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Physiological and Biochemical responses of two high yielding Groundnuts Cultivars (*Arachis hypogaea L.*cv. KCG-6 and GPBD-4) of Karnataka with Contrasting Drought tolerance.

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ABSTRACT

Water stress resulted in a significant modification in the level of anti oxidative metabolism. An antioxidant is a molecule that inhibits the oxidation of other molecules. A pot culture experiment in two different groundnut (Arachis hypogaea L.) cultivars (KCG-6 and GPBD-4) of groundnut seedlings were studied using biochemical and histochemical methods to estimate accumulation levels of antioxidant such as Ascorbic Acid Content, antioxidative enzymes such as ascarbate peroxidase (APX) Superoxide dismutase (SOD) and Guaiacol Peroxidase (GPX) and ROS levels. Plants were grown in pots under controlled environmental conditions, and subjected to water stress regimes characterized as control, mild, moderate and severe stress represented by 100, 75, 50 and 25% soil moisture for 12 days. However, the percent increase of antioxidants as well as anti oxidative enzymes was higher in cv KCG-6 and lower in cv. GPBD-4. The water stress resulted in increase in free radicals ($O_2^{-\cdot}$, Content, H_2O_2) in both cultivars, but more significantly in cultivar GPBD-4 than KCG-6. Profile analysis of anionic isoenzymes and total enzymes activity assay of superioxide dismutase (SOD), guaiacol peroxidase (GPX) and asorbate peroxidase (APX) revealed qualitative changes occurred during water stress in both cultivars. The accumulation of ROS, antioxidants and anti oxidative enzymes in relation to water stress of these cultivars was discussed. .The present study indicated that cv. KCG-6 is water stress tolerant than cv. GPBD-4.

Key words: Groundnut (*Arachis hypogaea* L.), Water stress, Antioxidant enzymes, antioxidant parameters, APX, SOD, ROS, GPX, $O_2^{-\cdot}$, Content, H_2O_2

INTRODUCTION

Water stress is a major abiotic stress factor affecting yield and quality of rain fed groundnut worldwide and has been focus of much research. Water stress cause great injury to plants by altering the metabolic processes. A huge variation in water stress tolerance was observed between different crop plants. The effects of water stress largely depend on soil properties and plant species.

Plant responses to water stress is complex phenomenon involving developmental changes as well as physiological and biochemical mechanisms.

An important feature of water stress is the generation of reactive oxygen species(ROS), such as superoxide anion (O_2), hydrogen peroxide($_{H2O2}$) and the hydroxyl radical (OH) that have proven to be important agents in the origin of tissue injury after the exposure of plants to a wide variety of water stress. Antioxidant enzymes such as superoxide dismutase (SOD), guiacol peroxidase (GPX) and ascorbate peroxidase (APX) (Nakano and Asada 1981; Cho and Par 2000; Verma and Dubey 2003; Mittler 2002) are known to be very important for plants in order to protect cellular membranes and organelles from the damaging effects of ROS generated by water stress. Legumes are reported to be tolerant to water stress. There has been a considerable need in finding suitable cultivars that are able to grow on water stress. Owing to the numerous economic importance of this crop as it is the choice oilseed-food-feed-fodder forage crop grown on 35.5 million ha across 82 countries in the world. It is very important to investigate impacts of water stress on growth, physiological and biochemical aspects. However selection of cultivars would be great importance with lesser impact of plant metabolism. Groundnut growing areas fall under sem-arid tropical region of the world. Groundnuts are often subjected to drought stresses for different duration and intensities. Understanding how groundnut plants despite water stress factors proved to be the only crop that will ensure some income to farmers and how plant responds to water stress is a prerequisite for developing strategies for crop improvement in drought tolerance. Hence, we made an attempt to understand the antioxidative metabolism of two groundnuts cultivars of Karnataka KCG-6 and GPBD-4 to water stress. In addition there are no publication on the effect of water stress on morphological, physiological and antioxidative responses of groundnut cultivars KCG-6 and GPBD-4 of Karnataka.

MATERIALS AND METHOD

The seeds of groundnut (*Arachis hypogaea* L. cv. KCG-6 and GPBD-4 were procured from Agricultural Research Station, Chintamani and Dharwad of Karnataka. The seeds were sterilized with 5 mins with frequent shaking and thoroughly washed with tap water. The disease freed and uniform size seeds were sown in earthen pots (60 x 50 cm) containing air-dried 8 kgs of red loamy soil and farm yard manure in 3:1 proportion. The pots were watered once a day with tap water. These pots were kept in the department botanical garden in a wire net enclosure under natural photo period of about 12-14 hours with a temperature of $28 \pm 4^{o}C$.

After 15 days, the seedlings were thinned to two per pot. The pots were maintained for month. One-month-old plants with approximately equal height were selected as experimental materials. Pots were divided into 4 sets. Plants in one set of pots were watered daily and maintained to field capacity are termed as controls. Remaining three sets of pots were used for stress treatments. Water stress was induced by adding required volume of water daily in the morning to give 75%, 50%, and 25% of field capacity and the stress levels were characterized as mild, moderate and severe stress treatments respectively. After induction of stress, the pots were maintained for another 12 days and the experimental data were collected at different time intervals i.e. on day-4, 8 and 12. After inducing water stress the leaf material for experimental data were collected at different time intervals i.e on day 4, 8 and 12. Ascorbic acid was analyzed by the spec method described by Roe and Keuther, (1943).

Extraction of ascorbic acid

The measurement of total ascorbic acid groups were carried out as described in Cakmak and Marshner (1992).0.5 g fresh leaf samples were extracted with 5 ml of 5% metaphosphoric acid, and centrifuged at 15,000x g for 15 min. for the assay of AsA, the reaction mixture contained 0.2 ml aliquot, 0.5 ml 150 mM phosphate buffer (pH 7.4) containg 5 mM EDTA, 0.1 ml 10 mM DTT and 0.1 ml 0.5% (w/v) N-ethylmaleimide (NEM) to remove excess DTT. To this, 0.4 ml 10% ethanol and 0.2 ml 3% FeC1 were added to develop colour. The mixtures were then incubated in awater bath at 40 C for 40 min and the colour produced was read at 525 nm. Ascorbic acid was extracted from 1 g of the plant sample using 4% TCA and the volume was made up to 10 ml with the same. The supernatant obtained after centrifugation at 2000 rpm for 10 minutes was treated with a pinch of activated charcoal, shaken vigorously using a cyclo mixter (REMI CM 101) and kept for 5 minutes. The charcoal particles were removed by centrifugation and aliquots were used for the estimation. Standard ascorbic acid ranging between 0.2-1.0 ml and 1.0 ml of the supernatant were taken. The volume was made up to 2.0 ml with 4% TCA. DNPH reagent (0.5 ml) was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37^0 C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5 ml of 85% sulphuric acid, in cold. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. The tubes were cooled in ice and the absorbance was read at 540nm in a spec. a standard graph was constructed using an electronic calculator set to the linear regression mode. The concentration of ascorbic acid in the samples were calculated and expressed in terms of µ mol/g of sample.

For the assay of SH groups, the reaction mixture contained 0.5 ml aliquot of the supernatant, 2.5 ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.5 ml 6 mM 5-5' dithiobis-(20nitro-benzoic acid). Following incubation at room temperature, the colour produced was measured at 412 nm with a spectrophotometer. Reduced glutathione (GSH) was used as a standard. The concentration of NP-SH compounds in the samples were calculated and expressed in µ mol/g dry weight.

Extraction procedure for SOD, APX and GPX

For biochemical assay, the leaf samples of 12^{th} day after stress imposition were collected and used for the antioxidant enzyme assays, and antioxidant enzyme isoforms.

The antioxidant enzymes were extracted and estimated from the leaves of groundnut (cultivar KCG-6 and GPBD-4) subjected to control and water stress levels.

Extraction

Lypophilized-powdered plant tissue was homogenized with ice-cold 100 mM potassium phosphate buffer, pH 7.0 containing 0.1 mM EDTA. The homogenate was filtered through muslin cloth and centrifuged at 16,000 g for 15 min. the supernatant fraction was used as crude extract for assaying SOD, POX and CAT enzyme activity and lipid peroxidation assays. All enzyme assay operations were carried out at 4^o C. An aliquot of 0.1 ml enzyme extract was used for the determination of the protein content.

Reactive oxygen species content

Superoxide anion content

Levels of $O_2^{\cdot -}$ were detected based on their ability to reduce nitro blue tetrazolium (NBT) as described in the method of Doke (1983). Fresh leaves were cut into fragments and immersed in 10 mM potassium phosophate buffer, pH 7.8, containing 0.05% NBT and 10 mM NaN_3 , and left for 1

h at 37^{o} C. after incubation, 2 ml of the reaction solution was heated at 85^{o}C for 15 min and cooled rapidly. Optical density was measured at A_{580} and $O_2^{\cdot\,-}$ Content was expressed as an increase in OD per g^{-1} dry weight.

Hydrogen peroxide content

The H_2O_2 content of the leaves was measured according to the method described by Singh et al. (2006), with slight modifications. First, fresh leaf tissue was homogenized in a pre-chilled mortar using pestle with 2.5 ml of 0.1% TCA and the hogogenate was centrifuged at 10,000xg for 15 min. to a 0.5 ml aliquot of the supernatant, 0.5 ml of 100 mM phosphate buffer (pH 7.6) and 1 ml of 1 M KI were added. The optical density was measured at A_{390}, the concentration of H_2O_2 was calculated using H_2O_2 standard graph and expressed as µmol H_2O_2 g^{-1} dry weight

RESULTS AND DISCUSSION

The two groundnut cultivars differed from each other in terms of morphological, physiological and antioxidative responses to water satress.

Effect of water stress on ascorbic acid content

Ascorbic acid content

The antioxidant ascorbic acid content was assayed in the leaves of control and water stressed plants and results are presented in table 1.

Ascorbic acid content was increased in leaves with increase in stress intensity and duration. Ascorbic acid content did not appreciably increased at mild stress treatment in both cultivars on day-4, but recorded a significant elevation in its activity at moderate and severe stress treatments in both cultivars. A similar trend, but with a greater elevation in ascorbic acid content was noticed on day-8 and day-12. Nevertheless, the magnitude of increase in was relatively more in cultivar KCG-6 than in GPBD-4 at all stress regimes on all days of sampling. Thus, in cultivar KCG-6 on day-12, severe stress treatment brought about 20.4 higher ascorbic acid content over the respective control (15.46). While in cultivar GPBD-4, on day-12, at severe stress approximately 18.40 fold increases in Ascorbic acid content was observed as compared to the control 14.80 **(Table 1**).

Table1: Total ascarbic acid content (µ mol/g. Fw) in two groundnut cultivars under control different stressed condition on day-12

Day	KCG-6				GPBD-4			
	Control	**Mild**	**Moderate**	**Severe**	**Control**	**Mild**	**Moderate**	**Severe**
4	15.46 (100)	15.9	16.4	16.6	14.80 (100)	14.90	15.5	16.20
8	15.98 (100)	16.80	17.60	18.50	14.86 (100)	15.80	16.46	17.80
12	16.06 (100)	16.8	17.26	20.4	15.10 (100)	16.23	16.90	18.40

Reactive Oxygen Species (ROS)

Effect of water stress ($O_2^{-\cdot}$), Content

Superoxide radical ($O_2^{-\cdot}$), Content

ROS are cytotoxic causing oxidative damage. Oxidization targets include DNA, proteins and lipids.Thus, in cultivar KCG-6 on day-12, severe stress treatment brought about 1.111 less $O_2^{-\cdot}$, over the respective control (0.292). While in cultivar GPBD-4, on day-12, at severe stress approximately 1.527 more $O_2^{-\cdot}$ was observed as compared to the control 0.203 (**Table 2**).

Day	KCG-6				GPBD-4			
	Control	**Mild**	**Moderate**	**Severe**	**Control**	**Mild**	**Moderate**	**Severe**
4	0.202	0.35	0.703	1.111	0.203	0.372	1.101	1.527
8	0.230	0.51	0.810	1.131	0.251	0.65	1.202	1.650
12	0.275	0.77	0.901	1.551	0.280	0.80	1.315	1.952

Effect of water stress H_2O_2

H_2O_2 (µ mol H_2O_2 /g^{-1} Fw)

Thus, in cultivar KCG-6 on day-12, severe stress treatment brought about 1.681 less H_2O_2 over the respective control (1.11). While in cultivar GPBD-4, on day-12, at severe stress approximately 2.141 more $H_2O_2$was observed as compared to the control 0.951(**Table 3**).

Day	KCG-6				GPBD-4			
	Control	**Mild**	**Moderate**	**Severe**	**Control**	**Mild**	**Moderate**	**Severe**
4	1.11	1.28	1.511	1.681	0.951	1.393	1.852	2.141
8	1.30	1.45	1.715	1.789	0.965	1.425	1.936	2.256
12	1.65	1.95	1.856	1.997	0.985	1.520	1.985	2.365

Antioxidative enzymes

Effect of water stress on antiooxidative enzymes

Ascarbate peroxides APX activity (µ mol H_2 O_2 reduced/min)

Antioxidative enzyme (APX) Ascarbate peroxidate in cultivar KCG-6 on day-12, severe stress treatment brought about 1.196 higher APX over the respective control (0.755). While in cultivar GPBD-4, on day-12, at severe stress approximately 0.582 fold increases in APX was observed as compared to the control 0.639 (**Table 4**).

Day	KCG-6				GPBD-4			
	Control	**Mild**	**Moderate**	**Severe**	**Control**	**Mild**	**Moderate**	**Severe**
4	0.58	0.65	0.68	0.72	0.54	0.61	0.69	0.74
8	0.61	0.71	0.79	10.0	0.58	0.68	0.76	0.92
12	0.63	0.84	0.99	14.6	0.60	0.72	0.85	10.4

Effect of water stress on SOD enzymes

SOD enzymes activity in leaves (units/min/mg^{-1} protein)

Thus, in cultivar KCG-6 on day-12, severe stress treatment brought about 54.583 less SOD over the respective control (23.53). While in cultivar GPBD-4, on day-12, at severe stress approximately 23.48 more SOD was observed as compared to the control 20.916(**Table 5).**

Day	KCG-6				GPBD-4			
	Control	**Mild**	**Moderate**	**Severe**	**Control**	**Mild**	**Moderate**	**Severe**
4	23.53	27.308	54.613	54.583	20.916	28.371	30.86	23.48
8	25.55	29.428	56.756	56.756	23.850	32.392	32.90	25.55
12	30.70	32.520	58.852	60.979	25.975	35.398	35.98	29.85

Effect of water stress on Guaiacol Peroxidate (GPX)

Guaiacol Peroxidate (GPX) ((µ mol H_2O_2 reduced/min/mg protein)

Thus, in cultivar KCG-6 on day-12, severe stress treatment brought about 0.602 less GPX over the respective control (0.35). While in cultivar GPBD-4, on day-12, at severe stress approximately 0.357 more GPX was observed as compared to the control 0.839(**Table 6**).

Day	KCG-6				GPBD-4			
	Control	**Mild**	**Moderate**	**Severe**	**Control**	**Mild**	**Moderate**	**Severe**
4	0.35	0.379	0.581	0.602	0.839	0.896	0.534	0.357
8	0.50	0.450	0.625	0.734	0.845	0.900	0.580	0.455
12	0.75	0670	0.875	0.980	0.950	0.925	0.602	0.525

Discussion

Morphological and biochemical changes in response to water stress treatment have been studied by several investigators. Groundnut plant was reported to be drastically affected by ROS. H_2 O_2 and $O_2^{\cdot -}$ are two kinds of reactive oxygen species causing oxidative stress in plants.

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acids, or polyphenols.

Plant possess a well organized ROS scavenging systems comprising enzymatic such as SOD, APX, GR, GPX and CAT, and non enzymatic antioxidants such as AsA, GSH, NP-SH and PCs. A coordinated function of these systems plays an important role in scavenging ROS and maintaining redox status of the cell (Cho and Seo 2005). In the current study, the ROS levels in groundnut are controlled by a complex enzymatic and non-antioxidant systems. The present result show an increase of AsA and NP-SH coupled with enhanced SOD, GPX and APX activities in Groundnut cultivars when subjected to water stress could be attributed to the increased $O_2^{\cdot -}$ and H_2O_2 radical concentration. Increased activity of APX may efficiently scavenge H_2O_2 to protect oxidative damage, SOD, APX and GPX in general show simultaneous induction and decline, which may be due to their co-regulation as reported earlier (Sharma and Dubey).

Insufficient levels of antioxidants or inhibition of the antioxidant enzymes such as SOD, APX and GPX cause oxidative stress and may damage or kill cells.

A better knowledge of physiology is required in order to understand why some plant species and varieties are more drought resistant than others.

CONCLUSION

In conclusion exposures of groundnut cultivars to water stress resulted in increases of antioxidants and decreased levels of ROS in both cultivars but more increased levels of antioxidant contents and decreased levels of ROS was found in cv. KCG-6 than cv. GPBD-4. root and shoot growth, fresh and dry weights of roots and leaves and leaf area. Based on morphological parameters, in the present investigation, cultivar KCG-6 with a smaller inhibition of root and shoot growth, biomass accumulation and leaf area may supports its better adaptive potential under water stress.

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