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Variability in drought stress-induced physiological, biochemical responses and expression of DREB2A, NAC4 and HSP70 genes in groundnut (Arachis hypogaea L.)

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ABSTRACT

Drought stress is one of the most important factors of physiological stress and the major constraint on crop productivity which limits plant growth and metabolism. The goal of this work was to study the differential response of groundnut genotypes to drought stress at the physiological, biochemical, and molecular levels during summer in the field. Thirty days old seedlings of six genotypes of groundnut viz. Kadiri 9, Narayani, Dharani, JL24, TPT-3, and Kadiri 6 were subjected to drought stress by withdrawing irrigation for 15 days. The results suggested a significant influence of drought stress on the physiological and biochemical levels in all the groundnut genotypes. A substantial decrease for physiological parameters viz. membrane stability index, chlorophyll stability index, and relative water content was observed under moderate and severe stress conditions compared to control across all genotypes. The high proline accumulating genotypes also exhibited lower lipid peroxidation under all stress periods. In Kadiri 9, Narayani, and Dharani genotypes, the antioxidant enzyme activity of superoxide dismutase, catalase, peroxidase, and glutathione reductase was significantly higher than JL24, TPT-3, and Kadiri 6 genotypes under all stress regimes. Among the genotypes tested, Kadiri 9, Narayani, and Dharani retained higher growth, yield, and seed quality characteristics showing tolerance for drought stress. qPCR analysis revealed stress-responsive existence of the selected genes, heat shock protein 70 (*HSP70*), dehydration-responsive element binding-2A (*DREB2A*), and no apical meristem, ATAF1/2, cup-shaped cotyledon 2 (*NAC4*) with 30 fold increase in the level of expression in Kadiri 9 compared to Kadiri 6.

1. Introduction

Groundnut (*Arachis hypogaea* L.) is an essential oilseed cash crop that contains 44 - 56% edible oil and 22 - 28% protein grown on 26.71 million hectares of land area with a yield of 1.68 t per hectare generating 44.86 million metric tons in 82 countries (Hamdy et al., 2019). Asia is the world's largest groundnut growing area accounting for 65.1 percent of global production (FAOSTAT, 2017). In India, with the production of 6.70 million metric tons per annum (USDA Foreign Agricultural Service, 2016), it is grown on 5.34 million hectares of land with yield of 1.25 t per hectare. Large countries which produce groundnut including China, India, Nigeria, and the US are facing extreme crop irrigation water shortages, which in the future will restrict worldwide groundnut production (Long et al. 2010). Plants are often subjected to abiotic stress due to both natural climatic conditions and raw agricultural practices (Iqbal, 2018). More than 50% of the production area is in arid and semi-arid areas, where drought stress often varies in duration and intensity (Reddy et al., 2003).

Drought stress is one of the major environmental stresses that reduces crop production dramatically almost every year. Food crop productivity is highly enviable due to the growing population worldwide and the need to broaden initiatives for optimum growth. Drought stress tolerance is present in most plants, but its magnitude varies from one species to other. Reproductive stage is the most vulnerable stage in plants during drought stress compared to pre- and post- reproductive stages (Akram et al., 2018). Drought stress has important effects on plant morphology, physiology, and biochemistry, and restricts plant growth and productivity in both dry land and irrigated crops (Liu et al., 2015). Attempts to improve crop drought tolerance through routine breeding programs have indicated limited attainment as a result of quality attributes. Drought resistance function in plants involves both avoidance and stress tolerance.

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Drought stress prevents germination by reducing water imbibition and decreases seed intensity (Kaya et al., 2006). Drought stress inhibits plant growth by affecting various metabolic processes such as photosynthesis, ion absorption, respiration, and metabolism of carbohydrates (Li et al., 2011). Recently physiological characteristics such as relative water content and electrolyte leakage have been related to drought tolerance in groundnut (Puangbut et al., 2011). Among the physiological parameters, osmolyte proline accumulation is one of the most effective methods to help plants counter the severe effects of water deficits. Oxidative stress can cause plant damage to DNA, lipid peroxidation, and protein oxidation (Blokhina and Fagerstedt, 2010). Lipid peroxidation (LPOX) in all living organisms is a very deleterious process (Gill and Tuteja, 2010). Probability of membrane LPOX with increased cellular disturbance contributes to oxygen accumulation in plant tissue. LPOX is therefore an index of oxidizing damage under stress. In plants, reactive oxygen species (ROS) including single oxygen ($^1O_2$), superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) are developed under oxidative stress (Sharma et al., 2012; Foyer and Noctor, 2011). Drought stress had a significant influence in antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and glutathione reductase (GR) (Sanchez-Rodriguez et al., 2012). Drought stress decreases growth and metabolic activities resulting in reduced crop attributes for agronomy and yield (Furlan et al., 2012).

There has been little advancement in molecular breeding because of the polygenic regulation of many resistance traits to drought. Plants have developed various resistance mechanisms to drought stress to cope with changing climate conditions through differential expression of genes that encode transcription factors (TFs), essential enzymes in biosynthetic pathways, and proteins associated in stress sensing and cell signaling (Shinozaki and Dennis, 2003). In response to various abiotic factors, heat shock factors (HSFs) play a major role in plants by controlling the expression of stress-responsive genes such as heat shock proteins (*HSPs*) and transcription factors (TFs) like *DREB, NAC* (Pruthvi et al., 2014; Wang et al., 2004). Drought causes several changes in gene expression, so it is important to recognize possible candidate genes that express in conditions of drought stress. While the effectiveness of molecular-based approaches in the production of drought-tolerant cultivars has not been completely realized, the technology holds promise and could ultimately facilitate the identification of genomic regions for the tolerance of drought to be recombined in breeding (Zhao et al., 2008).

Global groundnut production in the main areas of production is threatened by recurrent drought conditions, which are expected to increase with climate change. Drought resistant groundnut varieties are the perfect remedy to safeguard the crop from adverse drought effects. Groundnut occupies a substantial position in oil production and the improvement of the yield of groundnut genotypes under the stress of drought, and agroclimatic situations has key task for researchers to deal with the scenario. A major aim of groundnut breeding programs is therefore to classify and select the genotypes with an increased resistance to drought stress. With the aim of enhancing the candidate drought responsive genes in groundnut, three candidate genes *HSP70, DREB2A*, and *NAC4* were selected to study the expression pattern under all the drought stress regimes using quantitative real-time PCR (qPCR). We have assessed the consequences of physiological, biochemical, and molecular changes in plants grown in the field during the summer months.

2. Materials and methods

2.1. Plant materials and experimental design

Seeds of Kadiri 9, Narayani, Dharani, JL24, TPT-3, and Kadiri 6 groundnut genotypes have been obtained from Regional Agricultural Research Station, Tirupati, India. All the six groundnut genotypes are well known Spanish type and maturity ranging from 95 - 105 days with varying degrees of drought tolerance. Field trial was performed at the Biotechnology department, Sri Padmavati Mahila Visvavidyalayam, Tirupati during the April 2017/18 summer season. The experiment was conducted with a split-plot design of control (fully irrigated) and treatment (continuously withholding water for fifteen days) with three replicates in a randomized complete block system. After 30 days of sowing (the early reproductive stage), water was withheld in treated group and the fully expanded fresh leaf samples were collected from stressed along with their respective controls at 35th, 40th, and 45th days characterized as mild, moderate, and severe stress for the physiological, biochemical, and molecular characterization of groundnut. Weather data was collected from Meteorological Climate Data Center, Regional Agricultural Research Station, Tirupati for the duration of the experiments. Traits of yield and seed quality were assessed at harvest.

2.1.1. Membrane stability index, chlorophyll stability index and relative water content

Membrane stability index (MSI) was calculated using Towill and Mazur (1975) process determining absorbance at 273 nm and percent leakage was calculated by (initial absorbance/final absorbance) × 100. Chlorophyll Stability Index (CSI) was estimated according to Murthy and Majumdhar (1962) by reading the optical density at 663 nm and 645 nm. CSI (%) = total content of chlorophyll (treated)/ total content of chlorophyll (control) × 100. Relative water content (RWC) was assessed according to Barrs and Weatherly (1962) method with the following formula, RWC (%) = (FW-DW)/(TW-DW) × 100.

2.1.2. Free proline and malondialdehyde in leaf samples

Free leaf proline content was calculated with slight modifications to Bates et al. (1973) by measuring the intensity at 520 nm. Lipid peroxidation was assessed by measuring the formation of malondialdehyde (MDA) content using thiobarbituric acid (TBA) as defined in Heath and Packer (1968) by measuring the intensity at 532 nm using UV Visible Spectrophotometer.

2.1.3. Measurement of antioxidant enzyme activities in leaf samples

The extract was prepared as defined in Chakraborty et al. (2016) for all antioxidant enzymes and the absorbance was measured using UV spectrophotometer by kinetics method. Activity of superoxide dismutase (EC 1.15.1.1) was determined by the process described by BeauChamp and Fridovich (1971) by the enzyme inhibition of photochemical reduction of nitroblue tetrazolium (NBT) The absorbance was measured at 560 nm and one unit of enzyme activity was described as the amount of SOD that generated a 50% NBT reduction percent inhibition compared to tubes that lack enzyme. Peroxidase (EC 1.11.1.7) activity was calculated as illustrated in Putter (1974). The oxidation rate of the guaiacol was recorded at 436 nm. Catalase (EC 1.11.1.6) activity was assayed by measuring a decrease in H_2O_2 absorbance at 240 nm as a result of H_2O_2 intake (Luck, 1974). The glutathione reductase (EC: 1.6.4.2) activity was calculated using Mavis and Stellwagen (1968) method, and the absorption was measured at 340 nm.

2.1.4. Total free amino acids, total carbohydrates, protein and oil content

The amount of free amino acids was assessed by the process of Hodge and Hofreiter (1962) using ninhydrin reagent and glycine as standard. Total carbohydrate content was determined by anthrone reagent as per the method of Balasubramanian and Sadasivam (1987). Oil and protein contents were estimated in control and stressed groundnut genotypes using All Grain Analyzer Instrument (Zeutec, Germany).

2.2. RNA isolation and primer design

Total RNA was isolated from the most tolerant Kadiri 9 and most susceptible Kadiri 6 genotypes collected during mild, moderate, and severe stress periods using Sajeevan et al. (2014). A Nanodrop (GE health care, USA) was used to assess the quality and integrity of RNA in 2% agarose gel electrophoresis. Three candidate genes namely *HSP70* (EZ733089), *DREB2A* (DQ333948), and *NAC4* (HM776131) were selected from the previous studies and expressed sequence tags (EST) sequences published in Genbank NCBI. Using the Primer 3 Plus program (Untergasser et al., 2007), qPCR primers with Taqman probes were designed with the following criteria such as length between 19 - 22 nucleotides, annealing temperature ranged from temperature 60 - 65 °C, GC content between 45 - 55%, and product size of 90 - 110 bp (Table S1).

2.2.1. Reverse transcriptase PCR and real-time PCR

1 μg of total RNA was reverse transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). RT-PCR was first performed in a 20 μl reaction for PCR containing 100 ng cDNA with 1 U of Taq polymerase (Thermo Fisher Scientific, USA) in 1X reaction buffer, 2.5 mM $MgCl_2$, 250 μM dNTP mix, 0.2 μM primers for each gene and RT reaction template. The RT-PCR cycling conditions were programmed as follows: 94 °C for 5 min followed by 40 cycles of 15 s at 95 °C, 15 s at 61 °C for 1 min 72 °C for 1 min with a final extension of 72 °C for 10 min (Eppendorf, Germany). PCR products were then detected in 2.0% gel electrophoresis. qPCR analysis was performed on Real Time PCR (Quiagen Rotor Gene, USA) containing 5 μl of 2 × one step Master mix buffer (Eurofins, USA), 0.5 μl of 20X primer probe mix, 0.1 μl of RT mix, 3 μl of total RNA (100 ng) and RNase-free H_2O in 10 μl reaction volume. The cycling conditions of qPCR were 50 °C for 15 min (reverse transcription) followed by 40 cycles of 5 s PCR activation at 95 °C, 15 min denaturation at 95 °C and 15 s annealing at 60 °C. Melting curve analysis from 59 °C to 95 °C was done to confirm the specificity of the PCR products. After 40 cycles, the melting curve analysis was included to verify the specificity of the primer by heating from 58 °C to 95 °C with fluorescence measured within 15 min. The expression of the genes *NAC4, HSP70, DREB2A* was studied in control and stressed Kadiri 9 and Kadiri 6 genotypes. Relative gene expression levels in response to stress were calculated using the $2^{-\Delta\Delta Ct}$ approach (Livak and Schmittgen, 2001), and for normalization, glucose-6-phosphate 1-dehydrogenase (*G6PD*) was used as reference gene (Reddy et al., 2013).

2.3. Statistical analyses

Data was analyzed for variance analysis (ANOVA) and the mean differences were determined using the Duncan multiple range test at $p<0.05$ (IBM SPSS Statistics v. 16.0 Software). The values are the mean ± SD of five independent determinants in each group. For each genotype the correlation coefficient of all physiological, biochemical, and yield data was performed in R using mean value. Using principal component analysis, the relationship between yield and physio-biochemical traits was further determined. Using R package (R Core Team, 2012), principal component analysis (PCA) results were visualized between the two main components, PC1 and PC2 biplots constructed.

3. Results

3.1. Physiological responses to drought stress

Membrane stability index, relative water content and chlorophyll stability index are indices for calculating the degree of resistance to drought. A considerable high MSI, RWC, and CSI was observed in Kadiri 9, Dharani, and Narayani and decreased with the intensity of drought stress compared to control (Fig. 1). The percent decrease of MSI was observed significantly in Kadiri 9 under all three stress periods ranging from 35.2 - 39.5% followed by Narayani (36.5 - 38.9%) and Dharani (38.2 - 42.2%) compared to control ranging from 34.4 - 37.9%. MSI was low in TPT-3 (42.15 - 60.85%). The percent decrease of RWC was recorded significantly in Kadiri 9 genotype even at severe stress period (66.3%). The highest reduction was recorded in TPT-3 (50.3%) followed by Kadiri 6 (54.7%) and JL24 (61.1%). The

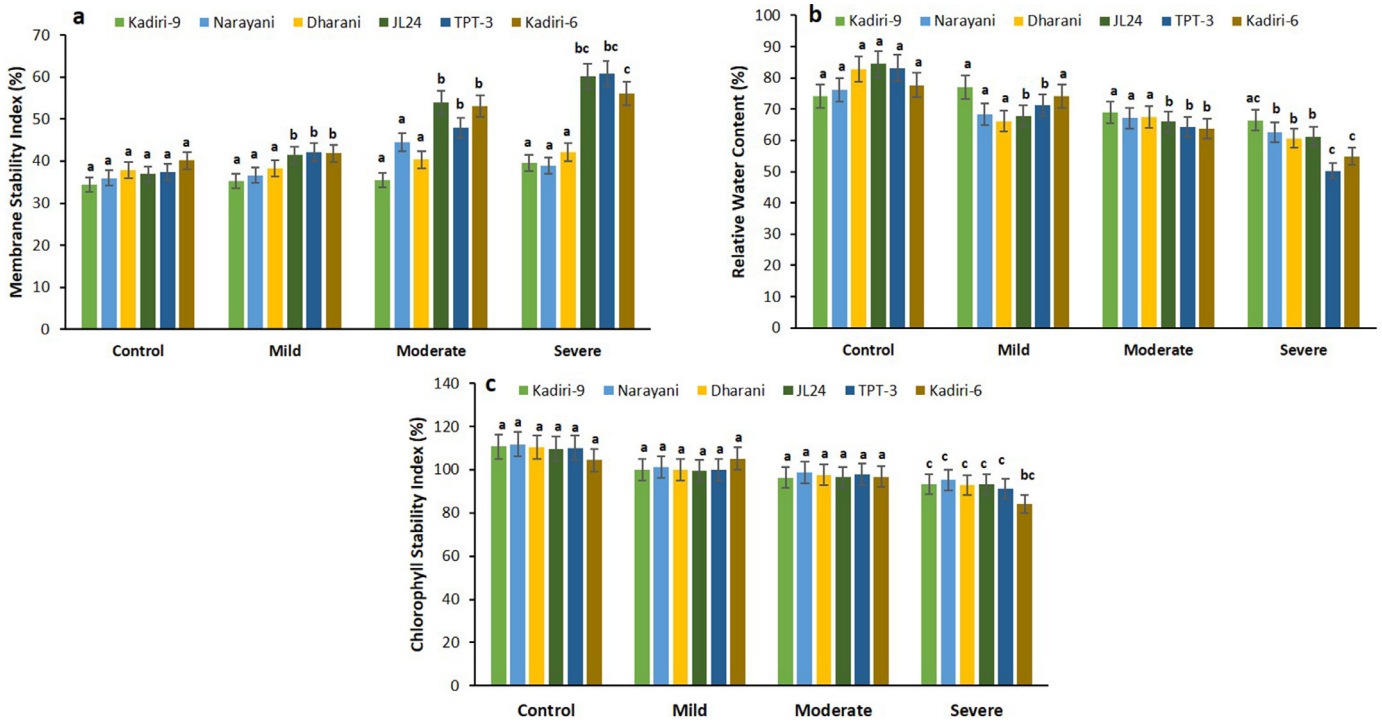

Fig. 1. Effect of drought stress on physiological traits.

percent decrease of CSI, 92.7% was observed significantly in Dharani while genotype Kadiri 6 recorded higher decline of 84.19% under severe drought stress compared to control.

3.2. Biochemical responses to drought stress

Leaf proline content increased significantly with the severity of the drought stress compared to control plants. In our findings, leaf proline content of control plants was found to be 55 - 441.67 μg/g fr. wt. Imposing water stress resulted in increased proline content of more than 3 to 5 folds, i.e., 609 to 1436.11 μg/g fr. wt. The highest proline content was accumulated in Kadiri 9, Narayani under severe stress condition. MDA contents were elevated in all six groundnut genotypes under all stress periods. Compared to control genotypes, Kadiri 6 recorded significant highest rate of lipid peroxidation (61.2 nmol/g protein) whereas Dharani showed lower levels of induction (43.1 nmol/g protein) under severe stress (Fig. 2).

All the six genotypes showed a significant increase in antioxidant activity of SOD, POX, CAT, and GR compared to control, but in severe stress period a significant decrease in CAT activity was observed (Fig. 3). Significant increased SOD activity was observed in Kadiri 9 (6.98 - 7.5 μg/g protein) and Dharani (6.18 - 7.46 μg/g protein) along with increase in drought stress. Like SOD, POX activity also increased marginally with the highest recorded in Dharani (10.92 - 13.6 μg/g protein), Kadiri 9 (11.38 - 13.3 μg/g protein), and lowest activity in Kadiri 6 (10.32 - 12.84 μg/g protein). An increase in CAT activity was observed in all six genotypes with the highest activity in Dharani (4.28 - 6.48 μmol H_2O_2/min/mg protein). Significant decrease in CAT activity was observed in TPT-3 (1.39 μmol H_2O_2/min/mg protein) and JL24 (2.62 μmol H_2O_2/min/mg protein) under severe stress condition. GR activity also increased marginally with the increase in stress duration and highest in Kadiri 9 (32.76 μmol $NADP^+$ /min/mg protein) whereas lowest in JL24 (27.36 - 28.0 μmol $NADP^+$ /min/mg protein). These results indicated that Dharani, Kadiri 9, and Narayani genotypes can tolerate better under drought stress by synthesizing several antioxidant enzymes.

3.3. Yield and seed quality components

Tirupati's average seasonal rainfall (April-July) was c.11 mm and is extremely variable during December and January with 54% relative humidity. The averages for maximum daily and minimum temperatures are 42 ± 5 °C in summer, respectively. The plants were carefully uprooted after harvesting (120 days) and thoroughly washed with running tap water and morphological characters like root, shoot lengths, fresh, dry weights, number of lateral, primary, secondary, tertiary branches, number of pods, kernels, and total fatty acids, total carbohydrate, protein, and oil were recorded. Drought stress at early reproductive stage significantly reduced yield components of all the selected groundnut genotypes studied and was highly significant at (p<0.05) (Table S2). Drought stress decreased pod number in all genotypes except in Dharani which indicates that in these genotype pollination and fertilization were not influenced by drought stress. Higher pod number was recorded in JL24 and Kadiri 6 under well-watered conditions, while in Dharani, JL24, and Kadiri 6 under drought stress performed best. The seed number was highest in genotypes Dharani, Kadiri 6, and Kadiri 9 under well-watered condition whereas under drought stress, seed number was highest in Dharani which resulted in higher average genotype performance. Yield performance of Dharani and Kadiri 6 were superior under both control and drought stress conditions. The genotype Dharani with highest seed yield and Kadiri 9, TPT-3 with low seed yield registered lowest reduction whereas Narayani recorded highest reduction. The oil content decreased significantly under drought stress condition and genotypes Narayani, Dharani, and JL24 recorded higher oil content with higher improvement under stress condition. While the oil yield of groundnut genotypes reduced drastically due to drought stress and highest reduction was recorded in TPT-3. The protein content of genotypes increased with drought stress and highest improvement was recorded in Kadiri 9 and Narayani whereas it decreased in Dharani, Kadiri 6, and TPT-3. In the present study, the concentration of total carbohydrate and total fatty acid was found to decrease under all drought stress conditions. In Dharani and Narayani, TC and TFA content was found to be more significant than JL24 and TPT-3.

3.4. Simple correlation coefficient analysis

3.4.1. Physiological and biochemical traits

Linear correlation analysis established the relationship between key physiological and biochemical features in the early reproductive stage of groundnut plants during drought stress (Fig. 4). The antioxidant enzymes showed significant correlations among each other under drought stress. In control conditions, MSI or RI had a strong significant positive correlation with proline (r = 0.86) followed by POX with SOD (r = 0.84) whereas, CSI had a strong significant negative correlation with proline (r= −0.95**). Under mild stress, POX had strong significant correlation with SOD (r = 0.93 **) followed by RWC with GR (r = 0.85**), and RI had a strong significant negative correlation with POX (r= −0.99**) followed by SOD (r= −0.97**). Under moderate stress, RWC had a significant positive correlation with SOD (r = 0.98**) and proline with SOD (r = 0.83**) whereas, MDA had strong negative correlation with proline (r= −0.89**) followed by SOD (r= −0.84**), and MSI with RWC (r= −0.85**). Proline had a strong significant positive correlation with SOD (r = 0.97**), CAT with proline (r = 0.86**) and POX with SOD (r = 0.85**) whereas, MSI had strong negative correlation with proline (r= −0.98**) followed by SOD (r= −0.93**), and MDA with SOD (r= −0.85**) followed by proline (r= −0.81**) under severe stress.

3.4.2. Yield and seed quality traits

Yield and seed quality traits showed significant correlations among each other under drought stress (Fig. S1). In control conditions, number of pods had strong significant positive correlation to

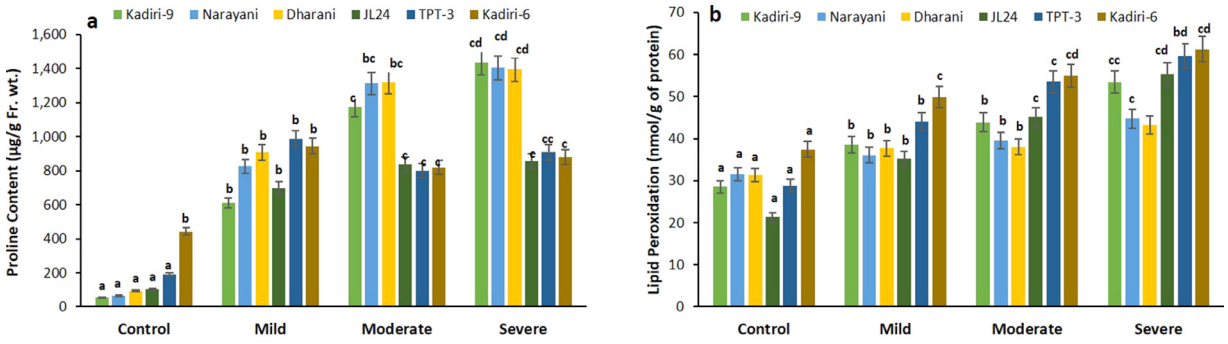

Fig. 2. Effect of drought stress on biochemical traits.

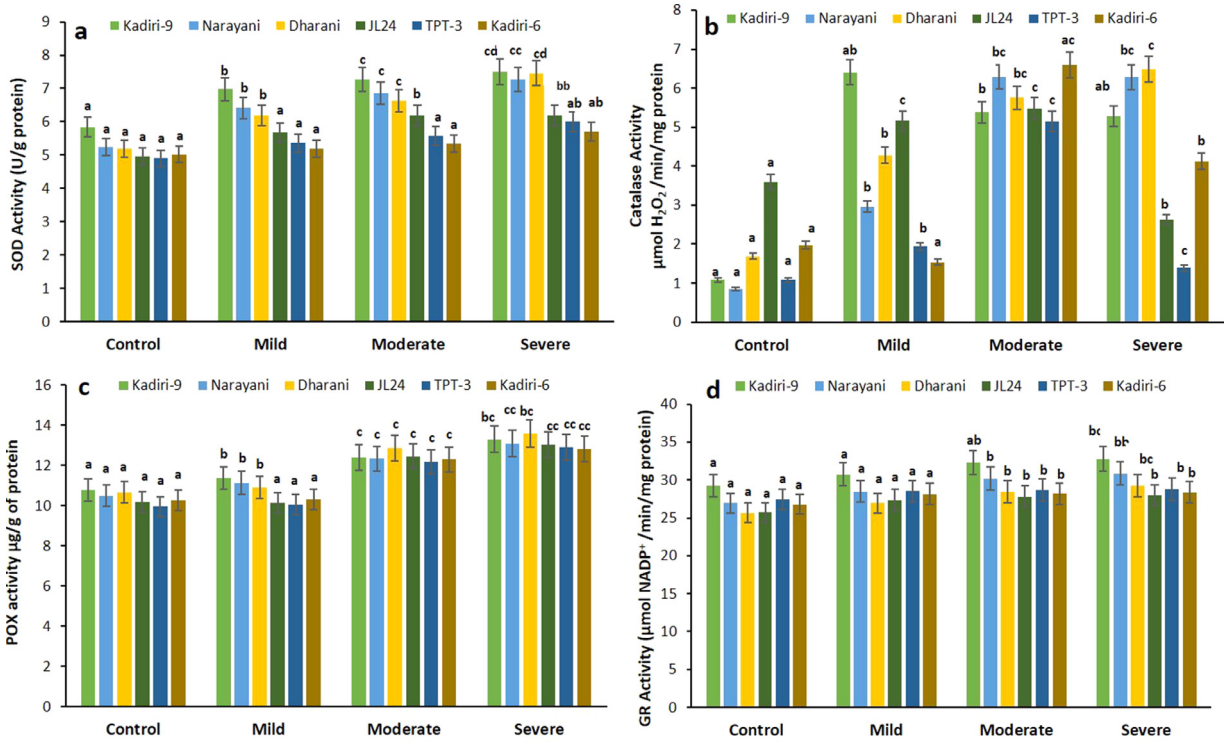

Fig. 3. Effect of drought stress on antioxidant enzyme activity.

number of kernels (r = 0.97**) followed by shoot length with plant height (r = 0.9**), TFA with TC (r = 0.9**), and strong negative correlation in TC with oil (r= - 0.85**) followed by fresh weight with secondary branches (r= −0.83**). Under drought stress, shoot length had strong significant positive correlation with plant height (r = 0.99**) followed by TFA with TC (r = 0.97**), fresh weight with number of kernel (r = 0.95**), and strong negative correlation in root length with protein (r= −0.81**), followed by plant height with number of pods (r= −0.78**), and shoot length with number of pods (r= −0.76**).

3.5. Multivariate analysis

3.5.1. Physiological and biochemical traits

Principal component analysis (PCA) showed that the first six PCs had Eigenvalues greater than 1 in control conditions. The first and

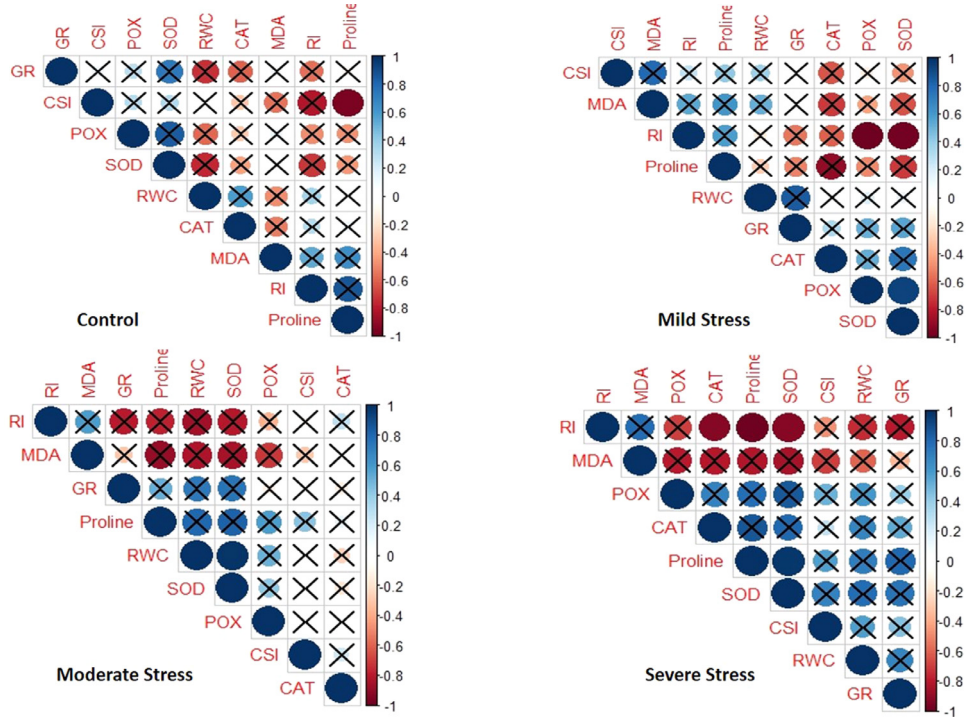

Fig. 4. Correlation between control and treated (mild, moderate, and severe stress) physiological and biochemical traits in six groundnut genotypes.

second major components clarified the phenotypic variability of 48.6 and 31.2% and the key contributors to these two PCAs were CSI, POX, SOD, GR, RWC, and CAT. In treated, the first six PCs had Eigenvalues more than one and the first two PCs clarified 56.2 and 26.5% phenotypic variance respectively. Under mild stress, CSI, POX, SOD, GR, RWC, and CAT were the major contributors to the first two components as in control. The first five PCs had more than 1 Eigenvalues under moderate stress. The first and second major factor clarified phenotypic variability of 56.7 and 18.2 percent and the key contributors to these two PCAs were GR, RWC, SOD, proline, and POX. The first seven PCs had more than 1 Eigenvalues under severe stress. The first and second principal components clarified the phenotypic variability of 74.4 and 9.7%, and the major contributors to these two PCAs were GR, RWC, proline, CAT, SOD, POX, and CSI (Fig. 5).

3.5.2. Yield and seed quality traits

Principal component analysis (PCA) showed that the first six PCs had Eigenvalues greater than 1 in control conditions. The first and second major components clarified the phenotypic variability of 31.3 and 25.5%, and the key contributors to these two PCAs were number of pods, number of kernels, oil content and lateral branches. In treated, Eigenvalues had more than one and first two PCs clarified 47 and 21.2% phenotypic variance respectively. Number of tertiary branches, number of secondary branches, plant height, and oil content were the main contributors to these two PCAs (Fig. S2).

3.6. Expression analysis

Primer specificity was verified with agarose gel electrophoresis using RT-PCR and melting curve analysis (Fig. S3). *HSP70, DREB2A*, and *NAC4* genes were upregulated in both Kadiri 9, and Kadiri 6 genotypes under stress conditions. In Kadiri 9, *DREB2A* and *NAC4* genes were significantly upregulated to more than 30 fold increase in expression respectively in mild and moderate stress conditions, whereas expression level decreased to 26, 14 fold when compared to control in severe stress. A 15 fold increase was observed in *HSP70* gene in mild and moderate stress and decreased to 10 further in severe stress condition when compared to control in Kadiri 9 (Fig. 6). In Kadiri 6, *DREB2A*, and *NAC4* genes were significantly upregulated

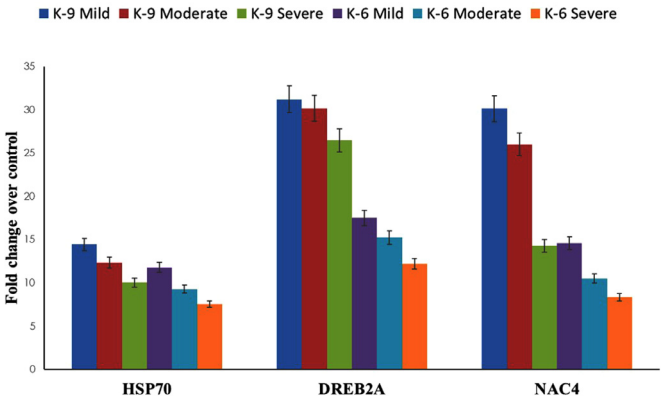

Fig. 6. Quantitative expression analysis of three candidate genes at different stress periods (mild, moderate, and severe) in Kadiri 9 and Kadiri 6 genotypes.

in mild, moderate stress conditions to 17, 14 fold in expression respectively and decreased to 12, 8 fold in expression when compared to control. In Kadiri 6, *HSP70* gene expression level was upregulated to 11, 9 fold in mild and moderate stress conditions, and further decreased to 7 fold in severe stress compared to control.

4. Discussion

The results of this study revealed a broad variability in selected groundnut genotypes in terms of growth, physiological, biochemical, molecular, yield, and seed quality components during the summer season under drought stress condition.

All the physiological parameters such as membrane stability index, relative water content, and chlorophyll stability index of selected six groundnut genotypes reduced under drought stress in mild and moderate stress conditions, and the decrease was more pronounced in severe stress conditions. A significant effect of water stress is typically on the alteration of the cellular membrane, resulting in complete dysfunction, and it is widely agreed that preserving the integrity and stability of the membranes under stress is a major component of stress tolerance in plants. According to

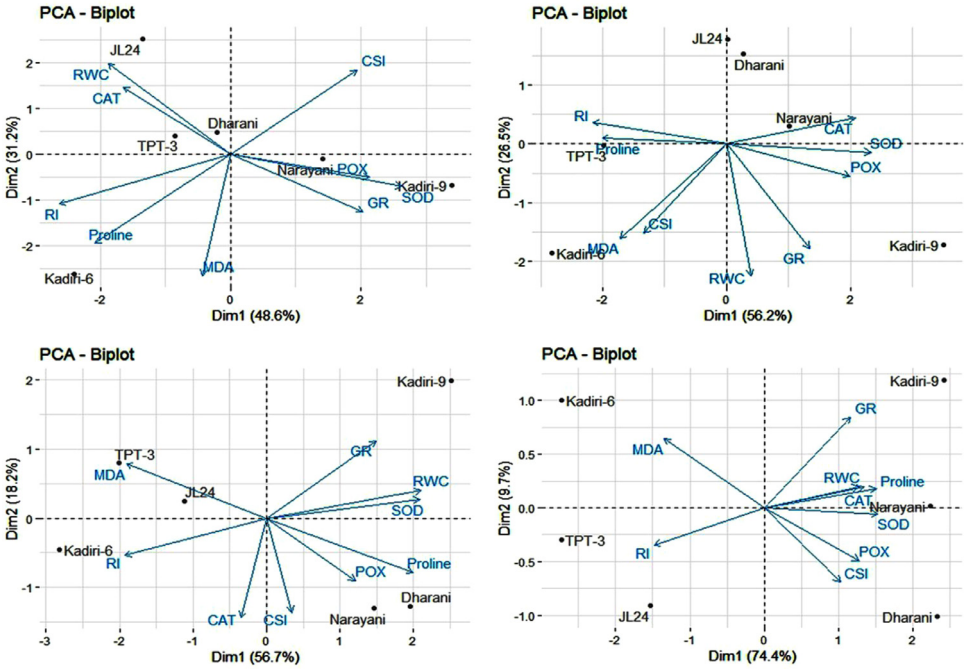

Fig. 5. Principal component analysis - Biplot of control and treated (mild, moderate, and severe stress) physiological and biochemical traits in six groundnut genotypes.

Akcay et al. (2010) and Shinde et al. (2010), drought induced relative injury leads to the development of susceptibility in several groundnut genotypes. Genotypes with higher MSI have improved adaptations under drought stress, and the genotypes Kadiri 9, Dharani, and Narayani with better MSI are likely to have favorable adaptations under stress in the current investigation. Drought stress decreased RWC in the leaves of both susceptible and tolerant genotypes, and the decrease was more significant in TPT-3 and Kadiri 6 compared to tolerant genotypes Dharani, Narayani, and Kadiri 9. Beltrano et al. (2006) showed that the genotypes which retain high levels of leaf water under water stress are less affected, and able to sustain normal growth and yield. This differential response is an adaptation by groundnut plants to avoid excessive dehydration while tapping moisture available in roots deep in the soil (Kokkanti et al., 2019a). CSI was high in Dharani, Narayani, and Kadiri 9 compared to Kadiri 6, the lowest CSI recorded under drought stress. According to Manivannan et al. (2007), loss of chloroplast membrane integrity due to lipid peroxidation is attributed to the stress induced reduction.

Drought stress peroxidation of cell membrane lipids resulted in a substantial increase in the MDA content, an indicator of toxic reactive oxygen species (Akcay et al., 2010). In the present investigation, MDA content increased in all groundnut genotypes as compared to control with the intensity of drought stress. It was significantly higher in Kadiri 6 and TPT-3 compared to other genotypes. Such genotypes also showed lower chlorophyll content reduction and higher proline levels that can help preserve the integrity of the membrane relative to other genotypes. The findings of this analysis follow Turkan et al. (2005) in beans, and Shinde et al. (2018); Furlan et al. (2015) in groundnut. In the current study, rapid accumulation of proline was observed in both susceptible and resistant genotypes with an increase in the severity of drought stress. The higher concentration of proline during drought stress indicates an efficient mechanism for osmotic adjustment that maintains cellular homeostasis (Vijayalakshmi et al., 2012). Genotypes Dharani, Narayani, and Kadiri 9 are expected to have better stress tolerance potential in the present study with higher proline accumulation under drought stress, indicating that compatible osmolytes contribute to the increased drought tolerance. Higher accumulation of proline was reported in groundnut cultivars due to drought stress by several authors (Shinde et al., 2018; Solanki and Sarngi, 2014; Sharada and Naik, 2011).

Drought stress induced an increase in the activity of antioxidant enzymes (SOD, CAT, POX, GR) compared to control in all genotypes. SOD, POX, CAT, and GR activity under drought stress was higher at all times than the control group and increased with the period of water stress reaching the maximum level at day 15. These parameters were highly significant for tolerant genotypes, indicating that moisture levels had significant influence on these biochemical parameters, and response of individual genotype to these conditions varied significantly. Similar results were obtained in different crop plants (Iqbal et al., 2019; Wang et al., 2019; Ren et al., 2016). Significant variations in SOD activity in all six genotypes were observed at control condition. The increased activity of SOD and POX enzymes in tolerant genotypes might play a key role in the moisture deficit stress defense mechanism indicating better protection against super oxide anion. Whereas, lower activity of susceptible genotypes suggests more oxidative damage in terms of cell membrane injury and lipid peroxidation. The findings are consistent with studies from Bhalani et al. (2019); Bhardwaj and Yadav (2012), and Shinde and Laware (2015); Furlan et al. (2015) on the increase of antioxidant enzymes, SOD and POX in horsegram, and peanut under drought stress conditions. CAT activity increased in the present study with the increase in the severity of drought stress. The increase in CAT activity was uniform in all the genotypes except, JL24, TPT-3, and Kadiri 6 where there was drastic decrease in the activity of CAT at severe stress conditions. The decreased activity of CAT was explained by Hertwig et al. (1992), who suggested that in the presence of intense sunlight, undergoes photo-inactivation followed by degradation. Cao et al. (2017) and Upadhyaya et al. (2005) reported a decrease in catalase activity under drought stress. Therefore, the Dharani and Narayani genotypes with higher activity of antioxidant enzymes are predicted to have greater potential for stress tolerance. GR activity also increased in all six genotypes, with the severity of the drought stress period. Increased activity of GR in the present study might suggest the recharging of oxidized glutathione to reduced glutathione. Comparatively higher activity of Kadiri 9 and Narayani shows the involvement of GR in efficient elimination of hydrogen peroxide.

The negative impacts of drought on yield depend largely on the extent of the stress, range of temperature exposed, and the stage of plant development. There was wide variability in the performance of available genotypes under these conditions. The average genotype performance was high in Dharani at both moisture levels. Kokkanti et al. (2019b) also reported that Dharani, Kadiri 9, and Narayani were tolerant to PEG induced drought stress at seedling stage. Babu et al. (2011) reported that the stress from the moisture deficit significantly reduced the number of mature groundnut genotypes pods and JL24 were highly affected. This study contrasted with our current investigation that JL24 was not affected by the number of pods under drought stress. Kakani et al. (2015) and Kambiranda et al., (2011) also confirmed that drought stress is significantly impacting groundnut yield and yield parameters. Seed number reduced under drought stress condition from well-watered controls. Drought stress at flowering stage decreased seed number significantly in bambara groundnut (Vurayai et al., 2011). The average genotype performance was highest with Dharani at both moisture levels and recorded highest seed yield. Similar reproductive response patterns were reported in bambara groundnut genotypes (Laary et al., 2012). According to Akram et al., 2018; Kakani et al. (2015); Shinde et al. (2010), and Puangbut et al. (2009), there were significant yield losses in groundnut under drought stress.

ANOVA showed that drought stress significantly influenced oil quality and significant variation in genotypes occurred, while the response of selected genotypes did not significantly differ in conditions of drought stress. The genotype Dharani recorded highest oil yield under both moisture levels as it registered higher seed yield coupled with better oil content. The reduction of oil yield was low in TPT-3 however the per seed oil yield was also low. The oil content is affected by decreased concentration of digestible carbohydrates during drought stress, which affects the fatty acids composition in the developing seeds (Bellaloui et al., 2013). The protein content was highest in Kadiri 9 under drought stress and the genotype Narayani recorded lowest protein content under well-watered condition however recorded highest under drought stress condition. Vaidya et al. (2015) and Chakraborty et al. (2013) also reported that influence of drought stress on protein content differed with groundnut genotypes. Dharani and Narayani recorded high TC and TFA content, and the reduction in carbohydrates in the present study may indicate the conversion of carbohydrates to lipids and proteins. Similar changes of reduced carbohydrate and fatty acid were reported by Chakraborthy et al. (2016); Gulluoglu et al. (2016), and Kandoliya et al. (2015) in groundnut under drought stress.

In the present study, PCA analysis revealed that physiological as well as biochemical parameters, cluster together indicating MDA, proline, MSI, SOD, and CAT are strong correlated. Previous studies have also highlighted the correlation of high activity of antioxidant enzymes with resistance to drought stress in peanut plants (Faye et al., 2015). Yield and seed quality traits were strongly correlated, and emerged as better indicators in differentiating genotypes for drought tolerance. Analysis of PCA and correlation also confirmed that Dharani, Kadiri 9, and Narayani performed better even under extreme drought conditions suggesting drought tolerance.

The molecular basis for tolerance of drought stress and plant growth through stress-dependent transcriptional regulation enables plants to adapt quickly to different climatic conditions. Drought stress tolerance occurs at the developmental, physiological, biochemical, and molecular levels that activate various genes, regulated by different transcription factors (Claeys and Inze, 2013; Ohama et al., 2017). Variation in the differential expression of candidate genes responsive to stress illustrates the essence of the plants stress responses. Validation of drought stress tolerant genes in plants may provide insight into their roles, making their use easier to increase crop productivity and yield. Some of the HSP genes expression is reported to be induced both by heat and drought stress (Wang et al., 2004). *HSP70* gene was up regulated in both Kadiri 9, Kadiri 6 genotypes under mild and moderate stress but the expression level was decreased more in Kadiri 6 under severe stress indicating susceptibility to drought. Banavath et al. (2018) reported the activation of *HSP70* gene during drought stress in groundnut. The present report indicates that many HSPs are up regulated due to the efficiency of upstream regulatory mechanisms such as signal transduction leading to multiple TFs expression.

TFs such as *DREB2A* and *NAC4* play a major role in regulating plant growth and metabolism (Banavath et al., 2018; Nakashima et al., 2012). *DREB2A* and *NAC4* were significantly up regulated in Kadiri 9 and Kadiri 6 genotypes under mild and moderate stress compared to control. Under severe stress, decline in expression level was pronounced more in Kadiri 6 than Kadiri 9. Several authors reported that DREB2A plays an important role in regulation of drought and heat responses in arabidopsis and groundnut (Kokkanti et al., 2019c; Mizoi et al., 2019; Pruthvi et al., 2014; Morimoto et al., 2013; Sakuma et al., 2006). According to Pandurangaiah et al. (2014), NAC4 was up regulated during drought stress in groundnut. The findings obtained explicitly point to a relationship between the genes expressed and leaf water content. The present report suggests that the activation of transcription factors *DREB2A, NAC4* regulate the expression of genes encoding several stress responsive genes like *HSP70*, and enhancement of tolerance mechanisms like ROS scavenging capacity imparting drought tolerance to groundnut genotypes. The expression of these genes is initiated even under severe drought stress in the resistant genotype Kadiri 9, thus underlining its significance in the use of crop yield enhancement programs in breeding.

5. Conclusion

The present study reveals the major impact of drought stress on MSI, proline, MDA content, SOD activity, and yield traits of groundnut genotypes. Dharani, Kadiri 9, and Narayani performed better than JL24, TPT-3 and Kadiri 6 under all drought stress regimes in the field during summer. Simple coefficient and principal component analysis also confirmed that Dharani, Kadiri 9, and Narayani performed better under drought conditions. Data generated by qPCR partially resolve genes, *DREB2A, NAC4*, and *HSP70* that play a key role in regulating the groundnut's response to drought stress. Further validation of above genes in tolerant genotype, Kadiri 9 will lead to a deeper understanding of the drought stress response mechanisms to enhance stress response. This comprehensive insight helps in developing elite superior groundnut genotypes showing high tolerance to drought stress.

Author contributions

RRK and RU designed the experiments. RRK carried out all the experimental work. RU supervised the work. RRK, VH, and AG analyzed the data, and RRK drafted the manuscript. All authors approve the final version of the draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2021.09.025.

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