount of water present in leaf tissues (Sampathkumar *et al.*, 2014). Maintenance of high RLWC is always associated with drought resistance and considered as a valuable indicator of plant water status as compared to other water potential parameters (Keles and Oncel, 2004). Severe drought leads to excessive production of reactive oxygen species (ROS) such as superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl

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radicals ($\cdot\text{OH}$) and singlet oxygen ($^1\text{O}_2$) which are responsible for peroxidation of membrane lipids, bleaching of photosynthetic pigments, protein denaturation and DNA mutation (Mohammadi *et al.*, 2011). Among these, H_2O_2 produced in the chloroplast is especially toxic because it can inhibit the Calvin-cycle enzymes even at low concentrations thereby reducing the photosynthetic carbon assimilation (Abu-Muriefah *et al.*, 2014). Therefore, removal of this toxic compound through the activation of anti-oxidant defense system is very important for plants to alleviate the adverse effects of drought. Under water deficit condition, higher H_2O_2 scavenging capacity reflects better tolerance capacity of plants to drought. Lipid peroxidation in the cell membranes is considered as one of the most challenging and detrimental effect of osmotic stress (Thankamani *et al.*, 2003). The degree of lipid peroxidation is measured in terms of MDA (malondialdehyde) content which is the break down product of lipid peroxidation in cellular membranes and can be used as an important index to examine the incident of oxidative membrane damage (Ozkur *et al.*, 2009).

Plants produce a complex anti-oxidant defense system in a regular fashion to protect cellular components from the damaging effect of ROS. This system includes antioxidant enzymes- SOD, glutathione reductase (GR), CAT and peroxidase, and low-molecular antioxidants such as ascorbic acid, glutathione, α -tocopherol, flavonoids and carotenoids (Sgherri *et al.*, 2000). Hence, enhanced activity of antioxidant enzymes are often observed in plants under stress condition (Khan and Naqvi 2010). O_2^- can be dismutated into H_2O_2 by SOD (the first defense against ROS) in the chloroplast, mitochondrion, cytoplasm and peroxisome. CAT, APX, POD are the enzymatic anti-oxidants that catalyse the conversion of H_2O_2 to water and O_2 and thus help in ameliorating the detrimental effects of oxidative stress (Abedi and Pakniyat, 2010).

The selection of suitable plant characters within a short time at critical crop growth stages emerges as a major challenge to the plant breeders in drought-prone areas (Talebi *et al.*, 2013). A better understanding of genotypic variability to water stress can aid in identifying drought tolerant genotypes to maintain better yield under climate change. Performance of crop plants under drought has been extensively studied. But in spite of being a high value crop of Indian agriculture, black gram has gain very little attention regarding its response pattern towards drought. A number of literatures are available on how the concentration of H_2O_2 changes under water deficit condition but, to our knowledge, no information is available on changes in H_2O_2 scavenging activity/ capacity of pulse crops in response to soil water deficit. The major objectives of this investigation was, therefore, to understand the responses of four commonly grown black gram genotypes in terms of leaf water balance, carotenoid and TSP content, degree of lipid peroxidation in terms of MDA content, H_2O_2 scavenging capacity, antioxidant enzyme activity (SOD and CAT) and finally seed yield under osmotic stress applied at different growth stages. Further, we tried to identify the most vulnerable growth stage to drought and the marker traits that can be useful for selection of drought tolerant genotypes of black gram.

MATERIALS AND METHODS

Experimental site

The experiment was conducted during March–Jun, 2013 at the experimental field of Tezpur University campus located at north bank plain zone of Assam (26°14' N and 92°50' E) at Tezpur, India. The maximum and minimum average temperature recorded was 32 °C and 20 °C respectively. Average rainfall recorded was 195 mm with a relative humidity of 79.45%. The experimental site is characterized by silt loam textured soil being slightly acidic in nature (pH 5.85).

Plant material and experimental design

Four popular black gram (*Vigna mungo* L.) genotypes of Assam- T9, PU 19, USJD 113 and KU 301 were used as test materials and were collected from Regional Agricultural Research Station (RARS), Shillongoni, Nagaon (Assam), India.

The site was ploughed with the help of a tractor. Fertilizers were applied @ 15:35:10 kg NPK ha⁻¹ according to the package of practice of Assam Agricultural University. A temporary rain shed (42×32 m) was constructed in the field with PVC (polyvinyl chloride) film (of about 0.15 mm thickness and 85% of transmittance) to avoid rainfall. In control plants, soil water potential (Ψ_s) of -0.15 to -0.20 bar was maintained with irrigation throughout the growing season while in the treated plots -0.70 to -0.75 bar of the same was recorded after withholding of irrigation for 15 consecutive days.

The experiment was conducted in factorial randomized block design with three replications under stress and non-stress conditions. Seeds were sown in the field on 5th of March, 2013 maintaining the requisite gap of 10 and 30 cm between plants and rows respectively. Control plants were grown with proper irrigation throughout the growing period.

Drought treatments for all the genotypes were arranged as follows:

- T₁– withdrawal of irrigation for 15 days at vegetative stage (25 days after sowing)
- T₂– withdrawal of irrigation for 15 days at flowering stage (35 days after sowing)
- T₃ – withdrawal of irrigation for 15 days at pod filling stage (45 days after sowing)

Soil analysis

Soil samples were collected from the experimental field, processed and analysed for various physico-chemical parameters (e.g. bulk density, soil pH, water holding capacity, soil available nitrogen and organic matter content). Soil tensiometers were used to record soil water potential. Gravimetric method was also employed to over check the moisture content of soil at weekly interval throughout the crop growing period.

Plant analysis

Fully expanded youngest leaves (from three plants of each treatment including control) were sampled at 15 days interval to assess RLWC, carotenoid, TSP, MDA,

antioxidant enzyme activity and H_2O_2 scavenging capacity of stressed plants with respect to their relative controls.

RLWC in leaves was estimated according to Lin and Ehleringer (1982). Fresh weight of leaf samples were taken and were allowed to float in distilled water in a petri dish for 12 h. After 12 h, turgid weight was recorded and finally the leaves were dried at 70 °C for 48 h. Dry weight of leaf samples were noted and RLWC was calculated by the following formula

$$RLWC = [(fresh\ mass - dry\ mass) / (turgid\ mass - dry\ mass)] \times 100.$$

Carotenoid content in leaves was determined by homogenizing 200 mg of fresh leaves collected from top of the canopy with 10 ml of 80% acetone and centrifuged at 1238 g for 10 min. The supernatant was kept aside and the residue was again extracted with 5ml of 80% acetone and centrifuged. A final volume 50 ml was made with 80% acetone. The optical density of the supernatant was measured at 645, 663 and 480 nm in ultra-spec double beam spectrophotometer (UV-1700 series, Pharma Spec, Japan). Carotenoid content was calculated using the formula given by Kirk and Allen (1965) and were expressed as $mg\ g^{-1}$ fresh weight.

$$Carotenoid = A_{480} + 114(A_{663}) - 638(A_{645}) \times V / (1000 \times W),$$

where, A_{480} = Absorbance of the extract at 480 nm; A_{645} = Absorbance of the extract at 645 nm; A_{663} = Absorbance of the extract at 663 nm; V = Volume of the sample; W = Fresh weight of the tissue.

TSP was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard. 500 mg of fresh leaves were homogenized in 0.1M of phosphate buffer (pH 7.5). The extract was centrifuged at 10,000 g for 20 min at 4 °C and supernatant obtained was used for protein determination and enzyme assay.

SOD activity was assayed by recording its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by Dhindsa *et al.* (1981). 3 ml of reaction mixture was prepared by mixing 0.1 ml of 1.5 M Na_2CO_3 , 0.2 ml of 200 mM methionine, 0.1 ml of 3mM EDTA, 0.1 ml of 2.25 mM NBT, 1.5 ml of 100 mM potassium phosphate buffer (pH 7.5), 1 ml of distilled water and 0.05 ml of enzyme samples. The tube without enzyme was taken as control. Reaction was started by adding 0.1 ml 60 μ M riboflavin and placing the tubes below a light source of two 15 W fluorescent lamps for 15 min. The reaction was stopped by switching of the light and covering the tubes with black cloth. Absorbance was recorded in ultra-spec double beam spectrophotometer at 560 nm. SOD activity of the extract was expressed as $U\ min^{-1}\ mg^{-1}$ protein.

$$\text{SOD activity (U min}^{-1} \text{ mg}^{-1} \text{ protein)}$$

$$= \frac{\% \text{ Reduction in color between blank and sample} \times \text{Dilution factor} \times 60}{50 \times \text{Incubation time} \times \text{mg protein in sample}},$$

where, Dilution Factor = Biomass taken (in mg)/ Volume of extraction buffer, 50% reduction in colour was considered as one unit of enzyme activity expressed in enzyme U mg⁻¹ protein min⁻¹.

CAT was assayed as per the method of Aebi (1984). The disappearance of H₂O₂ was monitored at 240 nm ($\epsilon = 40\text{mM}^{-1} \text{ cm}^{-1}$) in the reaction mixture consisting of 50 mM K-phosphate buffer (pH 7.0), 33mM H₂O₂ and enzyme extract. The activity of CAT was expressed as μ mole H₂O₂ scavenged min⁻¹ mg⁻¹ protein.

MDA content was estimated following the method given by Heath and Packer (1968) and Dhindsa *et al.* (1981) with some modifications. For this, 1 ml of extracted enzyme solution was added to 2 ml of a reaction solution containing 20% (v/v) trichloroacetic acid (TCA) and 0.5% (v/v) thiobarbituric acid (TBA). The solution was placed in a water bath at 95 °C for 30 min and then transferred to an ice water bath. It was then centrifuged at 10,000g for 10 min and absorbance of the supernatant was read at 532 and 600 nm using ultra-spec double beam spectrophotometer. Nonspecific absorbance at 600 nm was subtracted from that at 532 nm, and MDA content was calculated using this adjusted absorbance and the extinction coefficient of 155 mM⁻¹cm⁻¹ and was expressed as μ mol g⁻¹ fresh weight.

Hydrogen peroxide scavenging activity of leaf extracts was determined by the method of Ruch *et al.* (1989) with slight modification. For this, 1 ml of the extract prepared for enzyme estimation was dissolved in 3.4 ml of 0.1M phosphate buffer (pH 7.4) and mixed with 0.6 ml of 40 mM H₂O₂ solution. Absorbance of the reaction mixture at 230 nm was determined 10 minutes after H₂O₂ addition against a blank solution containing the phosphate buffer without H₂O₂ that was replaced by equivolume of buffer. The percentage of hydrogen peroxide scavenging were calculated as

$$\% \text{ Scavenged } [H_2O_2] = [(A_0 - A_1) / A_0] \times 100,$$

where, A₀ was the absorbance of the control (without sample extract) and A₁ was the absorbance in presence of the sample extract.

On maturity, harvesting was done from an area of 1m² of each replication; weighed and seed yield for each genotype at different treatments including the control was recorded. DTI was used as a measure of tolerance capacity to drought in terms of minimization of reduction in yield caused by water stress in comparison to control condition and was calculated as

DTI = (Yield_{drought} × Yield_{control}) / Mean yield of all genotypes under control condition (Baroowa *et al.*, 2012).

Statistical analysis

Results were analysed by analysis of variance (ANOVA) and LSD values were calculated for genotypes, treatments and the interaction between genotypes and treatments. Mean values were taken from the measurements of three replicates and standard error (SE) of the means was calculated. Duncan's multiple range tests (DMRT) was performed at $p \leq 0.05$. All the statistical analyses were done using the Statistical Package for Social Sciences (SPSS) for Windows (version 16.0).

RESULTS

Relative leaf water content (RLWC)

RLWC values of black gram genotypes grown under control and stress conditions are presented in [Figure 1A](#). Application of drought significantly decreased ($p \leq 0.01$) RLWC in stressed plants as compared to their respective control. This drought-induced reduction in RLWC was highest in those plants exposed to water stress during flowering stage (T_2). A considerable difference among the genotypes was also observed for RLWC. Genotype T9 maintained higher RLWC at all the growth stages. In contrast, lowest value of RLWC was recorded in treated plants of USJD 113.

Carotenoid

Carotenoid content of black gram leaves was highly influenced by water stress at all the growth stages. Withdrawal of irrigation for 15 consecutive days resulted in significant reduction ($p \leq 0.01$) in carotenoid and it was more pronounced in the plants suffered from osmotic stress during flowering stage ([Figure 1B](#)). The rate of decline in this photosynthetic pigment varied with genotypes and highest reduction was observed in USJD 113 (54.95–66.67%). Better maintenance of carotenoid was noted in T9 followed by KU 301 and PU 19 irrespective of treatments.

Total soluble protein (TSP)

Under well-watered condition, the values of TSP were significantly higher ($p \leq 0.05$) than the plants suffered from scarcity of water ([Figure 2A](#)). Drought-induced reduction in TSP occurred to a greater extent in treatment T_2 (drought applied at flowering stage). Irrespective of growth stages, genotype T9 experienced lowest reduction of TSP (5.84–12.64%) followed by KU 301 (16.01–22.93%), PU 19 (17.99–29.59%) and USJD 113 (29.17–44.93%).

Membrane lipid peroxidation (MDA)

We noted a significantly higher ($p \leq 0.01$) accumulation of MDA under water deficit stress in the studied genotypes which indicate higher degree of MDA ([Table 1](#)). Highest increment of MDA was observed in genotype USJD 113 (118.98–241.05%) in all the treatments. In contrast, T9 recorded lower increment of MDA (20.46–70.39%) after imposition of stress. Under water limited condition, highest increment in MDA were observed in T_1 plants (stressed during vegetative stage) and it was in the order $T_1 > T_2 > T_3$.

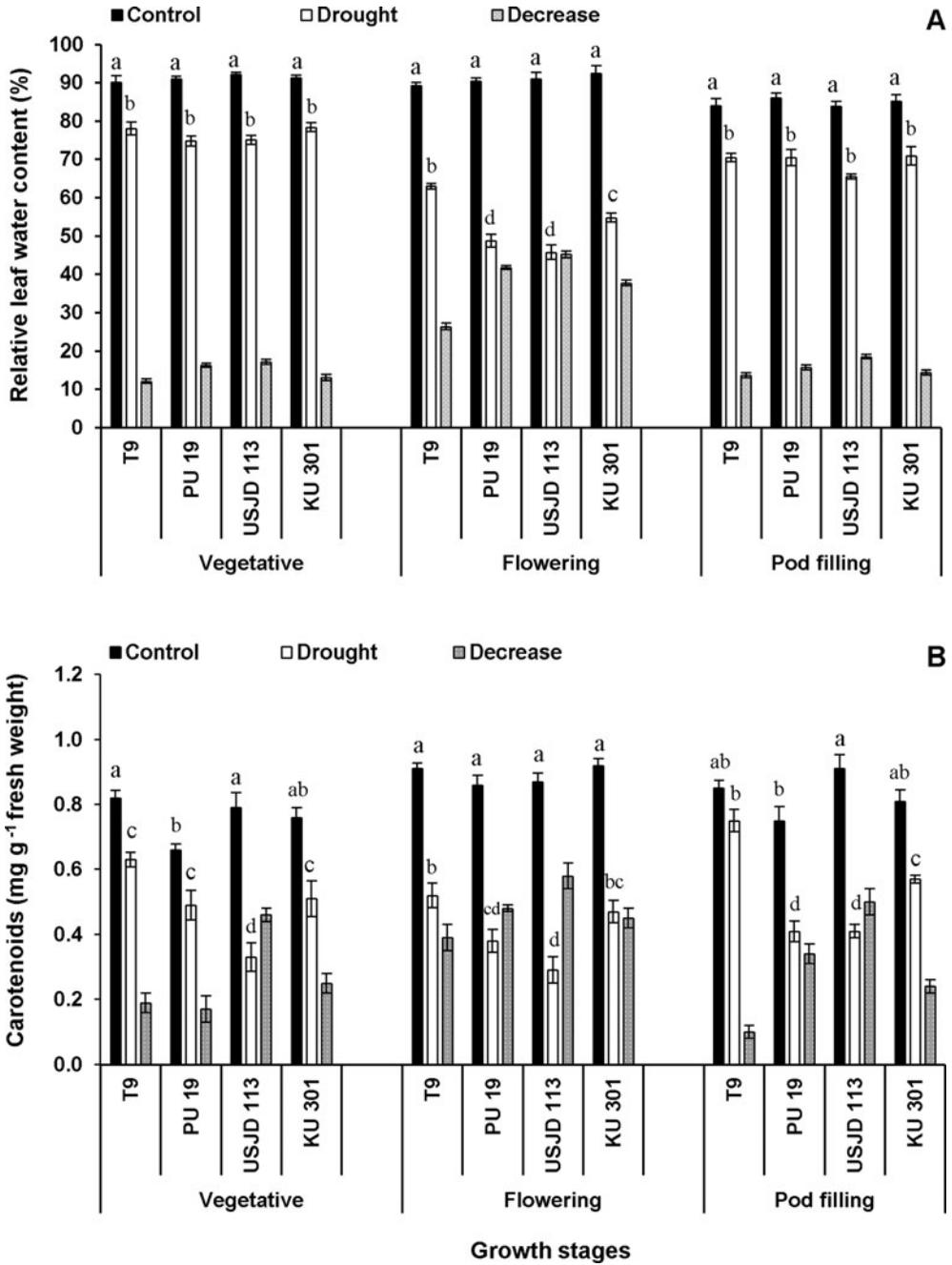


Figure 1. Drought induced changes in relative leaf water (A) and carotenoid (B) content of black gram (mean ± SE, values followed by different letters indicate significant differences among the genotypes within a stage of crop growth period at $p \leq 0.05$).

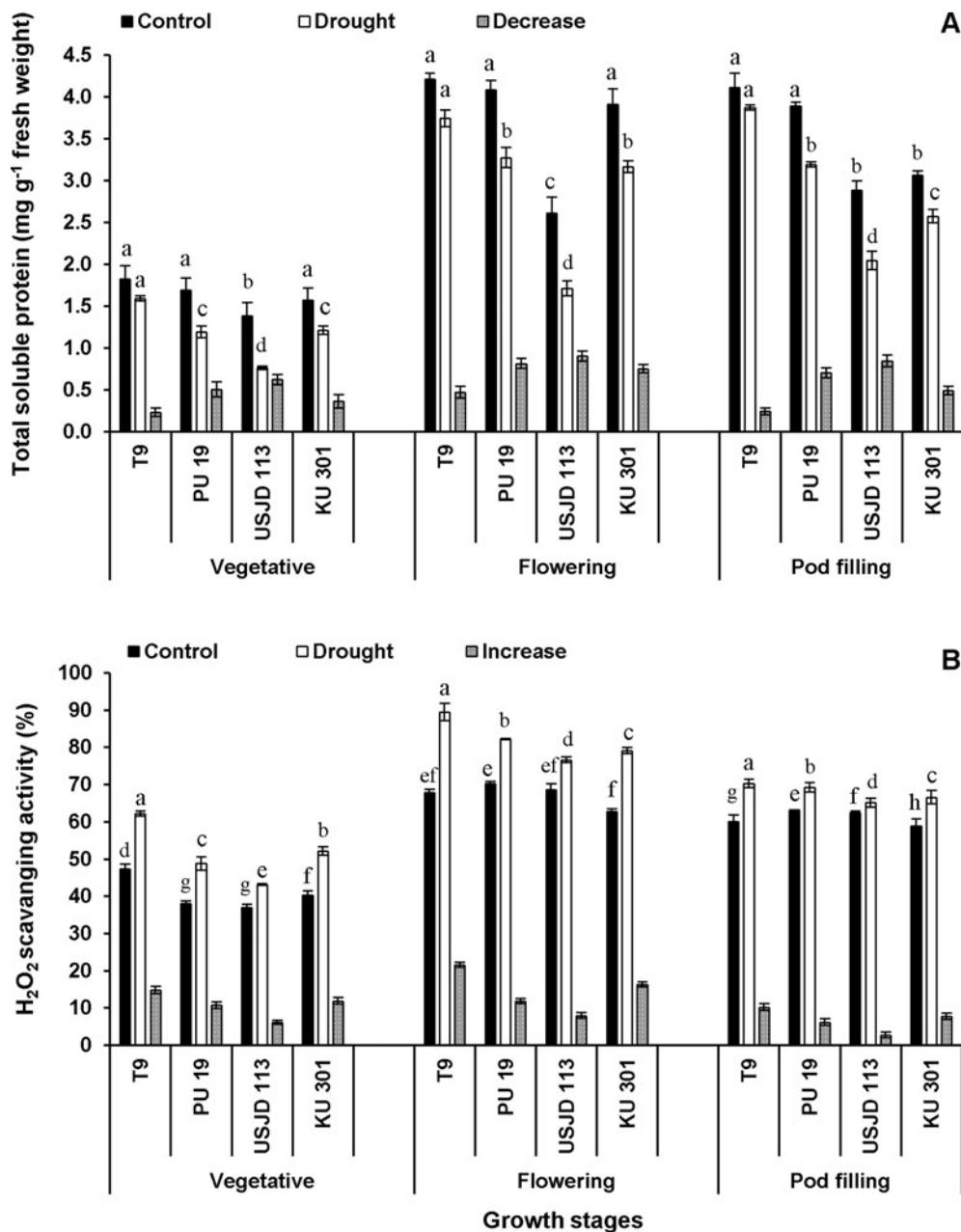


Figure 2. Drought induced changes in total soluble protein content (A) and hydrogen peroxide scavenging activity (B) of black gram (mean \pm SE, values followed by different letters indicate significant differences among the genotypes within a stage of crop growth period at $p \leq 0.05$).

Table 1. Effect of osmotic stress on membrane lipid peroxidation (MDA), super oxide dismutase (SOD) and catalase (CAT) activity of black gram (mean \pm SE, values followed by different letters indicate significant differences among the genotypes within a stage of crop growth period at $p \leq 0.05$).

Growth stages	Genotypes	MDA (μ mole g^{-1} fresh weight)			SOD (U min^{-1} mg^{-1} protein)			CAT (μ mole H_2O_2 scavenged min^{-1} mg^{-1} protein)		
		Control	Drought	% Increase	Control	Drought	% Increase	Control	Drought	% Increase
Vegetative	T9	0.91 \pm 0.03e	1.55 \pm 0.04d	70.39	15.78 \pm 0.66cd	17.72 \pm 0.38b	12.29	0.49 \pm 0.03c	0.99 \pm 0.07a	100.81
	PU19	0.89 \pm 0.03e	1.95 \pm 0.05b	119.06	17.19 \pm 0.59bc	17.95 \pm 0.49b	4.42	0.34 \pm 0.03c	0.70 \pm 0.05b	105.26
	USJD 113	0.73 \pm 0.05e	2.48 \pm 0.04a	241.05	14.76 \pm 0.40d	15.04 \pm 0.48d	1.90	0.35 \pm 0.07c	0.66 \pm 0.03b	87.75
	KU 301	0.91 \pm 0.11e	1.76 \pm 0.06c	95.27	18.78 \pm 0.45b	19.77 \pm 0.44a	5.27	0.40 \pm 0.07c	0.88 \pm 0.11ab	119.35
Flowering	T9	1.08 \pm 0.10d	1.48 \pm 0.04c	36.84	20.14 \pm 1.13c	25.93 \pm 0.61a	28.75	0.88 \pm 0.17b	1.09 \pm 0.05a	24.69
	PU19	1.34 \pm 0.14d	2.01 \pm 0.06b	50.37	21.36 \pm 0.16bc	22.12 \pm 0.61b	3.56	0.71 \pm 0.05b	0.84 \pm 0.02b	18.12
	USJD 113	1.04 \pm 0.13d	2.27 \pm 0.05a	118.98	17.43 \pm 0.61e	18.65 \pm 0.54d	7.00	0.69 \pm 0.02b	0.78 \pm 0.11b	11.35
	KU 301	1.27 \pm 0.03d	1.87 \pm 0.03b	47.59	23.43 \pm 0.55b	25.48 \pm 0.47a	8.75	0.73 \pm 0.08b	0.91 \pm 0.03ab	24.25
Pod filling	T9	0.86 \pm 0.05b	1.05 \pm 0.09b	20.46	20.43 \pm 0.55ab	21.86 \pm 1.13a	7.00	0.73 \pm 0.02a	0.79 \pm 0.01a	8.22
	PU19	1.02 \pm 0.07b	1.28 \pm 0.04b	25.02	20.97 \pm 1.09ab	21.27 \pm 0.15a	1.43	0.53 \pm 0.01b	0.58 \pm 0.02b	10.25
	USJD 113	0.91 \pm 0.06b	1.99 \pm 0.10a	119.07	18.93 \pm 0.64b	19.02 \pm 0.63b	0.48	0.65 \pm 0.01ab	0.68 \pm 0.01ab	5.26
	KU 301	0.85 \pm 0.07b	1.11 \pm 0.12b	30.13	22.46 \pm 1.18a	22.79 \pm 0.60a	1.47	0.59 \pm 0.01b	0.65 \pm 0.01ab	11.24

H₂O₂ scavenging activity

A sharp increase in H₂O₂ scavenging activity was observed in plants experiencing drought for 15 consecutive days (Figure 2B). This enhancement was more pronounced at flowering stage followed by vegetative and pod filling stages. In all the treatments, genotype T9 showed highest capacity to scavenge H₂O₂ (16.97–31.67%). The increment in H₂O₂ scavenging activity followed the order of T9 > KU 301 > PU 19 > USJD 113 in all the treatments.

Antioxidant enzyme activity

Stress applied at vegetative, flowering and pod filling stages activated the antioxidant system in all the genotypes. The activity of SOD and CAT increased considerably under stress condition as compared to their respective control (Table 1). Highest enhancement in the activity of SOD was noted in T₂ plants (stressed at flowering stage) while, T₁ plants (stressed at vegetative stage) of all the genotypes exhibited the highest increase of CAT activity. Under drought, highest enhancement of CAT activity was noted in genotype KU 301 (11.24–119.35%) while lowest of the same was recorded in USJD 113 (5.26–87.75%). On the other hand, enhancement of SOD activity was most prominent in genotype T9 followed by KU 301, PU 19 and USJD 113.

Seed yield

Imposition of drought had a significant ($p \leq 0.01$) negative impact on seed yield of black gram regardless of growth stages (Table 2). The yields of all the genotypes in non-stress environment clearly out yielded in the stress environment. Plants subjected to drought at flowering stage (T₂) produced lowest seed yield in comparison to the plants subjected to drought at vegetative and pod filling stages (Table 3). The reduction of yield was highest in USJD 113 (26.48–60.99%) and was lowest in T9 (12.10–33.91%). Genotype T9 also gave highest value of (DTI) than rest of the genotypes and it followed the order of T9 > KU 301 > PU 19 > USJD 113.

DISCUSSION

This study was carried out to evaluate the mechanism of drought tolerance in four commonly grown black gram genotypes of Assam (India). All the genotypes showed differential response pattern to soil moisture deficit which was observed by the changes in RLWC, carotenoid, TSP, MDA, H₂O₂ scavenging capacity and the activity of antioxidant enzymes- SOD and CAT.

Deficiency in soil moisture significantly affected the water balance of black gram plants. Plants experiencing drought recorded considerably lower values of RLWC with respect to their control (Figure 1A). Being a measure of plant water status, RLWC reflects the metabolic activities in tissues. Maintenance of RLWC helps in sustaining photosynthetic capacity of plants which ultimately leads to greater yield (Sinclair and Ludlow, 1986). Therefore, it can be considered as the most meaningful index for identifying genotypes with complementary differences in dehydration tolerance. In an earlier study, Zlatev (2005) found that drought tolerant bean cultivars had greater capacity to maintain RLWC under water-limited condition. In our experiment,

Table 2. Mean square and LSD values for relative leaf water content (RLWC), carotenoid, total soluble protein (TSP), MDA, H₂O₂ scavenging activity and SOD and CAT activity of black gram.

Growth stages	Sources of variation	H ₂ O ₂ scavenging activity						
		RLWC	Carotenoid	TSP	MDA	SOD	CAT	
Vegetative	Genotype (G)	3.694 ^{ns}	0.033**	0.411**	0.153**	237.168**	19.826**	0.075**
	Treatment (T)	1232.093**	0.424**	1.097**	6.997**	706.986**	5.900*	1.021**
	G × T	6.852 ^{ns}	0.026**	0.042 ^{ns}	0.344**	19.542**	0.735 ^{ns}	0.011 ^{ns}
	Error	4.115	0.004	0.038	0.010	0.042	0.732	0.012
LSD _{0.05}	Genotype (G)	2.471	0.077	0.237	0.122	0.250	1.042	0.133
	Treatment (T)	1.747	0.054	0.168	0.086	0.177	0.737	0.094
	G × T	3.495	0.109	0.336	0.172	0.353	1.474	0.189
Flowering	Genotype (G)	77.739**	0.024**	3.863**	0.198**	73.051**	45.413**	0.072*
	Treatment (T)	8538.667**	1.349**	3.205**	3.147**	1241.857**	36.040**	0.137*
	G × T	100.609**	0.010*	0.052 ^{ns}	0.190**	51.247**	7.859**	0.005 ^{ns}
	Error	6.198	0.003	0.047	0.021	5.172	1.212	0.020
LSD _{0.05}	Genotype (G)	3.033	0.067	0.264	0.177	2.770	1.341	0.172
	Treatment (T)	2.145	0.047	0.187	0.125	1.959	0.948	0.122
	G × T	4.289	0.094	0.373	0.250	3.918	1.897	0.244
Pod filling	Genotype (G)	16.049 ^{ns}	0.051**	2.881**	0.271**	13.752**	13.519**	0.022**
	Treatment (T)	1486.643**	0.522**	1.927**	1.122**	267.601**	1.728 ^{ns}	0.004 ^{ns}
	G × T	5.354 ^{ns}	0.043**	0.099*	0.313**	14.733**	0.552 ^{ns}	0.000 ^{ns}
	Error	7.619	0.003	0.025	0.019	0.031	2.000	0.004
LSD _{0.05}	Genotype (G)	3.363	0.067	0.193	0.168	0.214	1.723	0.077
	Treatment (T)	2.378	0.047	0.136	0.119	0.152	1.218	0.054
	G × T	4.755	0.094	0.272	0.237	0.303	2.436	0.109

^{ns} = non-significant.

* = significant at 0.05 probability level.

** = significant at 0.01 probability level.

Table 3. Effect of osmotic stress on seed yield and DTI of black gram genotypes (mean ± SE, values followed by different letters indicate significant differences among the genotypes under same treatment at $p \leq 0.05$).

Genotypes	Control	Seed yield (q ha ⁻¹)						DTI
		T ₁	% Reduction	T ₂	% Reduction	T ₃	% Reduction	
T9	10.09 ± 0.15a	8.87 ± 0.23a	12.10	6.67 ± 0.38a	33.91	7.11 ± 0.40a	29.48	7.48
PU 19	10.01 ± 0.23a	8.01 ± 0.11b	19.95	5.17 ± 0.18b	48.35	5.89 ± 0.25b	41.19	6.23
USJD 113	10.72 ± 0.30a	7.88 ± 0.05b	26.48	4.18 ± 0.08c	60.99	5.27 ± 0.07b	50.82	6.07
KU 301	9.94 ± 0.23a	7.77 ± 0.12b	21.89	5.59 ± 0.23b	43.81	6.08 ± 0.13b	38.82	6.32
LSD _{0.05}		Genotype (G)		Treatment (T)		G × T		
		0.315		0.315		0.630		

maintenance of higher RLWC by T9 resulted in lesser yield loss which proves its tolerance capacity to drought. On the other hand, USJD 113 showed its susceptibility as it recorded highest reduction in RLWC and consequently seed yield in all the treatments.

In the present study, the reduction in carotenoid content may be due to either slow synthesis or fast breakdown as suggested by Smirnoff (1993). During drought, the

excess generation of ROS results in oxidative stress that damages cellular membranes through peroxidation of lipids and causes degradation of many important components of cell including carotenoids (Monakhova and Chernyad'v, 2002). The observed higher concentrations of MDA under stress condition in our experiment support this. Maintenance of higher level of carotenoids throughout the growing period had a positive impact on seed yield. Therefore, the recorded lowest reduction of carotenoid in T9 can be attributed to its tolerance capacity to drought.

Plants subjected to soil moisture deficiency exhibited significant reduction ($p \leq 0.05$) of TSP in all the treatments (Figure 2A). Our results are in accordance with the findings of Abdel-Nasser and Abdel-Aal (2002) working with sunflower seedlings. Drought-induced overproduction of ROS caused damage in cell membranes which disrupted protein structures leading to reduction in yield. ROS are responsible for protein denaturation or they may also affect the process of protein synthesis (Schwanz *et al.*, 1996). Fattahi Neisiani *et al.* (2009) suggested that under moisture stress, the decomposition of protein is essential for production of amino acids to contribute osmotic adjustment by acting as osmoticum. Therefore, the reduction of TSP is often considered as an adaptive mechanism to overcome the adverse effects of drought.

Under osmotic stress, the MDA contents of all the genotypes increased significantly (Table 1) in comparison to the plants grown in well-watered environment. Higher accumulation of this product was previously recorded in many species like bread wheat and maize exhibiting osmotic stress (Fattahi Neisiani *et al.*, 2009; Khan and Naqvi, 2010). Like other environmental stresses, drought can bring about lipid peroxidation through the activation of toxic O_2 molecules that can then attack fatty acids chains in thylakoid membrane. This results in enhanced formation of MDA with subsequent damage to membrane (Abu-Muriefah *et al.*, 2014). Pandey *et al.* (2010) suggested that genotypes which produce lower level of MDA under stress have better capacity for moisture stress tolerance. Hence, in our experiment, genotype T9 showed its tolerance capacity by recording lowest increment in MDA thereby preventing membrane damage. The increment in MDA was most prominent in the genotype USJD 113 which indicates higher degree of membrane damage triggered by osmotic stress.

Regardless of growth stage, hydrogen peroxide scavenging activity was found to increase significantly ($p \leq 0.01$) when plants were subjected to drought for 15 consecutive days (Figure 2B). In plant cell, H_2O_2 is produced by univalent reduction of $O_2^{\cdot-}$. Excessive production of H_2O_2 under stress condition leads to the occurrence of oxidative stress due to its ability to inactivate anti-oxidant enzymes by oxidizing their thiol groups (Gill and Tuteja, 2010). To hold out in this unfavourable condition, it is essential for plants to scavenge this free radical. During drought, the increased activities of antioxidant enzymes are associated with higher scavenging of H_2O_2 . We observed a significant increase ($p \leq 0.05$) in SOD and CAT activity under stress conditions (Table 1) which reveals that the H_2O_2 scavenging capacity of black gram is closely associated with the activation of anti-oxidant defense system triggered by enhanced activities of SOD and CAT. Our results resembles with the findings of Masoumi *et al.*

(2010) while working with soybean. In earlier studies also, higher increment in the activities of SOD, GR, APX, GPX, GST and POD under drought were recorded in tolerant varieties of common bean and wheat (Bhardwaj and Yadav, 2012; Hameed *et al.*, 2013).

SOD is considered as the most efficient anti-oxidant enzyme. It plays a key role in quenching active oxygen by working as a catalyst to carry out the dismutation of O_2^- into H_2O_2 which are further eliminated by CAT, POD and other anti-oxidant enzymes (Fu and Huang, 2001). In all the studied genotypes, highest increment of CAT activity was observed in the plants stressed during vegetative stage (T_1). The increased rate of metabolism and production of new organs in this stage leads to excess generation of ROS under water-limited environment. Up-regulation of CAT averts cytosolic H_2O_2 build up and protect plant cell from oxidative damage (Prochakova *et al.*, 2001). Therefore, in water-limited conditions, higher activity of SOD accompanied by increased CAT activity is highly desirable to fulfil the high demand of quenching H_2O_2 . In the present study, enhancement in the activity of antioxidant enzymes (SOD and CAT) was lowest in genotype USJD 113 regardless of treatments. These findings suggest the higher susceptibility of USJD 113 towards drought than rest of the studied genotypes. Our results are in confirmatory of the findings of Chen and Cao (2008). Lower level of MDA along with enhanced activities of SOD and CAT (Table 1) in T_9 can be linked to its ability to cope up with water scarcity by limiting the damaging effects of drought through up-regulation of antioxidant enzymes. Again, higher level of MDA and lower increment in anti-oxidant enzyme activities in USJD 113 could be one of the reasons of its susceptibility.

Osmotic stress imposed at vegetative, flowering and pod filling stages had significant ($p \leq 0.01$) impact on seed yield. Control plants of all the genotypes gave considerably better yield than the plants grown under water deficit condition (Table 3). The effect of water scarcity on yield is a highly complex phenomenon and involves processes like gametogenesis, fertilization, embryogenesis and seed development (Barnabas *et al.*, 2008). Zare *et al.* (2012) reported that limitation of soil moisture during reproductive stage is especially detrimental for crops in terms of economic yield. Because at this stage, the development of reproductive organs is under the control of photo-assimilate production and partitioning by the source tissues. Therefore, lack of moisture in active crop root zone during flowering and pod formation causes poor nutrient utilization and hindrance in flowering. As a result, the probability of transition from a developing flower to pod is reduced resulting in pod abortion (Kimber and McGregor, 1995). In our experiment, highest reduction in yield was observed when plants were subjected to stress during flowering stage (T_2) followed by pod filling (T_3) and vegetative (T_1) stage. Reduction in yield due to drought was also observed in our earlier studies on black gram and green gram (Baroowa and Gogoi, 2013; Baroowa and Gogoi, 2014). Lesser yield loss accompanied by higher value of DTI recorded in T_9 (Table 2) proves its tolerance capacity to soil moisture deficit. On the other hand, greater yield loss and lower value of DTI noted in USJD 113 can be attributed to its susceptibility to osmotic stress.

CONCLUSION

Remarkable impact of drought was observed in all the genotypes of black gram. Deficiency of soil moisture significantly reduced RLWC, carotenoid and TSP while it increased peroxidation of membrane lipid giving higher concentration of MDA. To counterpart this changes, all the genotypes increased their antioxidant defense capacity through enhancement in the activity of SOD and CAT for scavenging of drought-generated ROS including H_2O_2 . Among the studied genotypes, T9 was identified as the most tolerant genotype of black gram for Assam (India) and areas with similar environmental condition while USJD 113 showed its susceptibility towards osmotic stress. Flowering stage had been proved to be the most vulnerable growth stage towards drought. Genotypic variability, treatment difference and the interaction effect of treatment and genotypes was statistically significant ($p \leq 0.01$) for carotenoid, MDA content and H_2O_2 scavenging activity at all the growth stages and can be taken as marker traits for selection of drought tolerant genotypes of black gram. These findings suggest that black gram can be successfully cultivated even in drought-prone areas by coinciding the time of reproductive development with the period of water availability to minimise yield loss. These results may able to aid some important information in the field of black gram research which will be helpful for breeders also.

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