

Physiological responses of peanut (*Arachis hypogaea* L.) cultivars to water deficit stress: status of oxidative stress and antioxidant enzyme activities

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Abstract – From a field experiment, the changes in oxidative stress and antioxidant enzyme activities were studied in six Spanish peanut cultivars subjected to 25–30 days of water deficit stress at two different stages: pegging and pod development stages. Imposition of water deficit stress significantly reduced relative water content, membrane stability and total carotenoid content in all the cultivars, whereas total chlorophyll content increased at pegging stage but decreased at pod developmental stage. Chlorophyll *a/b* ratio increased under water deficit stress in most of the cultivars suggesting a greater damage to chlorophyll *b* rather than an increase in chlorophyll *a* content. Oxidative stress measured in terms of H₂O₂, superoxide radical content and lipid peroxidation increased under water deficit stress, especially in susceptible cultivars such as DRG 1, AK 159 and ICGV 86031. Relationship among different physiological parameters showed that the level of oxidative stress, in terms of production of reactive oxygen species, was negatively correlated with activities of different antioxidant enzymes such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase. In conclusion, the study shows that water deficit stress at pod development stage proved to be more detrimental than at pegging stage. The higher activities of antioxidant enzymes in the tolerant cultivars like ICGS 44 and TAG 24 were responsible for protection of oxidative damage and thus provide better tolerance to water deficit stress.

Keywords: antioxidant enzymes, *Arachis hypogaea* L., lipid peroxidation, oxidative stress, peanut, reactive oxygen species

Abbreviations: APX – ascorbate peroxidase, CAT – catalase, DAS – days after sowing, FW – fresh weight, GR – glutathione reductase, GSSG – oxidized glutathione, MSI – membrane stability index, POD – peroxidase, ROS – reactive oxygen species, RWC – relative water content, SOD – superoxide dismutase, SOR – superoxide radical, TBARS – thiobarbituric acid reactive substances

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Introduction

Water deficit stress is one of the major environmental constraints limiting agricultural productivity and plays a major role in the distribution of plant species across different types of environments (ASHRAF 2010). Two-thirds of the potential yields of major crops are usually lost due to adverse growing environments (CHAVES et al. 2009). Drought or water deficit condition can be defined as the absence of adequate moisture necessary for normal plants to grow and complete their life cycle (ZHU 2002). The lack of adequate moisture leading to water stress is the most common phenomenon in rain-fed areas, where erratic rainfall distribution and poor irrigation facilities prevails (WANG et al. 2005).

Peanut is an important legume crop grown in tropical and sub-tropical semi-arid regions of the world in rain-fed areas; the yield level is severely affected by shortage of soil moisture. In India, rainfall accounts for over 50% of variance in yield (CHALLINOR et al. 2003) and the average peanut yield in our country is very low because of moisture stress faced at various growth stages, irrespective of the other factors of the crop production package (SINGH et al. 2013).

Plants differ in their responses to water deficit stress. Those that are tolerant try to overcome the stress by modifying morpho-physiological and biochemical characters, while susceptible plants develop symptoms of stress (DHURVE et al. 2009). Under water deficit stress the production of reactive oxygen species is invariably increased, often leading to damage of the cellular and sub-cellular components in plants (SHAO et al. 2008). Oxidative stress occurs when the defensive capacity of plants is broken by the formation of free radicals. Further down, in the Fenton/Haber-Weiss pathway, toxic hydroxyl radical ($\cdot\text{OH}$) is produced inside the plants, which ultimately destabilizes the membrane lipids *via* lipid peroxidation leading to membrane injury (MITTLER 2002).

Under limited water supply, the photosynthetic process in the plant slows down considerably and the constant accumulation of photo-reducing power causes an excess of electrochemical energy in membranes. This extra energy is canalized through the Mehler reaction, which generates reactive oxygen species (ROS) mainly superoxide anion ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (HERNANDEZ et al. 2001), ultimately provoking the oxidative stress syndrome (KHOLOVA et al. 2009). These ROS are cytotoxic and highly detrimental to the cellular lipids, nucleic acids and proteins and the genotypes that are able to maintain a low steady state of the ROS are better adapted to tolerate stress conditions (BAJJI et al. 2001). Damage to cellular membranes and chlorophylls are reliable indicators for determination of the extent of damage to the plants due to oxidative stress (SAIRAM et al. 2000).

Plants generally scavenge and dispose of these reactive substances by the use of anti-oxidant defence enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) and other associated enzymes involved in the cellular detoxification mechanism. The SOD constitutes the first line of defence *via* detoxification of superoxide radicals to hydrogen peroxide; CAT and POD degrade hydrogen peroxide (SANTOS and ALMEIDA 2011). Cellular accumulation of H_2O_2 is restricted by its reduction to the non-toxic H_2O molecule, either through the action of catalase or ascorbate-glutathione cycle. Finally, NADPH-dependent reduction of oxidized glutathione (GSSG) is performed by glutathione reductase to replenish the cellular pool of reduced glutathione (GSH) (NOCTOR et al. 2002).

The protective roles of the antioxidant enzymes during drought stress have been reported in a number of crop plants including wheat (AGARWAL et al. 2005, SAIRAM and SAXENA 2005) and rice (SHARMA and DUBEY 2005), and also in peanut under simulated water deficit stress condition using various concentrations poly-ethylene glycol (CELIKKOL-AKÇAY et al. 2010). However, studies related to stage-specific response of peanut crop due to water deficit stress under field conditions are lacking. Hence, the objectives of this study were to quantify the levels of oxidative stress faced by peanut cultivars under water deficit stress at two different growth stages and to ascertain the role of antioxidant enzymes in imparting tolerance to drought.

Materials and methods

Plant material and growth condition

A field experiment was conducted during the summer season of 2011 (February–June) using six Spanish (bunch type) peanut cultivars namely SG 99, ICGS 44, ICGV 86031, TAG 24, AK 159 and DRG 1 with a maturity period of 110–120 days, at the research farm of the Directorate of Peanut Research, Junagadh, Gujarat (21°31'N, 70°36'E), India on a site of black clay soil (pH 7.8). Preliminary trials (data not shown here) showed that out of these cultivars, ICGS 44 and TAG 24 are tolerant, while SG 99 behaves moderately and rest of the cultivars are susceptible to water deficit stress. The experiment was laid out in split plot design with six cultivars and water deficit stress treatment and corresponding controls. The net plot size was 4 × 3 m with 9 rows per plot at 45 cm row to row and 10 cm plant to plant spacing.

For the present study, water deficit conditions were created by withholding irrigation at two different stages in two different plots, which were compared with the control (non-stressed) plot in each case. In the present study, the first sample was drawn from 60-day old plants which were subjected to water deficit stress by withholding irrigation for the previous 30 days (30–60 DAS), corresponding to the R1–R5 stage in peanut (BOOTE 1982) denoted as the 'pegging stage' (beginning of blooming to the beginning of seed formation). Similarly, the samples were collected from another set of 85-day old plants, subjected to water deficit stress for the previous 25 days (60–85 DAS), corresponding to the R5–R7 stage in peanut (BOOTE 1982) and denoted as the 'pod development stage' (beginning of seed formation to beginning of maturity). However, the control plot was irrigated to replenish 100% cumulative pan evaporation at weekly intervals. The stages selected for the present study were considered to be most critical in terms of water requirement in peanut (NAUTIYAL et al. 2012). To characterize the level of imposed stress, soil moisture content was measured from each plot at every stage by the gravimetric method [(fresh weight-dry weight)/dry weight × 100] (BLACK 1965). Recommended plant protection measures were followed to maintain a healthy crop stand.

Sampling for different physiological parameters, oxidative stress and antioxidant enzyme activities was done at 24–48 h before the withdrawal of stress at both the stages, from the third fully-matured leaf in control and stressed plants by randomly collecting samples in triplicate from 3 replicated plots.

Analysis of physiological parameters

Specific leaf area was calculated by dividing total leaf area by total leaf weight in at least 5 plant samples (NAGESWARA RAO et al. 2001). Leaf relative water content (RWC) was

estimated by recording the fresh and turgid weight, and dry weight (WEATHERLEY 1950). Membrane stability index (MSI) was estimated by measuring the electrical conductivity of leaf samples (100 mg) in 10 mL double distilled water by heating at 40 °C for 30 min and 100 °C for 10 min according to SAIRAM et al. (1997). For total chlorophyll and carotenoid estimation 50 mg leaf material was extracted in 10 mL dimethylsulfoxide (HISCOX and ISRAELSTAM 1979), incubated at 65 °C for 4 h, and then cooled to room temperature. Absorbance of the extract was recorded at 470, 645 and 663 nm. Chlorophyll and carotenoid content was calculated according to ARNON (1949), and LICHTENTHALER and WELLBURN (1985).

Determination of oxidative stress

Superoxide radical (SOR) content was estimated by its capacity to reduce nitroblue tetrazolium chloride (NBT) and the absorption of end product was measured at 540 nm (CHAITANYA and NAITHANI 1994). Briefly, one gram leaf tissue was homogenized in 10 mL of pre-cooled phosphate buffer (0.2 M, pH 7.2) containing 1 mM diethyldithiocarbamate to inhibit SOD activity and 10 µM diphenylene iodonium chloride. The homogenate was centrifuged at $10,000 \times g$ for 10 min at 4 °C and supernatant was immediately used for the measurement of superoxide radical. The reaction mixture contained 0.25 mL supernatant, 0.075 mM NBT, 25 mM Na₂CO₃, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 13.33 mM L-methionine and water to make the volume of 3 mL. The reaction mixture was incubated at 30 °C for 10 min and absorbance was recorded at 540 nm. Superoxide radical content was calculated according to its extinction coefficient $\epsilon = 12.8 \text{ mM}^{-1} \text{ cm}^{-1}$.

Hydrogen peroxide (H₂O₂) was measured through formation of the titanium-hydro peroxide complex (RAO et al. 1997). One gram leaf material was ground with liquid nitrogen and the fine powdered material was mixed with 10 mL cooled acetone in a cold room (10 °C). Mixture was filtered through Whatman No.1 filter paper followed by the addition of 4 mL titanium reagent and 5 mL ammonium solution to precipitate the titanium-hydro peroxide complex. Reaction mixture was centrifuged at $10,000 \times g$ for 10 min at 4 °C. Precipitate was dissolved in 10 mL of 2 M H₂SO₄ and then recentrifuged. Absorbance of the supernatant was taken at 415 nm against blank. Hydrogen peroxide contents were calculated by comparison with a standard curve drawn with known hydrogen peroxide concentrations.

The level of lipid peroxidation was measured in terms of thiobarbituric acid-reactive substances (TBARS) content (HEATH and PACKER 1968). Leaf sample (0.5 g) was homogenized in 10 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at $15,000 \times g$ for 15 min. To a 1.0 mL aliquot of the supernatant, 4.0 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA was added and the mixture was heated at 95 °C for 30 min and then cooled in an ice bath. After centrifugation at $10,000 \times g$ for 10 min the absorbance of the supernatant was recorded at 532 nm. The TBARS content was calculated according to its extinction coefficient $\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$. The values for non-specific absorbance at 600 nm were subtracted.

Antioxidant enzyme assays

Enzyme extract for SOD, APX, GR, POD and CAT was prepared by first freezing the weighed amount of leaf samples (1 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 ml extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA in case of SOD, GR, POD, CAT and 1 mM ascorbic acid in the case of

APX). Extract was passed through 4 layers of cheesecloth and filtrate was centrifuged for 20 min at $15,000 \times g$ and the supernatant was used as enzyme.

The total SOD (EC 1.15.1.1) activity was measured by the inhibition of the photochemical reduction of NBT by the enzyme (DHINDSA et al. 1981). The 3 mL reaction mixture contained 13.33 mM methionine, 75 μ M NBT, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate, 0.1 mL enzyme extract and water to make a final volume of 3.0 mL. The reaction was started by adding 2 mM riboflavin (0.1 mL) and placing the tubes under two 15 W fluorescent lamps for 15 min. Illuminated and non-illuminated reaction mixture without enzyme were used for calibration. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme that reduced the absorbance reading to 50% in comparison with tubes lacking enzyme per unit time.

The catalase (EC 1.11.1.6) activity was assayed by measuring the disappearance of H_2O_2 (AEBI 1984) in a reaction mixture (3 mL) consisting of 0.5 mL of 75 mM H_2O_2 and 1.5 mL of 0.1 M phosphate buffer (pH 7) on the addition of 50 μ L of diluted enzyme extract. The decrease in absorbance at 240 nm was observed for 1 min in a UV- visible spectrophotometer. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed. The initial and final contents of H_2O_2 were calculated by comparison with a standard curve drawn with known concentrations of H_2O_2 .

The peroxidase (EC 1.11.1.7) activity was measured in terms of increase in absorbance due to the formation of tetraguaiacol at 470 nm, and the enzyme activity was calculated as per extinction coefficient of its oxidation product, tetraguaiacol $\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (CASTILLO et al. 1984). The reaction mixture contained 50 mM phosphate buffer (pH 6.1), 16 mM guaiacol, 2 mM H_2O_2 and 0.1 mL enzyme extract. The mixture was diluted with distilled water to make up final volume of 3.0 mL. Enzyme activity is expressed as μ mol tetraguaiacol formed per min per mg protein.

The ascorbate peroxidase (EC 1.11.1.11) activity was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm (NAKANO and ASADA 1981). The 3 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2 , 0.1 mL enzyme and water to make a final volume of 3.0 mL in which 0.1 mL of H_2O_2 was added to initiate the reaction. Decrease in absorbance was measured spectrophotometrically and the activity was expressed by calculating the decrease in ascorbic acid content using a standard curve drawn with known concentrations of ascorbic acid and expressed as μ mol oxidised ascorbate $\text{mg}^{-1}_{\text{protein}} \text{ min}^{-1}$.

The glutathione reductase (EC 1.8.1.7) activity was assayed using the method of SMITH et al. (1988). The reaction mixture containing 66.67 mM potassium phosphate buffer (pH 7.5) and 0.33 mM EDTA, 0.5 mM 5,5-dithiobis-(2-nitro) benzoic acid in 0.01 M potassium phosphate buffer (pH 7.5), 66.67 μ M NADPH, 666.67 μ M oxidized glutathione (GSSG) and 0.1 mL enzyme extract. The mixture was diluted with distilled water to make up a final volume of 3.0 mL. The reaction was started by adding 0.1 mL of 20.0 mM GSSG. The increase in absorbance at 412 nm was recorded spectrophotometrically and the activity was expressed as micromole of GSSG reduced per mg protein per min.

Total soluble protein was determined according to the method of BRADFORD (1976), with bovine serum albumin as a calibration standard.

Statistical analyses

All the data recorded were the mean values of at least three independent assays with three replications each. The data was subjected to analysis of variance appropriate to the experimental design. The least significant differences at 5% probability level ($LSD_{p=0.05}$) were considered statistically significant (GOMEZ and GOMEZ 1984). Correlations (Pearson correlation coefficient) among different physiological parameters (RWC, MSI, total chlorophyll and carotenoid content), oxidative stress build-up (H_2O_2 and SOR production, and lipid peroxidation) and antioxidant enzyme (SOD, CAT, POD, APX, GR) activities under water deficit stress condition were analyzed. All the 14 physiological parameters analysed in the present study was further categorized by multivariate statistics i.e. principal component analysis (PCA) by SPSS program (version 16.0).

Results

Intensity of water deficit stress and soil moisture status

Water deficit stress imposed by the withholding irrigation for 30 days at pegging stage, reduced soil moisture content by 45% at 0–15 cm soil depth and 44% at 15–30 cm soil depth compared to irrigated control plot where optimum moisture level was maintained throughout the crop growth period. Similarly, at pod development stage the extent of stress was more severe as it recorded 69 and 50% decrease in soil moisture content at 0–15 and 15–30 cm soil depth, respectively (Fig. 1).

Physiological parameter

The relative water content (RWC) of leaves decreased in all the peanut cultivars with the imposition of water deficit stress during pegging as well as pod development stage (Tab. 1).

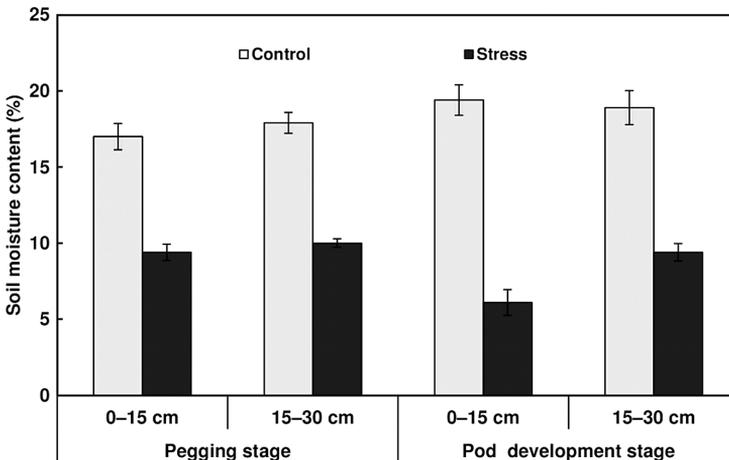


Fig. 1. Changes in soil moisture content at different depths in response to water deficit stress at pegging and pod development stages. Values are mean of three replicates \pm SE. $LSD_{0.05}$ values for pegging and pod development stages are 0.86 and 1.14, respectively, where $LSD_{0.05}$ value represents least significant difference value at 5% probability level.

Tab. 1. Changes in relative water content (RWC), membrane stability index (MSI) and specific leaf area (SLA) of peanut leaves at different developmental stages in response to water deficit stress. Values are mean of three replicates \pm SE; T – treatment; C – cultivar; C \times T – cultivar \times treatment interaction, NS – non-significant. $LSD_{(0.05)}$ – least significant differences at 5% probability level.

Cultivar	RWC (%)						MSI (%)						SLA (cm ²)																				
	Pegging stage		Pod development stage		Pegging stage		Pod development stage		Pegging stage		Pod development stage		Pegging stage		Pod development stage																		
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress																	
SG 99	94.2 \pm 2.6	91.7 \pm 0.3	90.2 \pm 2.7	87.2 \pm 0.2	89.4 \pm 0.6	80.7 \pm 2.3	71.4 \pm 2.8	62.2 \pm 1.6	146.0 \pm 1.7	125.9 \pm 2.8	158.8 \pm 2.8	139.2 \pm 1.6	ICGS 44	90.9 \pm 3.1	90.3 \pm 3.3	91.9 \pm 1.8	86.8 \pm 1.9	91.2 \pm 1.2	83.8 \pm 0.8	68.3 \pm 0.8	61.9 \pm 0.7	148.3 \pm 2.1	132.6 \pm 2.6	153.2 \pm 0.8	135.8 \pm 0.7								
ICGV 86031	91.7 \pm 1.9	85.7 \pm 3.2	91.2 \pm 1.1	83.5 \pm 1.8	83.8 \pm 1.8	73.3 \pm 2.5	73.3 \pm 3.7	63.2 \pm 0.9	129.1 \pm 1.8	109.7 \pm 2.4	144.4 \pm 3.7	118.7 \pm 0.9	TAG 24	91.2 \pm 2.2	88.1 \pm 1.5	88.7 \pm 1.3	86.1 \pm 0.8	85.8 \pm 2.1	82.4 \pm 0.5	73.4 \pm 2.1	65.3 \pm 0.9	149.7 \pm 3.4	135.3 \pm 3.4	160.5 \pm 2.1	140.5 \pm 0.9								
AK 159	91.3 \pm 2.6	87.7 \pm 2.1	91.6 \pm 1.5	83.9 \pm 2.9	84.5 \pm 1.4	81.0 \pm 0.6	66.7 \pm 1.8	54.3 \pm 1.5	141.4 \pm 2.9	124.7 \pm 3.2	153.0 \pm 1.7	133.6 \pm 1.5	DRG 1	91.8 \pm 1.6	86.4 \pm 2.2	92.1 \pm 0.9	82.8 \pm 1.2	82.0 \pm 0.3	73.9 \pm 2.0	67.2 \pm 2.0	52.3 \pm 2.2	144.0 \pm 6.8	121.6 \pm 2.7	158.1 \pm 2.1	134.3 \pm 2.2								
$LSD_{0.05}T$	1.74		2.56		NS		3.24		1.88		8.58		$LSD_{0.05}C$	2.39		2.71		4.28		3.43		9.93		$LSD_{0.05}C \times T$	NS		3.82		6.05		10.11		14.04

Though the difference in RWC within the cultivars at pegging stage was not significant, at pod development stage the differences were significant and the cultivar TAG 24 showed the least reduction in RWC compared to non-stressed plants. The average reduction in leaf RWC at pod developmental stage was 6.5%, which resulted in reduced growth and partial wilting of leaves in stressed plants (data not shown). Similarly, membrane stability index (MSI) was also reduced due to imposition of water deficit stress and the interaction effect of stress and peanut cultivars was found to be significant for all cultivars at both stages (Tab. 1). The cultivar ICGV 86031 and DRG 1 showed highest reduction in MSI at pegging and pod development stages, respectively. Imposition of water deficit stress significantly decreased specific leaf area (SLA) at both the stages (Tab. 1). The reduction in SLA was higher (> 15%) in ICGV 86031 and DGR 1 than in the rest of the cultivars at both pegging and pod development stages, whereas it was only 11% in ICGS 44.

Interestingly, the total chlorophyll content (mg g^{-1} fresh weight) in peanut leaves increased when water deficit stress was imposed at pegging stage. It was reduced in all the cultivars when stress was imposed at pod development stage. The highest reduction in total chlorophyll content under stress at pod development stage was observed in DRG 1 (14.4%), while the cultivar ICGS 44 showed the least reduction (8.9%) (Fig. 2A). However, the total carotenoid content reduced in all the cultivars under water deficit stress both at pegging and pod development stages with higher reduction at later stage. The cultivar SG 99 showed the highest reduction in total carotenoid content at both the stages followed by ICGV 86031 and AK 159 at pegging and pod development stages, respectively (Fig. 2B). Chlorophyll *a/b* ratio increased under water deficit stress in most of the cultivars except in ICGS 44 at pegging stage and TAG 24 and AK 159 at pod development stage (Fig. 2C).

Oxidative stress

Water deficit stress resulted in increased levels of oxidative stress in all the peanut cultivars. The H_2O_2 content increased significantly at both pegging and pod development stages, but, increased progressively at a later stage (86.5%). The cultivar ICGS 44 showed the least increase in H_2O_2 content from 0.99 (in the control) to $1.40 \mu\text{mol g}^{-1}$ FW (in stressed plants) at pegging stage and from 1.14 (in the control) to $1.72 \mu\text{mol g}^{-1}$ FW (in stressed plants) at pod development stage, followed by TAG 24 and SG 99 (Fig. 3A).

Superoxide radical (SOR) content also increased significantly under water deficit stress with more pronounced effect in cultivars DRG 1 and ICGV 86031, at both, pegging and pod development stages. On the other hand, TAG 24 (19.7%), ICGS 44 (19.9%) and AK 159 (24.5%) showed a smaller increase in SOR content at pod development stage (Fig. 3B). Similarly, lipid peroxidation, measured in terms of TBARS content, increased significantly under water deficit stress in all the cultivars, with higher increase at pod development stage (39%) than at pegging stage (28%) (Fig. 3C). The least increase in lipid peroxidation was observed in TAG 24 (15.6%) followed by ICGS 44 (17.7%), compared to non-stressed plants, while the highest TBARS content ($18.9 \text{ nmol mg}^{-1}$ FW) was found in DRG 1 at pod development stage.

Antioxidant enzyme activities

Water deficit stress, especially at later stages of growth significantly increased the activities of all the antioxidant enzymes. Under water deficit conditions, the highest increase

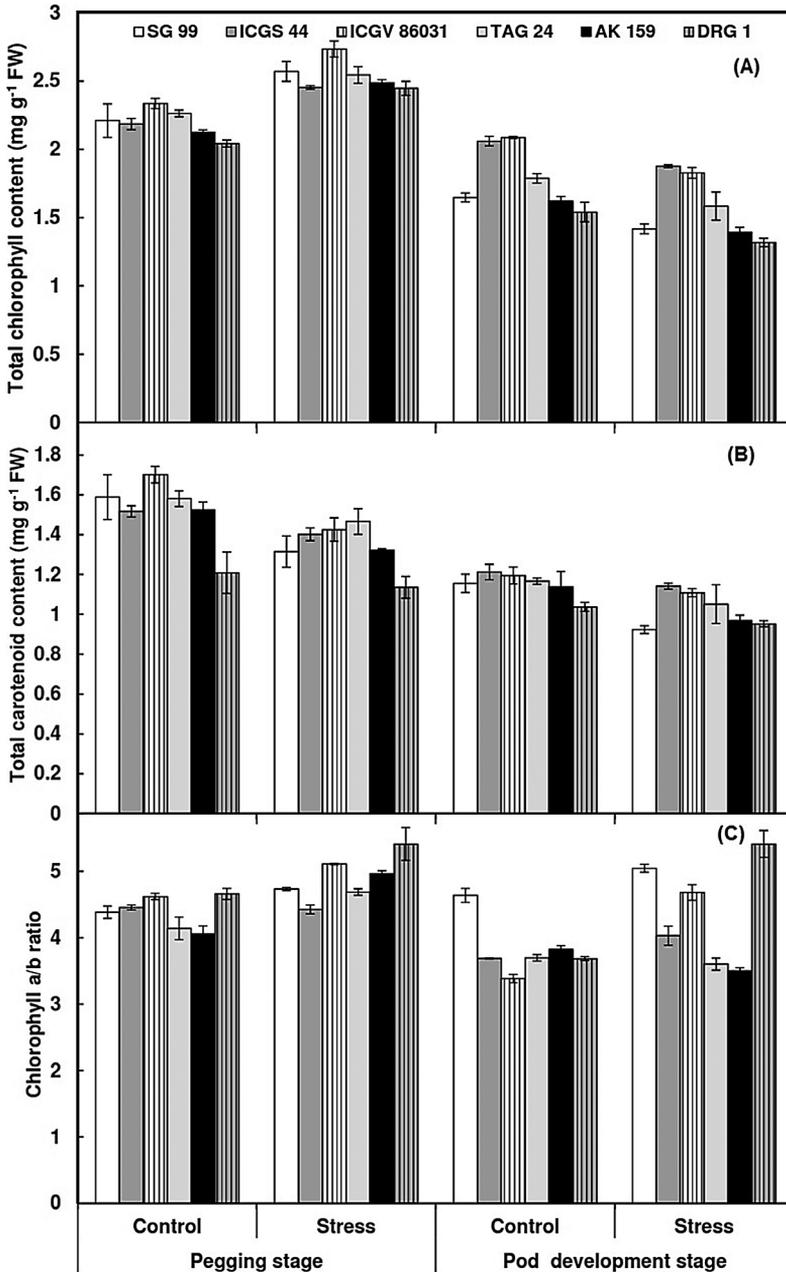


Fig. 2. Changes in (A) total chlorophyll content (mg g^{-1} fresh weight); (B) total carotenoid content (mg g^{-1} fresh weight); (C) chlorophyll *a/b* ratio in leaves of different cultivars of peanuts in response to water deficit stress at pegging and pod developmental stages. Values are mean of three replicates \pm SE. $\text{LSD}_{0.05}$ ($C \times T$) values for pegging and pod development stages are 0.23 and 0.32 for (A), 0.22 and 0.16 for (B), and 0.52 and 0.43 for (C), respectively, where $\text{LSD}_{0.05}$ ($C \times T$) value represent least significant difference value of cultivar and treatment interaction at 5% probability level.

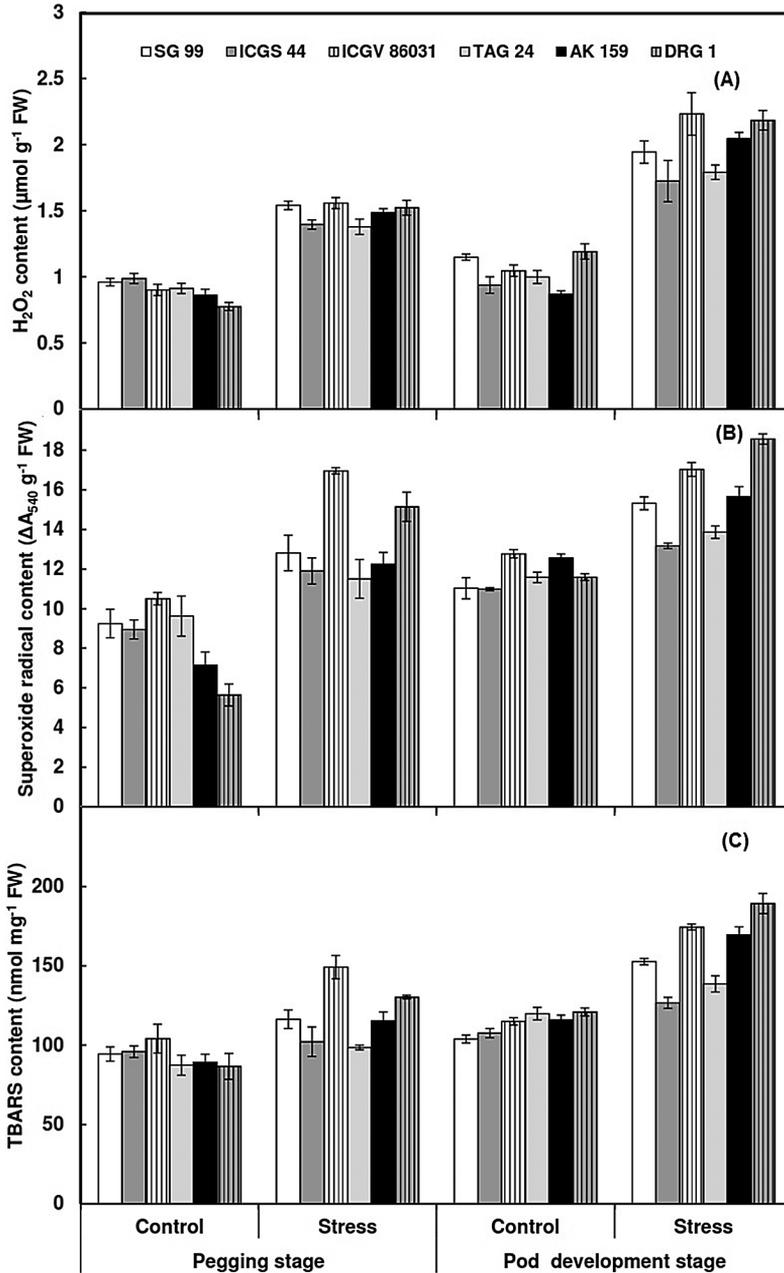


Fig. 3. Changes in (A) H_2O_2 content ($\mu\text{mol g}^{-1}$ fresh weight); (B) superoxide radical content (ΔA_{540} g^{-1} fresh weight); (C) lipid peroxidation (nmol of TBARS content mg^{-1} fresh weight) in leaves of peanut cultivars in response to water deficit stress at pegging and pod developmental stages. Values are mean of three replicates \pm SE. $LSD_{0.05}$ ($C \times T$) values for pegging and pod developmental stages are 0.13 and 0.20 for (A), 1.80 and 0.91 for (B), and non-significant and 1.42 for (C), respectively, where $LSD_{0.05}$ ($C \times T$) value represent least significant difference value of cultivar and treatment interaction at 5% probability level.

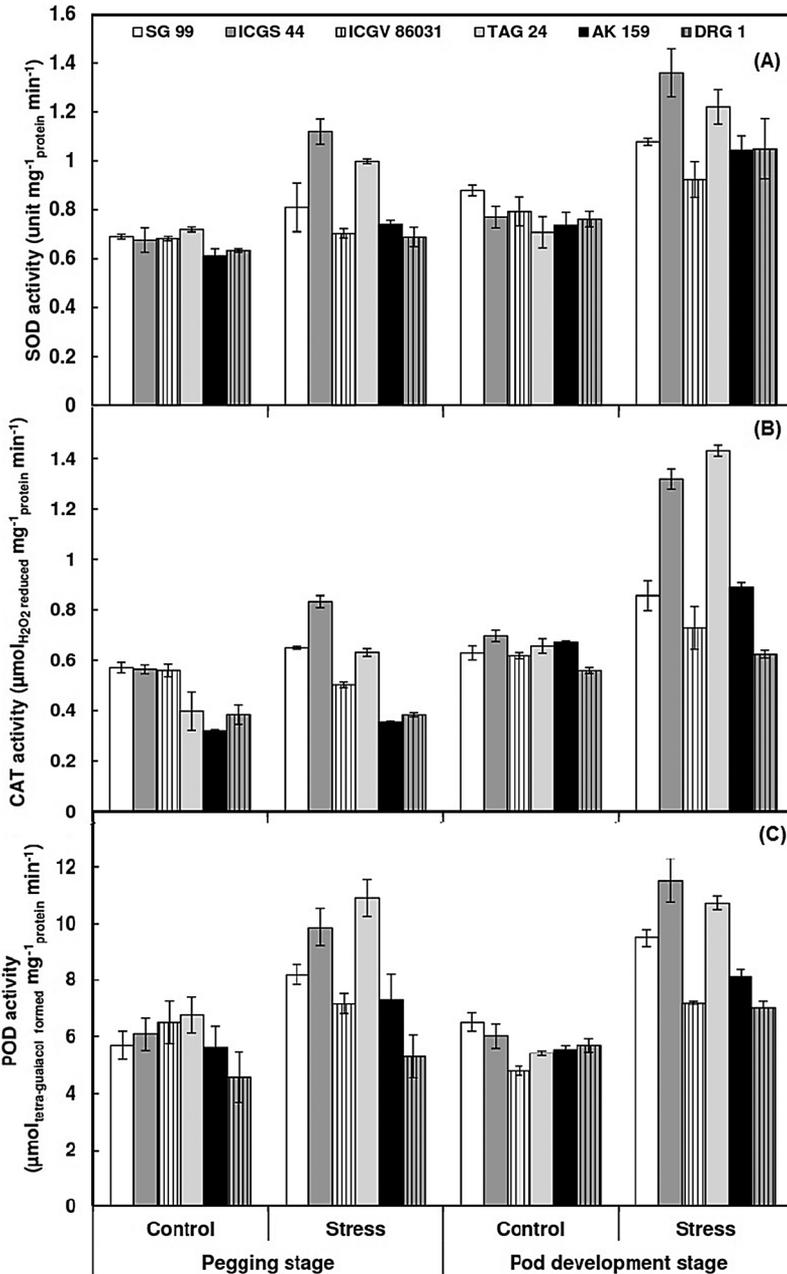


Fig. 4. Changes in (A) SOD activity (unit mg⁻¹ protein min⁻¹); (B) CAT activity (μmol_{H₂O₂ reduced} mg⁻¹ protein min⁻¹); (C) POD activity (μmol_{tetra-guaiacol formed} mg⁻¹ protein min⁻¹) in leaves of peanut cultivars in response to water deficit stress at pegging and pod developmental stages. Values are mean of three replicates ± SE. LSD_{0.05} (C × T) values for pegging and pod developmental stages are 0.11 and 0.20 for (A), 0.09 and 0.11 for (B), and non-significant and 0.97 for (C), respectively, where LSD_{0.05} (C × T) value represent least significant difference value of cultivar and treatment interaction at 5% probability level.

in total SOD activity was observed in ICGS 44 (1.12 unit $\text{mg}^{-1} \text{protein min}^{-1}$), TAG 24 (0.99 unit $\text{mg}^{-1} \text{protein min}^{-1}$) and AK 159 (0.74 unit $\text{mg}^{-1} \text{protein min}^{-1}$) in comparison to the respective controls (0.68, 0.72 and 0.61 unit $\text{mg}^{-1} \text{protein min}^{-1}$, respectively) (Fig. 4A) at pegging stage. However, water deficit stress during pod development stage increased SOD activity in all the cultivars and the highest increase (75%) was observed in ICGS 44 and TAG 24.

The CAT activity also followed the similar trend as of SOD. Imposition of water deficit stress at pegging stage increased CAT activity in relatively tolerant cultivars TAG 24 (58.4%) and ICGS 44 (47.7%), but reduced in susceptible cultivars ICGV 86031 and DRG 1 (Fig. 4B). At pod development stage, CAT activity increased in all the cultivars under water deficit stress, and in TAG 24 it was more than double than in non-stressed plants. The

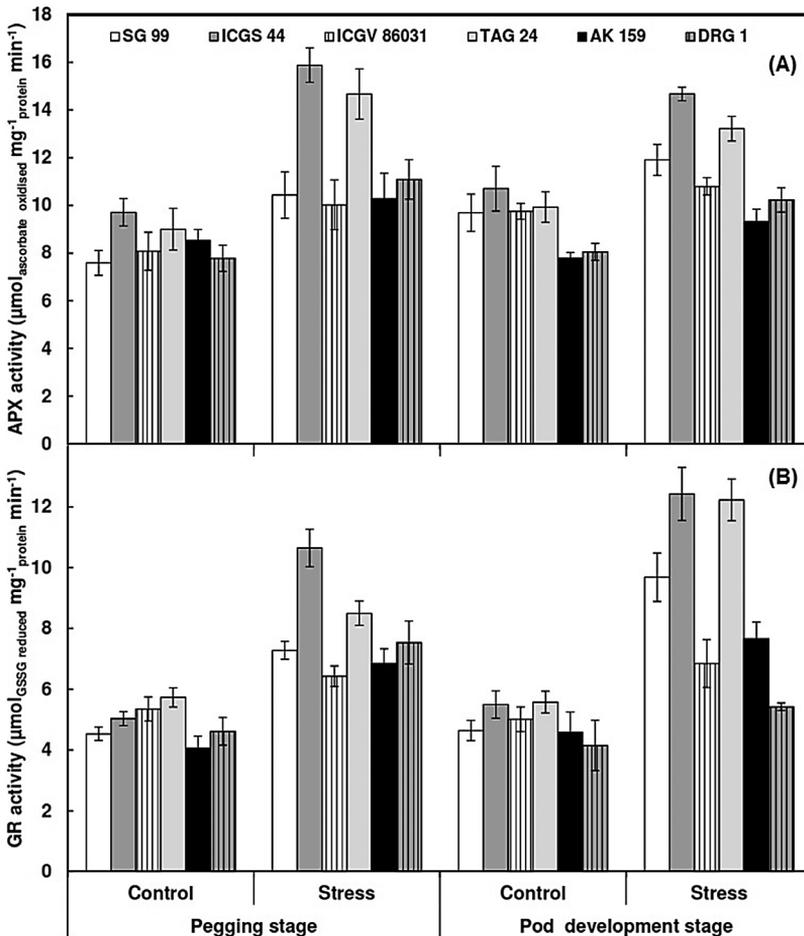


Fig. 5. Changes in (A) APX activity ($\mu\text{mol}_{\text{ascorbate oxidized}} \text{mg}^{-1} \text{protein min}^{-1}$); (B) GR activity ($\mu\text{mol}_{\text{GSSG reduced}} \text{mg}^{-1} \text{protein min}^{-1}$) in leaves of peanut cultivars in response to water deficit stress at pegging and pod developmental stages. Values are mean of three replicates \pm SE. $\text{LSD}_{0.05} (C \times T)$ values for pegging and pod developmental stages are 2.95 and 1.98 for (A), and 2.71 and 2.82 for (B), respectively, where $\text{LSD}_{0.05} (C \times T)$ value represent least significant difference value of cultivar and treatment interaction at 5% probability level.

POD activity increased with imposition of water deficit stress at both early and later stages of growth and ICGS 44 and TAG 24 recorded the highest increase (62%), followed by SG 99 (44%) at pegging stage (Fig. 4C). The POD activity was almost doubled in stressed plants of ICGS 44 and TAG 24 compared to the non-stressed plants at pod development stage.

Imposition of water deficit stress at both pegging and pod development stage invariably increased the activity of APX in all the cultivars, but the extent of change varied from cultivar to cultivar. Compared to non-stressed plants, the cultivar ICGS 44 and TAG 24 showed maximum increase (63%) in APX activity at pegging stage, while ICGS 44 recorded maximum increase (37%), from 10.7 μmol oxidized ascorbate per min per mg of protein in control to 14.7 μmol oxidized ascorbate per min per mg of protein, at pod development stage (Fig. 5A). Similarly, the activity of GR increased at both pegging and pod development stages in all the cultivars under water deficit stress. However, the changes were higher when the stress was imposed at pod development stage than at pegging stage. The cultivar ICGS 44 showed higher GR activity than other cultivars with 10.6 and 12.4 μmol GSSG reduced per minute per mg of protein at pegging and pod development stages, respectively (Fig. 5B).

Relationship among physiological parameters, oxidative stress and antioxidant enzyme activities under stress

Significant negative correlations (-0.900 , -0.735 and -0.881) were observed between MSI and H_2O_2 , SOR production and lipid peroxidation, and MSI and lipid peroxidation, respectively (Tab. 2), suggesting more damage to the membrane structure with rising levels of oxidative stress under water deficit condition. In general, negative correlations among H_2O_2 , SOR production and lipid peroxidation and antioxidant enzyme activities indicated

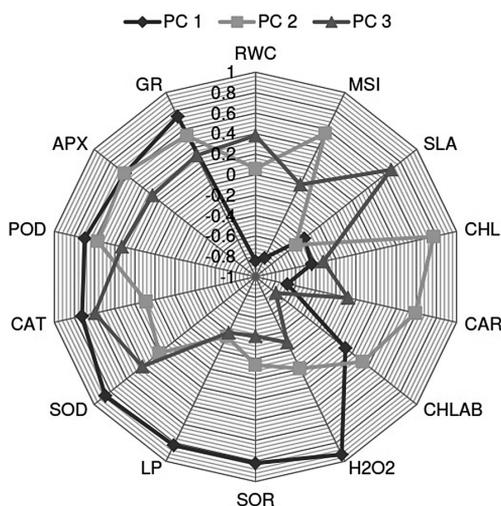


Fig. 6. Results of principal component (PC) analysis of different physiological parameters under water deficit stress in peanut cultivars, where RWC – relative water content, MSI – membrane stability index, SLA – specific leaf area, CHL – total chlorophyll content, CAR – total carotenoid content, CHLAB – chlorophyll *a/b* ratio, LP – lipid peroxidation, APX – ascorbate peroxidase, CAT – catalase, GR – glutathione reductase, POD – peroxidase, SOD – superoxide dismutase, SOR – superoxide radical.

Tab. 2. Relationship (Pearson correlation coefficient) between different physiological parameters, oxidative stress and antioxidant enzyme activities in peanut leaves under water deficit stress. APX – ascorbate peroxidase, CAT – catalase, GR – glutathione reductase, MSI – membrane stability index, POD – peroxidase, RWC – relative water content, SOD – superoxide dismutase, SOR – superoxide radical, LP – lipid peroxidation. * and ** represent level of significance at 5% and 1% probability, respectively.

	RWC	MSI	CHL	CAR	H ₂ O ₂	SOR	LP	SOD	CAT	POD	APX	GR
RWC	1.000											
MSI	0.809**	1.000										
CHL	0.629*	0.904**	1.000									
CAR	0.611*	0.883**	0.927**	1.000								
H ₂ O ₂	-0.781**	-0.900**	-0.856**	-0.827**	1.000							
SOR	-0.810**	-0.735**	-0.501*	-0.580*	0.770**	1.000						
LP	-0.860**	-0.881**	-0.726**	-0.749**	0.927**	0.939**	1.000					
SOD	-0.087	-0.434	-0.605*	-0.380	0.288	-0.154	0.050	1.000				
CAT	-0.056	-0.403	-0.534*	-0.375	0.258	-0.137	0.052	0.891**	1.000			
POD	0.332	0.062	-0.148	0.110	-0.186	-0.569*	-0.418	0.809**	0.761**	1.000		
APX	0.446	0.356	0.152	0.331	-0.439	-0.633*	-0.623*	0.620*	0.488	0.780**	1.000	
GR	0.313	0.028	-0.170	-0.067	-0.204	-0.518**	-0.407*	0.768**	0.841**	0.822**	0.775**	1.000

the role of antioxidant enzymes in lowering the level of oxidative stress in peanut cultivars under water deficit condition. Three principal components (PCs) of eigenvalue more than 1.0 were identified (Fig. 6). Among these PC 1 screened most variable parameters as H₂O₂ content (0.927), SOD activity (0.864), and RWC (−0.843) under imposition of water deficit stress. However, the PC 2 selected chlorophyll content (0.772), APX activity (0.626), and carotenoid content (0.591) as another set of parameters showing more variability as compared to rest of the parameters. The PC 3-screened sensitive parameters towards stress were chlorophyll *a/b* ratio (−0.747), SLA (0.681), and CAT activity (0.597) showing maximum variability as detected by PCA.

Discussion

Water deficit stress induces several physiological and biochemical changes in plants depending upon the time, intensity and duration of stress (PATTANGUAL and MADORE 1999). General effect of water deficit stress in plants is manifested in terms of osmotic stress which results in lower leaf water potential, turgor potential and stomatal conductance (BOOTE and KETRING 1990). This study demonstrated that water deficit stress led to differential responses in peanut cultivars when it was imposed at different stages of crop growth. The extent of reduction in RWC, MSI and SLA due to water deficit stress at the pegging stage was lower than at pod development stage. DHRUVE et al. (2009) also reported decrease in leaf water content and membrane stability under water deficit stress in peanut.

Interestingly, in the present study, the total chlorophyll content of the leaf increased in most of the cultivars when drought was imposed at the initial stage. But subsequent drought at a later stage reduced it significantly in the all the cultivars. Water deficit stress destroys the chlorophyll and prevents its biosynthesis as well (LESSANI and MOJTAHEDI 2002), which was reported by researchers in many crops, including peanut (SHARADA and NAIK 2011, ARJENAKI et al. 2012). It was also reported that drought increased chlorophyll content in sesame at the initial stage and that later it remained unchanged (MENSAH et al. 2006). Ability to synthesize more chlorophyll under water deficit condition at initial stage of growth is a good measure of the ability of peanut genotypes to cope with drought stress during initial stages of growth (ARUNYANARK et al. 2008). Here, the tolerant cultivars TAG 24 and ICGS 44 showed higher increase in chlorophyll content at pegging stage indicating their tolerance to water deficit stress.

Water deficit stress in general reduced the total carotenoid content, but this reduction was relatively less in tolerant cultivars ICGS 44 and TAG 24. As a part of light-harvesting complex carotenoids act as cellular protector of chlorophyll pigments and membrane structure by quenching triplet chlorophyll and removing oxygen from the excited chlorophyll-oxygen complex (YOUNG 1991). Carotenoids also play a protective role as molecular antioxidant in the cell by scavenging of singlet oxygen (KNOX and DODGE 1985) and hence their comparative levels in cultivars are of much importance in determining relative tolerance. Decrease in carotenoid content under water stress in the present investigation probably contributed to greater ROS production and further destruction of photosynthetic pigments.

Increased chlorophyll *a/b* ratio with decreasing total chlorophyll content under water deficit stress signifies greater damage to chlorophyll *b* rather than an increase in chlorophyll *a* (MAFAKHRI 2010). The present study showed a comparatively lower chlorophyll *a/b* ratio in tolerant than in susceptible cultivars, which implies more degradation to chlorophyll *b* in

the latter than increased synthesis of chlorophyll *a* per se. Increased chlorophyll *a/b* ratio under water deficit condition suggests the greater vulnerability of the chlorophyll *b* molecule under stress. The chlorophyll *a/b* ratio is an indicator of the antenna sizes of PS I and PS II. The core antenna contains only chlorophyll *a*, whereas the outer antenna contains both chlorophyll *a* and *b* (TAIZ and ZEIGER 2006). The lower chlorophyll *a/b* ratio in tolerant genotypes probably was obtained due to lesser damage to PS I and PS II, leading to better capacity to photosynthesize even under stressed condition. Increase in chlorophyll *a/b* ratio due to imposition of drought is also reported in other legumes (MAFAKHERI 2010).

Water deficit stress increases production of ROS that inactivate enzymes, damage cellular components (SHAO et al. 2008) and diminish the defense capacity of plants (ERICE et al. 2010). Plants possess antioxidant defense system against ROS, operative through the changes in activities of SOD, CAT, POD and enzymes of ascorbate and glutathione redox cycle (NOCTOR and FOYER 1998). In this study, oxidative stress (measured as SOR and H₂O₂ production) varied in the peanut cultivars facing water deficit stress at various stages; however the extent varied with the cultivars. The net production of H₂O₂ and SOR in the tolerant cultivars TAG 24 and ICGS 44 was relatively lower than in the susceptible ones due to better balance maintained between ROS production and the capacity of the defense mechanisms to protect the plant under severe stress. Severe drought depresses the activity of enzymes devoted to detoxify H₂O₂, which is in conformity with the greatest display of H₂O₂ concentration and peroxidation to membrane lipids in stressed leaves (FINI et al. 2012).

SOR and H₂O₂ accumulated due to water deficit stress resulted in membrane lipid peroxidation and destabilization of the cellular and subcellular membrane via production of toxic hydroxyl radical (MITTLER 2002). In the present study, the plants faced water stress, showing greater accumulation of SOR and higher lipid peroxidation than the plants grown under non-stressed condition. Here, the tolerant cultivar TAG 24 and ICGS 44 accumulated less SOR and also showed the least lipid peroxidation. Studies revealed positive correlation between leaf dehydration and increase in H₂O₂ and SOR concentration under sequential drought (JUBANY-MARÍ et al. 2010).

The SOD constitutes the primary step of cellular defence and dismutates superoxide radical to H₂O₂ and O₂. Further, the accumulation of H₂O₂ is restricted through the action of catalase in mitochondria, by the ascorbate glutathione cycle in chloroplast, where ascorbate peroxidase reduces it to H₂O. Finally, glutathione reductase catalyzes the NADPH-dependent reduction of oxidized glutathione to the reduced glutathione (NOCTOR et al. 2002). In this study, the SOD activity increased with imposition of water deficit stress during pod developmental stage with higher activities recorded in TAG 24 and ICGS 44 correlating well with relatively lower production of superoxide radical in these cultivars under water deficit stress. This might be the primary reason of low oxidative stress built-up in these cultivars leading to water deficit stress tolerance. Higher activity of SOD, in roots and leaves has been identified as an indicator of a redox status change under drought (SCHWANZ and POLLE 2001).

Downstream to SOD, CAT and POD are the most important enzymes involved in regulation of intracellular levels of H₂O₂ (PRASAD et al., 1995). Here, we observed relatively higher activities of CAT and POD in the tolerant cultivars, whereas hardly any induction was noticed in susceptible cultivars like DRG 1 and AK 159. This result is in accordance with the values for oxidative stress parameters, which were quite high in the susceptible cultivars. SHARIFI et al. (2012) reported that the tolerant wheat lines revealed high activity of

POD and CAT enzyme under drought conditions with higher yields, thus showing positive correlation between enzyme activity and yield in drought conditions.

The enzymes APX and GR complete the detoxification cycle of ROS inside cell via the Halliwell-Asada pathway and the activity of these enzymes determine the rate of dissipation of toxic substances (MITTLER 2002). The present study has shown higher activities of these enzymes under water stress, especially in tolerant cultivars, which is a desirable trait that may be associated with water deficit stress tolerance in peanut. Antioxidants such as ascorbate and glutathione are involved in scavenging H₂O₂ in conjunction with monodehydro ascorbate reductase (MDAR) and GR, which regenerate ascorbate (HOREMANS et al. 2000).

In conclusion, the study shows that water deficit stress in peanut imparted different physiological and biochemical responses at different growth stages. As a general response, the peanut cultivars showed decrease in RWC, membrane stability, specific leaf area and chlorophyll and carotenoid contents, but the extent of reduction varied with cultivars and developmental stage of the crop. At the initial stage, the impact of water deficit stress was lower, but with the advancement of crop growth stage a more pronounced effect was observed. The production of various reactive oxygen species and lipid peroxidation also supports this conclusion, showing a greater degree of oxidative stress when water deficit stress is imposed at pod developmental stage than at pegging stage. Higher activities of SOD, CAT, POD, APX and GR, as a whole, contributed to less oxidative stress in tolerant cultivars like ICGS 44 and TAG 24, establishing the role of antioxidant defence system in the ability of the peanut to tolerate water deficit stress.

Acknowledgements

Authors gratefully acknowledge the Director, DGR, for providing facilities, and Mr. C.B. Patel for technical support in raising the crop during the conduct of the experiment. The authors are also grateful to Dr. D. Bhaduri for her support rendered in statistical analysis.

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